

BLADDER CARCINOGENESIS AND THE BIOLOGICAL ACTIVITY OF SULFORAPHANE AND IBERIN

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Abstract

In this thesis, I sought to explore the biological mechanisms that may underlie the chemoprotective effects of broccoli consumption towards bladder cancer. In particular, I was interested in the effects of diet on the transition from superficial bladder cancer to a more invasive form of this disease. Initially, I characterised global gene expression profiles of superficial and invasive bladder carcinomas through tissues obtained from the Norfolk and Norwich University Hospital Tissue Bank. Non hierarchical cluster analyses was used to identify a suite of genes that characterised these two tumour types. Of particular interest was variation in genes involved in the synthesis of the extra cellular matrix, such as COL6A1 that was upregulated in the more invasive tumour type. This was subsequently verified by RT PCR with independent tissue samples. I then explored how the broccoli isothiocyanates, sulforaphane and iberin and their N-acetylcysteine conjugates were able to perturb gene expression in two bladder cell lines that differed in their cancer phenotype. I showed that not only were these ITCs able to upregulate phase II detoxification genes, which has previously been reported in other cell lines, but they, and their conjugates, were able to down regulate the COL6A1 gene. Studies on the effects of ITCs on gene expression were complemented with studies on cell migration and invasiveness. I then sought to see if any of the changes in gene expression observed in the cell lines could be observed in tissues obtained as part of a human intervention study. I designed and executed a small pilot study that enabled bladder tissue biopsies to be obtained before and after a four day intervention with broccoli. Global gene expression analyses again suggested alterations in genes determining the extracellular matrix, such as Tenascin-C. This study demonstrated the proof-of-principle that these types of intervention studies are possible, but was of an insufficient size to draw definite conclusions. Finally, I investigated in more details the expression of splice variants of Tenascin-C in relationship to bladder cancer grade. In conclusion, this study has suggested that dietary ITCs may perturb genes involved in the extracellular matrix and this may be an important component of understanding their chemopreventive activities, and has demonstrated the feasibility of human intervention studies with the analyses of target tissues as opposed to peripheral biomarkers.

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Abbreviations

ARE	antioxidant response element
BBB	blood brain barrier
BCA	bicinchoninic acid
BCG	bacilli calmette-guerin
BSA	bovine serum albumin
CYP	chromosome P450
CT	threshold cycle
CT	computed tomography
DSMO	dimethyl sulphoxide
dH₂O	distilled water
dChip	DNA-Chip Analyzer
DMEM	Dulbecco modified eagles medium
ECACC	European collection of cell cultures
EMEM	Eagle's Minimum Essential Medium
EMT	epithelial to mesenchymal transition
ECM	extracellular matrix
FDR	false discovery rate
FCS	foetal calf serum
GAG	glycosaminoglycan
genMapp	gene map annotator and pathway profiler
GLS	glucosinolate
GSH	glutathione
GST	glutathione-transferase
GSTM1	glutathione-transferase M1
GSTM3	glutathione-transferase M3
GSTP1	glutathione-transferase P1
GSTT1	glutathione-transferase TI
HPLC	high performance liquid chromatography
ICE	integrated communication environment
ITC	isothiocyanate
IVP	pyelogram
Keap-1	kelch-like ECH-associated protein 1
µl	microlitre

µm	micrometre
MMP	metalloproteinases
mg	milli-gram
ml	milli-litre
mM	millimolar
MAPK	mitrogen-activated protein kinase
MCC	mycobacterial cell wall DNA complex
MRI	magnetic response imaging
NAC	<i>N</i>-acetylcysteine
NNUH	Norfolk and Norwich university hospital
NQO	NAD (P₀) H: quinine oxidoreductase
NQO1	NADPH quinine oxidoreductase
NrF2	nuclear factor erthroid-derived 2-like 2
PAS	patient administration ststem
PEITC	phenethyl ITC
PBS	phosphate buffered saline
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
Secs	seconds
SE	standard error
SF	sulforaphane
TCC	transition cell carcinoma
TURB	transurethral resection of the bladder
TURBT	transurethral resection of the bladder tumour
Ta	non-invasive papillary carcinoma
Tis	carcinoma <i>in-situ</i>
T1	tumour invades subepithelial
T2	tumour invades muscle
T2a	tumour invades superficial muscle
T2b	tumour invades deep muscle
T3	tumour invades pereivesical muscle
T3a	microscopically
T3b	macroscopically
T4	metastasis

Author's declaration

I declare that this thesis represents my own unaided work, with the exception of:

- **The RNA hybridisation of all Affymetrix U133 plus 2.0 array GeneChips, which was performed at the European Arabidopsis Stock Centre (NASC)**
- **Methylation studies of GSTM1 gene, this work was in collaboration with Mr Jim Bacon**
- **Total GST activity studies, this was in collaboration with Dr Rachel Hurst**
- **Tenascin- C mapping diagram which was created by Dr Caroline Furniss**
- **Synthesis of N-acetylcysteine, which was carried out by Dr Paul Needs**

Melanie Jane Dunk

Part of this work has been presented at the following scientific meeting:

SARS/UROLOGY Meeting, University of Bristol, Chemistry Lecture Theatres

Thursday 8th January 2009

Effect of a short-term broccoli intervention in volunteers with bladder cancer

Melanie Dunk, Robert Mills, Richard Ball and Richard Mithen.

Award given for best non-clinical urological scientific research presentation.

Chapter One

General Introduction

Chapter 1: General Introduction

1.1 The Bladder

The bladder is a hollow, muscular, and distensible organ that sits in the pelvis. Wastes are filtered out of the blood stream by the kidneys and enter the bladder via the ureters, figure 1.1, collecting in the bladder as urine. From the bladder, urine passes out of the body through a hollow tube called the urethra (van der Horst and Junemann 2004), figure 1.1.

The bladder has an outer wall of muscle and connective tissue. Nerves in the wall monitor bladder filling and signal the body to urinate. Like other organs the bladder's inner surface is lined with epithelial cells (van der Horst and Junemann 2004).

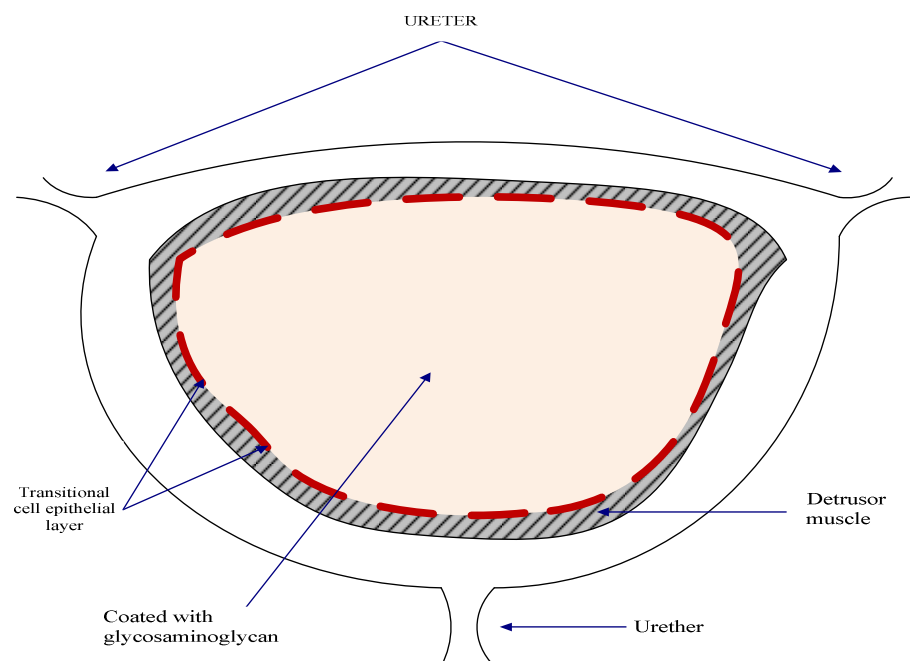


Figure 1.1 Anatomy of the Bladder

As illustrated in figure 1.1 the inner wall of the bladder is coated with a substance called glycosaminoglycan (Parsons 1986) (GAG). This is a mucus-like layer that help to protects the epithelial cells from acids and toxins in the urine and

has an antibacterial defence mechanism that operates by resisting bacterial adherence and infection (Parsons, Stauffer *et al.* 1988). The bladder and the urinary tract has generally been thought to be solely a storage and transit vehicle for urine made by the kidney, however recent *in-vivo* animal studies suggest that reabsorption of urea and other urine constituents also occur across the bladder and urinary tract epithelia (Spector, Yang *et al.* 2007).

1.1.1 Layers of the bladder

The bladder consists of four structurally distinct tissue layers:

Epithelium: The epithelium shown in figure 1.2, which lines the bladder and is in contact with the urine, is referred to as transitional cell epithelium. Most bladder



cancers originate from the cells of this transitional epithelium and hence is termed transitional cell carcinoma (TCC) (pathology2.jhu.edu/bladder_cancer 2007).

Figure 1.2 Transitional epithelium also known as urothelium. www.ouhsc.edu/histology.

Lamina propria: Under the epithelium is the lamina propria, a layer of connective tissue and blood vessels. Within the lamina propria, there is a thin and often discontinuous layer of smooth muscle called the muscularis mucosa. This superficial layer of smooth muscle is not to be confused with the true muscular layer of the bladder called the muscularis propria or detrusor muscle, figure 1.1.

Muscularis propria or detrusor muscle: This deep muscle layer consists of thick smooth muscle bundles that form the wall of the bladder. For purposes of staging bladder cancer, the muscularis propria has been divided into a superficial (inner) half and a deep (outer) half, figure 1.1.

Perivesical soft tissue: This outermost layer consists of fat, fibrous tissue and blood vessels. If a tumour reaches this layer, it is considered out to be of the bladder (pathology2.jhu.edu/bladder_cancer 2007).

1.2 Cancer

One in three individuals develop cancer during their lives and each year around 289,000 people are newly diagnosed with cancer. There are many different types of cancer, but as can be seen from figure 1.3 four of them, breast, lung, colorectal and prostate account for over half of all new cases (Statistics 2004). Breast cancer is the most common cancer in the UK despite the fact that it is rare in men. The 20 most commonly diagnosed cancers in the UK are shown in figure 1.3.

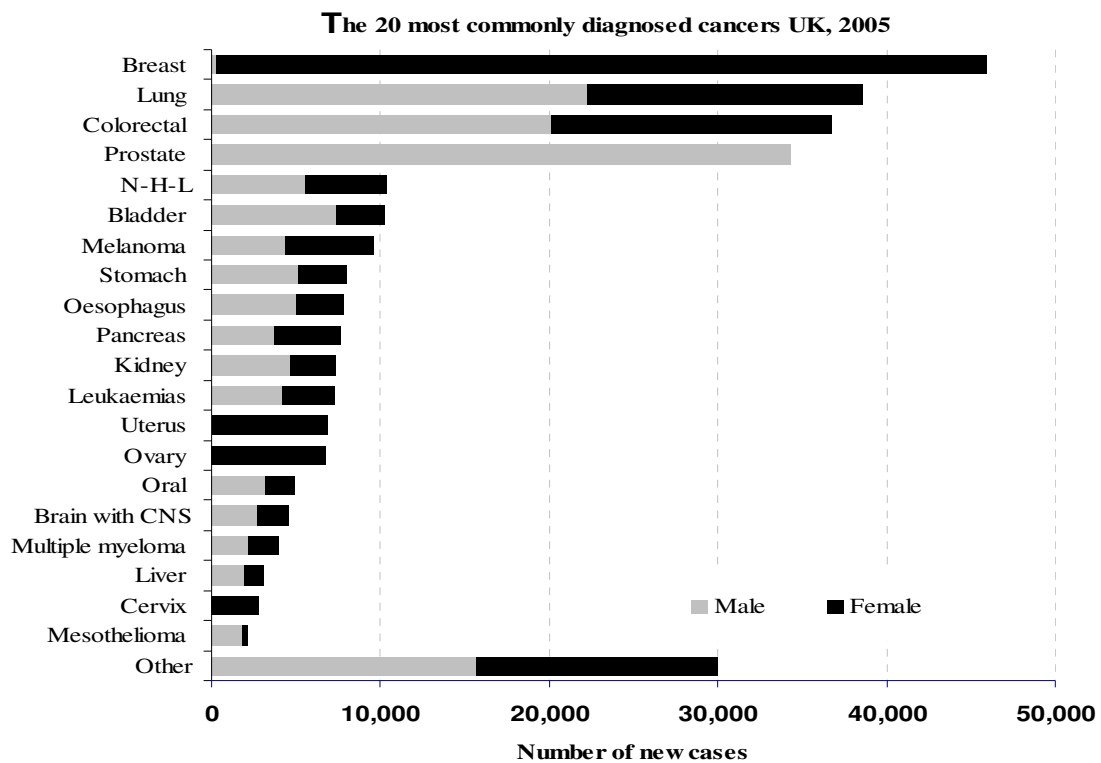


Figure 1.3 The 20 most commonly diagnosed cancers in the UK Adapted from www.cancerresearchuk.org

Although a wide variety of human diseases arise due to defects in gene regulation, the human disease that exhibits the most extensive modifications of gene expression is cancer (Gerdes 2002). Mutations affecting transcriptional and post transcriptional gene regulation may give rise to an abnormal cell. An isolated abnormal cell that does not proliferate more than its neighbours does no significant damage; but if its proliferation is out of control it will give rise to a neoplasm. If the neoplastic cells remains clustered together in a single mass the tumour is said to be benign. A tumour is classed as cancer only if it is malignant, that is if the cells have the ability to invade surrounding tissue (Gerdes 2002). Cancers are classified according to the tissue and cell from which they arise. Cancers arising from epithelial cells are termed carcinomas, and account for 90% of human cancers. This is perhaps

because epithelial cells line virtually every organ in the body and most of the cell proliferation in the body occurs in the epithelia; or perhaps because the epithelial tissues are most frequently exposed to the various forms of physical and chemical damage that facilitate the development of cancer.

This particularly applies to bladder cancer, as there is a constant assault on the bladder tissue from the waste and toxins contained in the urine. Most cancers have characteristics that reflect their origin, such as specific cell type, location in the body and structure of the tumour, and are often named in accordance. For example transitional cell carcinomas of the bladder are termed as such because they arise in the transitional cells of the bladder. Transitional epithelia are multi-layered cells that can contract and expand. These cells are predominately found in the urinary tract, especially around the bladder. They are also found in the ureters and superior urethra. These cells are cuboidal when the organ is not stretched. When the bladder fills up these cells are stretched and they look squamous, flat and irregular. They have the ability to change from cuboidal like epithelium to squamous like epithelium so as to accommodate fluctuations of volume of the liquid in the organ.

1.2.1 Invasive and metastatic cancer

The mechanisms facilitating progression from benign to invasive, and finally to metastatic carcinoma, remain largely elusive. Extraordinary demands are placed on epithelium-derived carcinoma cells to successfully metastasise, including separation from the epithelial collective, degradation of the surrounding matrix, migration and invasion through the basement membrane intravasation and survival in the circulation, extravasation at a secondary site, survival as a micrometastasis, and finally growth into overt metastases (Chaffer, Brennan *et al.* 2006; Przybylo and Radisky 2007). To

successfully complete these complex steps, cancer cells exhibit both mesenchymal- and epithelial-like properties at different times, or even at the same time (Chaffer, Brennan *et al.* 2006; Hugo, Ackland *et al.* 2007). The loss of epithelial characteristics and the acquisition of mesenchymal like migratory phenotype are crucial to the development of invasive carcinoma and metastasis (Lee, Dedhar *et al.* 2006; Chaffer, Thompson *et al.* 2007). Epithelial-mesenchymal transition (EMT) is a programmed development of cells characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell mobility. Initiation of metastasis involves invasion, which has many phenotypic similarities to EMT, including a loss of cell-cell adhesion mediated by E-cadherin repression and an increase in cell mobility. In both normal and pathological EMT this cell adhesion protein E-cadherin is critical to the differentiation and maintenance of the epithelial phenotype and loss of this protein is rate-limited for EMT (Arima, Inoue *et al.* 2008). It is now widely accepted that this developmental pathway is exploited in various disease states, including cancer progression. By adopting developmental EMT pathways, sessile epithelial carcinoma cells are transformed into cells with migratory and invasive capability, metastasis potential and resistance to chemotherapy (Thompson and Williams 2008; Wallerand, Robert *et al.* 2009) and anoikis, a form of apoptosis which is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (ECM) (Chaffer, Thompson *et al.* 2007). Several pathways, such as TGFbeta and RTK/Ras signalling, Ras-MAPK and Wnt, Notch-, Hedgehog- and NF-kappaB-dependent pathways, have been shown to be involved in regulation of EMT (Huber, Kraut *et al.* 2005; Christofori 2006). In particular, Ras-MAPK has been shown to activate two related transcription factors known as Snail and Slug (Przybylo and Radisky 2007; Arima, Inoue *et al.* 2008). Both of these proteins are transcriptional repressors of E-cadherin

and their expression induces EMT (Huber, Kraut et al. 2005; Przybylo and Radisky 2007). Snail is a transcription factor long studied for its role in physiological EMT but which is increasingly recognised as a factor involved in tumour progression and malignancy. Twist, another transcription factor, has also been shown to induce EMT, and is also implicated in the regulation of metastasis (Wallerand, Robert et al. 2009). Interestingly, urine analysis showed that the twist gene was frequently methylated in urine samples collected from bladder cancer patients, including those with early-stage and low-grade disease (Renard, Joniau et al. 2009). The study suggests a sensitive and specific, noninvasive approach for detecting primary bladder cancer and surveillance strategies for bladder cancer recurrence which is significantly better than that of cytology, refer to section 1.4.1, (Kim and Kim 2009).

1.2.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a family of more than 28 enzymes that were initially identified on the basis of their ability to cleave most elements of the ECM but have subsequently been found in many physiological processes that require tissue remodelling and have also been shown to be up regulated in many tumours (Orlichenko and Radisky 2008), where they stimulate tumour growth, invasion, and metastasis. These enzymes digest components of the ECM, as well as cell surface receptors for soluble factors and junction proteins involved in cell-cell and cell-ECM interactions (Przybylo and Radisky 2007).

Matrix metalloproteinases (MMPs) play an important role in tissue remodelling associated with various physiological and pathological processes such as cell proliferation, cell migration, cell differentiation, angiogenesis, morphogenesis, apoptosis, tissue repair, cirrhosis, arthritis and metastasis. The gelatinases (MMP-2

and MMP-9) are thought to be the most important in the metastatic process. MMP-2 and 9 have the ability to degrade type IV collagen, which is one of the major components of the basement membrane (de Vicente, Fresno et al. 2005; Shah, Shukla et al. 2009). They play a key role during invasion and metastasising of malignant cells and they have been shown to be associated with invasive phenotypes and poor prognosis in several tumours (Hazar, Polat et al. 2004; Li, Cao et al. 2004; Aref, Osman et al. 2007; Shah, Shukla et al. 2009), including bladder carcinoma (Xu, Hou et al. 2002), where the level of the MMP-2 mRNA was shown to be proportional to TCC grading and staging in human TCC. Contrary to these results, another study showed that MMP-2 over expression correlated with bladder cancer stage but not with grade (Vasala, Paakko et al. 2003).

There are many MMPs which are classified in to several groups. The most studied are the collagenases, the gelatinases, the stromelysins, and the membrane type MMPs (MT-MMPs). Apart from the six membrane types all other MMPs are secreted enzymes. The MMPs are initially synthesized as inactive zymogens with a pro-peptide domain that must be removed before the enzyme is active. The pro-peptide domain is part of the “cysteine switch”. These enzymes are inhibited by specific endogenous tissue inhibitor of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. In a recent study, serum levels of the inactive enzyme proMMP-2, the active enzyme MMP-2 and the inhibitor TIMP-2 were assessed in 84 patients suffering from bladder carcinoma. The results indicated that low circulating proMMP-2 and TIMP-2 levels significantly correlated with poor prognosis (Vasala, Kuvaja et al. 2008). The 5-year disease-specific survival rate was 46% in patients with high levels of proMMP-2 versus 23% in patients with low proMMP-2 levels ($p = 0.011$). Presumably, this is because the

high levels of proMMP-2 indicates the circulation of the inactive form as opposed to the active form of MMP-2 and the high level of TIMP-2 indicates a greater inhibitory effect of the MMP-2. They conclude by suggesting the results indicate that high levels of circulating proMMP-2 and TIMP-2 s are both associated with a better clinical course and that total proMMP-2 may be an independent prognostic marker of bladder cancer progression (Vasala, Kuvaja et al. 2008). MMP-2 and 9 activity in the urine of patients with bladder cancer has also been studied. MMP-2 and 9 activities were verified in the urine of 25 patients with bladder cancer; ten patients with Ta-T1, eight patients with T3 and seven patients with T4 TCC (refer to 1.5.2 for explanation of tumour grades). The urine of healthy volunteers, with no evidence of disease, were used as controls. The majority of cancerous urine samples showed MMP-9 lytic activity but only a few contained MMP-2 and in the healthy subjects only traces of MMP were detected. Moreover, MMP-9 content was enhanced in the urine from patients with high-grade and advanced-stage bladder tumours (T3 and T4 respectively) (Di Carlo, Terracciano et al. 2006).

1.3 Epidemiology of Bladder Cancer

Transitional cell carcinoma is the most common malignancy of the urinary bladder, urethra and renal pelvis with in excess of 10,000 new cases occurring each year in the UK. Incidences and mortality rates of bladder cancer vary about 10-fold worldwide, with the highest rates found in North America and Europe (Engel, Taioli *et al.* 2002; Yancik 2005). However differences between countries are caused by the differences in registration or reporting of low grade tumours (Kirkali, Chan *et al.* 2005), which are recorded as malignant by some cancer registries and as benign by others. This makes comparison between counties very difficult.

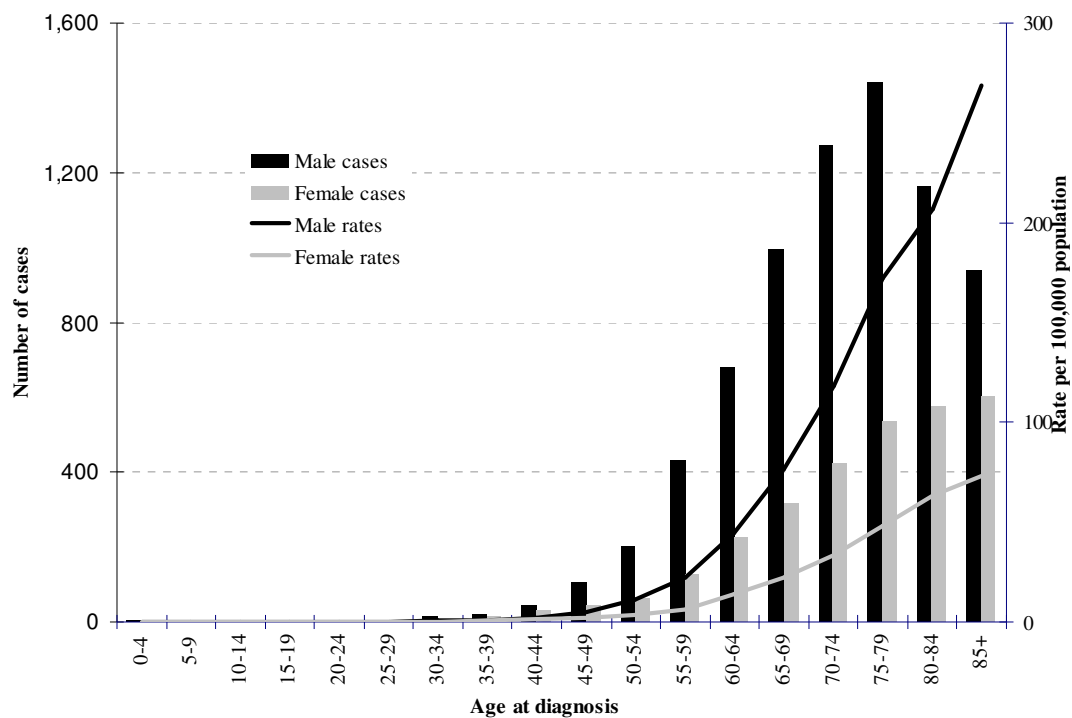


Figure 1.4 The number of new bladder cancer cases and age specific incidence rates by sex. Adapted from www.cancerresearchuk.org

Bladder cancer is the fourth most common cancer in men and the ninth most common cancer in women. In the UK more than 7000 new cases were diagnosed in men in 2005. This compares to nearly 3,000 cases in females - giving a male: female ratio of 2.5:1.0. As can be seen from figure 1.4, few cases of bladder cancer occur under the age of 50, but thereafter the rates rise with age to reach a peak in the oldest age groups (Statistics 2004). The surveys of cancer incidences and mortality suggest that parous women have a lower risk of bladder cancer than nulliparous women, probably because of hormones (Cantor, Lynch *et al.* 1992).

	England	Wales	Scotland	N.Ireland	UK
Cases					
Males	6091	619	468	132	7310
Females	2403	260	247	58	2968
Persons	8494	879	715	190	10278

Table 1.1 Number of new cases and rates of bladder cancer in the UK (2005). Adapted from www.cancerresearchuk.org

Although it can be seen from figure 1.4 and table 1.1 that bladder cancer is more common in males than in females, it has been suggested that the stage-adjusted survival of bladder cancer women is worse than in men (Mungan, Aben *et al.* 2000). It can be seen from the above graph that approximately 88% of male deaths and 90% of female deaths occur after the age of 65 years and as expected, for both men and women bladder cancer survival rates decrease with age. Norfolk has one of the largest admission rates for bladder cancer compared with other local health authorities in England, although this is probably due to the high ageing populations in Norfolk (Statistics 2004).

1.4 Diagnosis of bladder cancer

There are several methods for the detection of bladder cancer; by intravenous pyelogram (IVP), computed tomography (CT) and magnetic resonance imaging (MRI), however there is currently no inclusive test for the detection of bladder cancer and as such involves a collaboration of techniques and expertise. The most common procedure employed is cytology and cystoscopy examination, but both methods have their limitations (www.cancer.gov/cancertopics 2007). Cystoscopy is increasingly being demonstrated to miss both tumour *in-situ* (Tis) and superficial bladder cancer and urine cytology has high specificity, but has low sensitivity and significant variability in performance (Lotan and Roehrborn 2003). Both preparation and the experience of the investigator can affect performance of this test.

1.4.1 Cytology

A preliminary diagnosis may be made by using the oldest urine-based biomarker, cytology. Individual cells from the urine are microscopically looked at, by thinly spreading them onto a glass microscopic slide and staining, see figures 1.6 and 1.7. Although urine cytology has high specificity and high positive predictive value for high grade bladder cancers, it has low sensitivity, particularly for low-grade cancers; however it does help to establish a picture to aid diagnosis. More recent tests, such as the bladder tumour antigen (BTA) tests, the BTA stat (Polymedco, Redmond, WA) and BTA-TRAK (Polymedco); ImmunoCyt (Diagnocure, Quebec City, Quebec, Canada); the urinary nuclear matrix protein (NMP) test, NMP-22 BladderChek (Matritech, Newton, MA); and UroVysion (Abbott Molecular/Abbott Laboratories Inc., Des Plaines, IL) may have higher sensitivity and better negative predictive value.

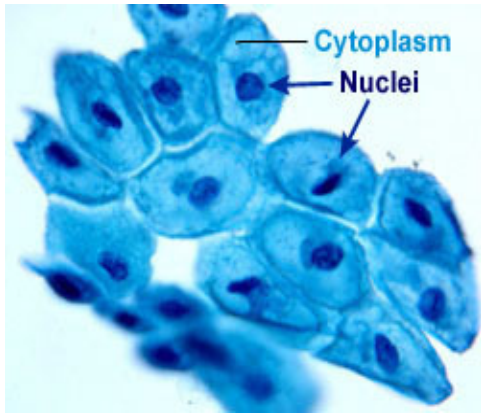


Figure 1.6 Normal urothelial cells: Normal urothelial cells have a differentiated and uniform appearance with abundant cytoplasm and small nuclei (pathology2.jhu.edu/bladder_cancer 2007).

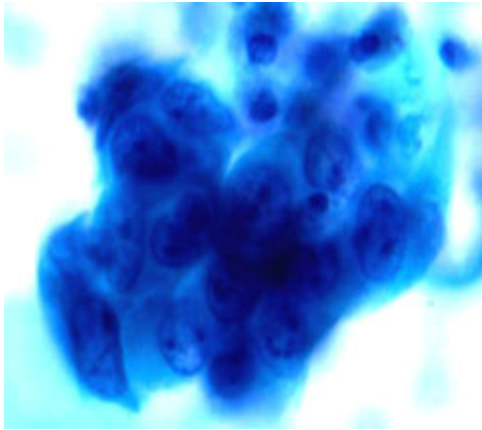


Figure 1.7 High grade bladder cancer: Bladder cancer cells are undifferentiated and enlarged with large dark nuclei (pathology2.jhu.edu/bladder_cancer 2007).

1.4.2 Flexible cystoscopy

A cystoscopy is an examination undertaken by a consultant urologist or specialist registrar of the inside of the bladder using a thin, flexible tube like telescope called a cystoscope. This instrument is carefully passed up the urethra and into the bladder. Cystoscopy is routinely performed as an outpatient or day case procedure, with no overnight stay in hospital. It is usually performed under local anaesthetic, which is given in the form of a gel placed into the opening of the urethra. Once the end of the cystoscope is in the bladder, sterile water is passed through it to fill the bladder up and make the whole of the lining visible. A tiny light and lens on the cystoscope enables the inspection of the bladder lining for any abnormal growths or disease, refer to figure 1.8. Cystoscopy remains the gold standard for the detection of bladder cancer. It enables both detection and minimal invasive treatment of disease that has not invaded deeply into the bladder wall. Nevertheless, cystoscopy has its limitations. Although it is generally recognized that cystoscopy often fails to detect carcinoma *in-situ*, more recent data with fluorescence demonstrate that conventional (white light) cystoscopy can also fail to detect papillary tumours (Jichlinski, Guillou *et al.* 2003; Kausch, Doehn *et al.* 2006; Jocham, Stepp *et al.* 2007) .

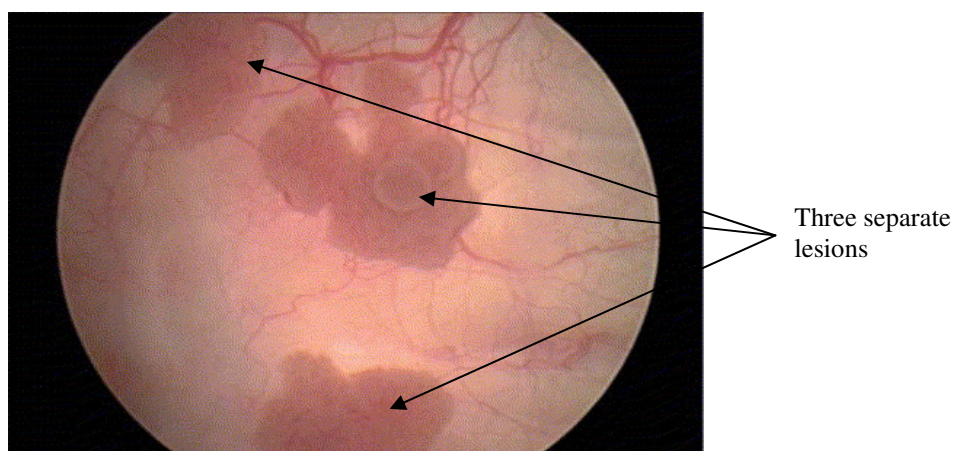


Figure 1.8 Shows a cystoscopy of three separate lesions of typical superficial transitional cell carcinoma with feeding blood vessels and papillary morphology (Brinkman, Buntinx *et al.* 2006).

There is inadequate evidence to determine whether routine screening for bladder and other urothelial cancers would have any impact on mortality. Although due to the high rate of bladder cancer recurrence, once an individual has suffered from bladder cancer they will attend regular surveillance cystoscopy. Patients are generally seen by an urologist every three months for the first one to two years, then every six months for another one to two years, and then once a year thereafter. At each visit, the urologist will perform cystoscopy with or without biopsy and obtain urine for urine cytology (Oosterlinck 2004).

1.4.3 Histological examination of bladder biopsy tissue

The diagnosis of bladder cancer is based on histological examination of cells from the bladder biopsy sample. It can be identified whether the tumour is benign or malignant and the type of tumour. This is essential because tumours of different types behave very differently and require different treatment regimens.

1.4.4 Norfolk and Norwich University Hospital Tissue bank and pathology/histopathology department

Norfolk and Norwich University Hospital NHS Trust is a large acute trust that provides a range of services to a population of approximately 500,000. The Norfolk and Norwich University Hospital has approximately 1015 beds and provides secondary and tertiary cancer services. It has cancer centre status for chemotherapy, radiotherapy and specialist oncology surgery. The Trust was given a one star rating in the NHS Performance Ratings 2003/2004, (Healthcare Commission), following a 2 star rating in 2003.

The NNUH histopathology department routinely receive tissue biopsies for the classification and grading of many cancers to aid diagnosis. Also they will routinely receive donations of non transplantable human organs, tissues and donations of surgical residue tissue from living patients undergoing surgical operations. The whole organ can be removed and donated for biomedical research, however more commonly just biopsies are removed along with routine biopsies for diagnosis. The information concerning the use of donated human tissue to be used for biomedical research is approved by the Norfolk Research Ethics Committee and East Norfolk & Waveney Research Governance Committee, which involves scientific investigations conducted within the hospital, university or other research laboratories, into the causes, treatment and prevention of ill-health.

Tissue received by the NNUH histopathology department from the hospital theatres would have been dissected into its constitutive parts of no more than 2cm³ and placed into an appropriate container. A request for the necessary work on these samples would have been requested on the ICE (Integrated Communications Environment) requesting system which is an electronic link between the wards, theatres, GP practices and the hospital pathology laboratories. On receipt of the sample in the pathology department, details of the patient (hospital number, date of birth, full name, tissue type and date of donation) would have been electronically entered into the Labtrack pathology computer system and a unique accession tracking reference number generated for each sample. If the sample was to be stored in the tissue bank then it would as be given a tissue bank number (07TB....). All patient related data is stored on this password protected dedicated database. The Labtrack database holds all haematology, biochemistry, histopathology, cytology and radiography reports for all patients and is accessible only to the medical and scientific

staff of the NNUH pathology department. Both Labtrack and the ICE computer systems are interfaced to the hospitals main patient administration system (PAS), allowing the donated tissue, patient and their results to be tracked and audited within the NNUH. Sections of the biopsy sample would have been either paraffin embedded and stained for histological examination or snap frozen in liquid nitrogen and stored in a -80°C tissue bank freezer. The freezing process would have taken no longer than 1 min per 2cm³ piece. Material can be stored in a -80°C freezer indefinitely.

1.5 Classification of cancer

1.5.1 Classification of tumours based on their tissue of origin

Tumours may be classified not only on their biological behaviour but also on their tissue of origin. Most tumours retain sufficient characteristics to determine the type of tissue from which they derived. Table 1.3 gives the names and definitions of cancers.

Cancer	Definition
Carcinomas	Most cancers (approx 80-90 %) are carcinomas. These start in the epithelial cells that line internal and external body surfaces. The commonest carcinomas are lung cancer, breast cancer and bowel cancer.
Sarcomas	Sarcomas begin in connective tissues of the body, such as muscle, bone, cartilage and fat.
Leukaemia	The leukaemias are cancers of the blood cells that grow in the bone marrow, which are found in the bloodstream.
Lymphomas	Lymphomas develop in the lymph nodes and tissues of the immune system.
Adenomas	Adenomas are tumours (often benign) that begin in glandular tissue, such as the pituitary gland or thyroid.
Blastomas	Childhood cancers often form in the embryonic tissues which remain. Tumours formed here are often named after the blastocytes, for example neuroblastoma, nephroblastoma.
Primary of unknown origin	This is where the tissue or origin cannot be determined

Table 1.2 Classification of tumours based on their tissue of origin. Information taken from Eastern Cancer Registration & Information Centre (ECRiC) Annual Report 2006 - 2007

1.5.2 Classification of bladder cancer

Tumour, node, metastatic (TNM) classification of urinary bladder cancer World Health Organization (WHO)(Oosterlinck, Lobel *et al.* 2002) gives a pathological stage of the tumour.

Superficial

- TX Primary tumour cannot be assessed
- T0 No evidence of primary tumour
- Ta Non-invasive papillary carcinoma
- Tis Carcinoma *in situ* ('flat tumour')
- T1 Tumour invades subepithelial connective tissue

Invasive (Non-superficial)

- T2 Tumour invades muscle
 - T2a Tumour invades superficial muscle
(Inner half)
 - T2b Tumour invades deep muscle (outer half)
- T3 Tumour invades perivesical tissue:
 - T3a Microscopically
 - T3b Macroscopically (extravesical mass)

Tumours are classified by the position of the tumour in the bladder; figure 1.9 shows the position of tumours in relation to the above stages of bladder cancer.

Approximately 90% of bladder cancers are transitional cell carcinomas (TCC) (Oosterlinck 2004). Clinically superficial TCC tumours (Ta, Tis, and T1) account for

approximately 75% to 85% of bladder neoplasms, while the remaining 15% to 25% are invasive (T2, T3 and T4) or metastatic lesions at the time of initial presentation (Oosterlinck, Lobel *et al.* 2002; Oosterlinck, Lobel *et al.* 2002; Oosterlinck 2004; Oosterlinck, Solsona *et al.* 2005; Sanchez-Carbayo and Cordon-Cardo 2007). In a recent study by Vrabie *et al.*, histological and immunohistochemical analysis revealed that 70% of the clinically superficial tumours were carcinomas infiltrating the lamina propria (T1), and almost 22.85% represent with Ta, while only 7.15% of cases presented with Tis (Vrabie, Petrescu *et al.* 2007).

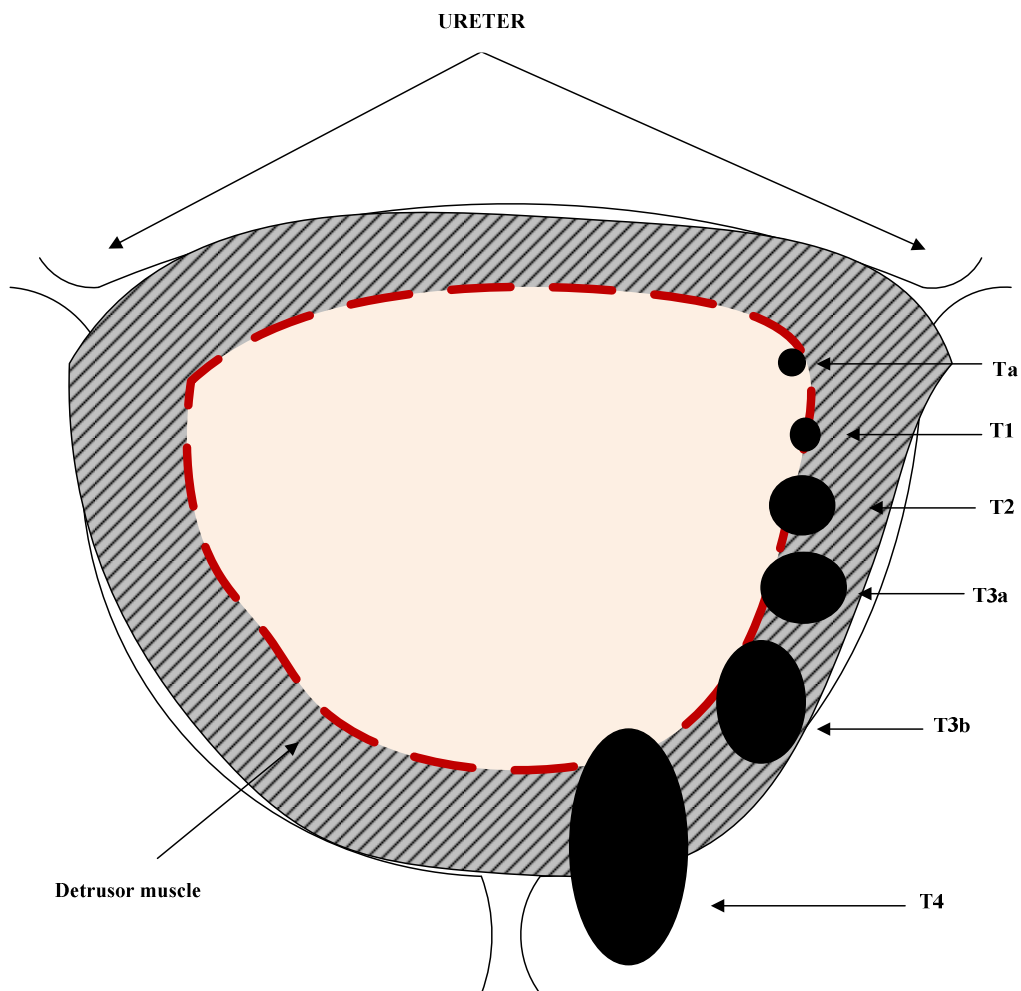


Figure 1.9 Diagram showing the position of tumour in relation to stages of bladder cancer.

1.5.3 Histopathological grading (Oosterlinck, Lobel *et al.* 2002)

Grade is described on a scale of 1-4

GX Grade of differentiation cannot be assessed

G1 Well differentiated

G2 Moderately differentiated

G3-4 Poorly differentiated/undifferentiated

A differentiated cell is one which appears most like the normal cells from the tissue of origin. An undifferentiated cell is one that looks least or less like the normal cells from the tissue of origin.

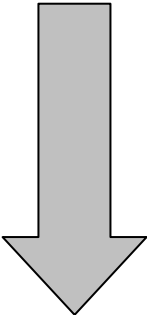
GRADE	% undifferentiated cells	BEST
1	<25	
2	>25 <50	
3	>50% <75%	
4	>75%	
		WORST

Figure 1.10 Diagram adapted from Eastern Cancer Registration & Information Centre (ECRiC) Annual Report 2006 - 2007 representing the significance of grade and percentage of cell undifferentiation in relation to 'best' and 'worst' outcome.

1.5.4 High rate of reoccurrence of bladder cancer

Disease prevalence is high in western nations due to the predominance of the chronic relapsing of bladder cancer that occurs in these countries and this single fact makes bladder cancer one of the most expensive cancers to treat (Lerner S P 2005).

The recurrence rate of treated superficial bladder cancer is very high. As table 1.3 shows, more than 60% of patients first presenting with superficial tumours will have one or more recurrences after initial treatment (Oosterlinck, Lobel *et al.* 2002; Oosterlinck, Lobel *et al.* 2002; Sanchez-Carbayo and Cordon-Cardo 2007), and 40% of these patients will later progress to a higher stage or grade and die of the disease (Oosterlinck, Lobel *et al.* 2002; Sanchez-Carbayo and Cordon-Cardo 2007). Over 50% of patients who first present with high grade TCC tumours will have one or more recurrences after the initial treatment (Oosterlinck, Lobel *et al.* 2002), however many of these patients will re-present with a higher grade of TCC and 25% will die from the disease (Oosterlinck, Lobel *et al.* 2002).

GRADES TO PROGRESSION AND RECURRENCE OF TCC		
	LOW GRADE (TA- T1 G1-G2) (N=42)	HIGH GRADE (T2-T4 G3-G4) (N=62)
RECURRENCE	64.1%	56.4%
ANY STAGE PROGRESSION	10.5%	27.1%
SUBMUCOSAL INVASION	2.6%	8.3%
DETRUSOR MUSCLE INVASION	5.3%	6.3%
METASTASES/DEATH	10.6%	25.0%

Table 1.3 Table represents grades of TCC (high; Ta-T1 G1-G2 and low; T2-T4 G3-G4) and the percentage of recurrence, progression, invasion into the submucosal, invasion into the muscle and metastases/death of TCC. Adapted from a report by the World Health organisation/International society of urological pathologist 2005 (Oosterlinck, Solsona *et al.* 2005).

A recent study conducted to determine the effect of polymorphisms in quinone reductase (NQO1) on bladder cancer disease, course and clinical outcomes showed interesting findings in relation to the recurrence rate and stage progression of the recurrence. A total of 546 patients with bladder cancer were studied over a five year period. Follow up of the patients showed that patients who presented and were treated for TaG1 showed no recurrence of bladder cancer over the 5 year period. Patients presenting with and were treated for TaG2 bladder cancer showed the highest recurrence rate. However, although patients presenting with and treated for TaG3 and T1 bladder cancer were shown to have a lower rate of recurrence than TaG2, they showed a high rate of stage progression and a shorter disease free survival then that of patients suffering with TaG2 bladder tumours. Most cases of T2+ died within the 5 year period (Sanyal, Ryk *et al.* 2007).

1.6 Treatment of Bladder Cancer

1.6.1 Transurethral resection of bladder tumour

Figure 1.11 gives a simplified outline of the possible treatment of TCC; however this will vary from hospital and/or consultants protocols. The standard initial treatment of superficial tumours includes cystoscopy with trans-urethral resection of the bladder tumour (TURBT). A transurethral bladder tumour resection is a procedure in which the tumour is removed from the bladder and does not involve making an incision in the body. The operation is referred to as 'transurethral' because it is performed through the urethra. A larger cystoscope is put into the urethra and up to the bladder. Water is again passed through the tube to fill the bladder allowing visualization and the tumour is shaved down a piece at a time until the entire bladder tumour is removed (Kirkali, Chan *et al.* 2005). Figure 1.11 represents the possible course of action following a cystoscopy investigation.

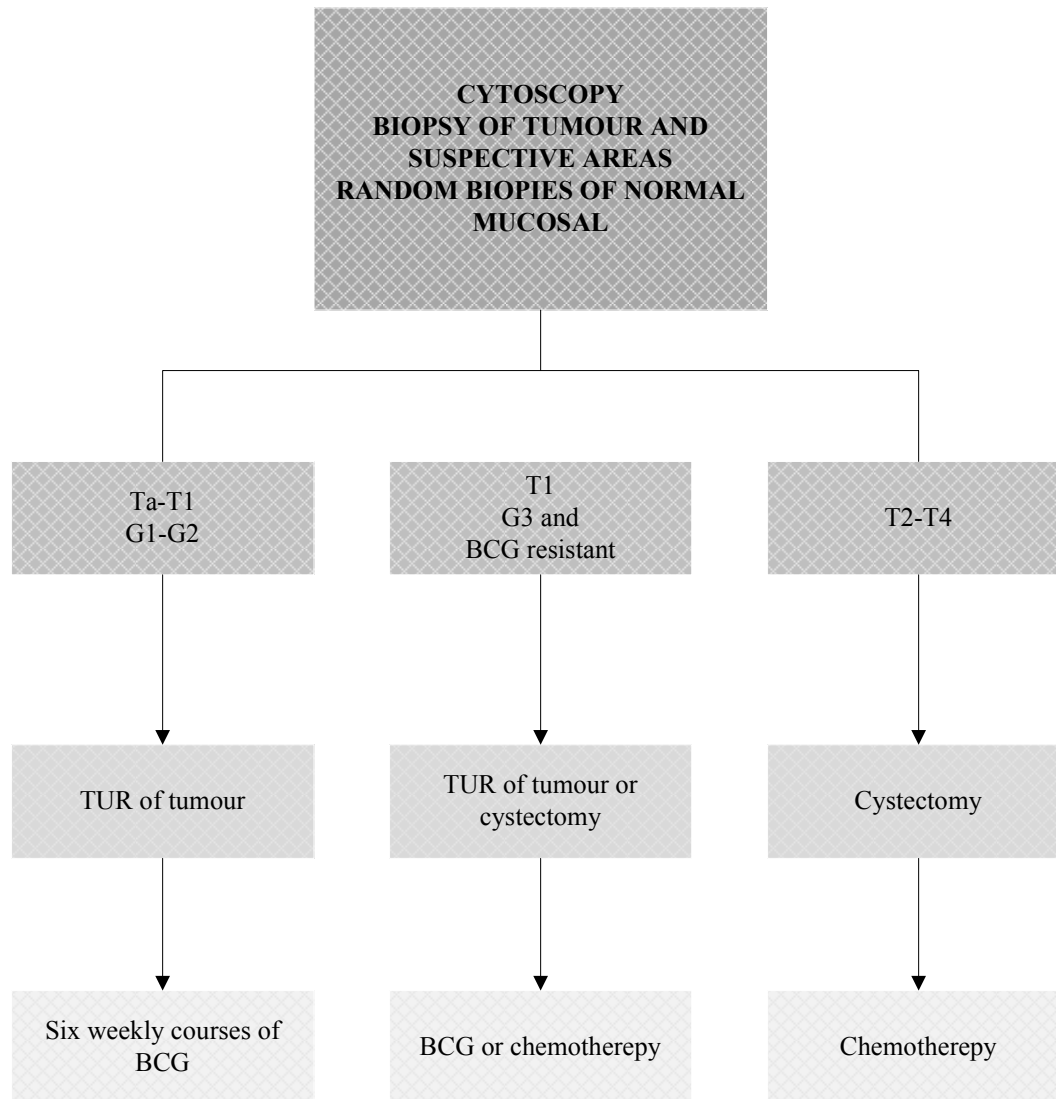


Figure 1.11 The flow diagram shows the possible course of action following a cystoscopy investigation. This will vary depending on the hospital and/or consultants protocols.

1.6.2 Adjuvant intravesical drug therapy

Adjuvant intravesical drug therapy after TURBT is commonly prescribed for patients with tumours that are large, multiple, high grade or superficially invasive, dependent on the hospitals policy. Intravesical therapy consists of drugs placed directly into the bladder through a urethral catheter at the time of resection, in an attempt to minimize the risk of tumour recurrence and progression. The most commonly used intravesical drugs is the immunotherapeutic agent Bacille Calmette-Guerin (BCG) and the

chemotherapy agent Mitomycin C. Maintenance therapy (repeated therapy on a regular basis) with BCG or Mitomycin C, administered intermittently following initial diagnosis and treatment of superficial bladder tumour, decreases the likelihood of recurrence (Schenkman and Lamm 2004). Dr. DL Lamm, a pioneer in the field of immunotherapy for bladder cancer since the early '80's, reports that patients with a solitary tumour that appears to be low grade can be best treated with single instillation of chemotherapy. Single instillations have been demonstrated to be effective with other chemotherapy agents such as Thiotepa, Adriamycin and Epirubicin, in these low grade patients. Also BCG is not necessary unless tumour recurrence becomes a problem for these low grade patients. The patients with CIS, Ta Grade 3 TCC, or lamina propria invasion (T1) are best treated with BCG. BCG has been proven to be superior to chemotherapy (Thiotepa, Adriamycin, Mitomycin, and Epirubicin) in comparative controlled studies and BCG, but not chemotherapy, is found to significantly reduce disease progression. Direct contact of BCG with the urothelium is essential. Fibronectin-binding proteins on the surface of the Mycobacteria bind to receptors on the surface of the urothelial cells, initiating intracellular signalling which results in cell mediated antitumor activity (Lamm 2000). Lamm *et al* analysed six studies comparing surgery alone and combined with BCG immunotherapy. This meta-analysis found a 42% significant difference in recurrence in favour of BCG treatment.

Some patients become resistant to therapy with BCG and currently there is a clinical trial to determine the efficacy and safety of intravesical Mycobacterial Cell Wall-DNA Complex (MCC) in patients with non-muscle invasive TCC.

Mycobacterial cell wall-DNA complex (MCC) is a bifunctional anticancer agent that induces cancer cell apoptosis and stimulates cytokine synthesis by immune cells.

1.6.3 Cystectomy

Cystectomy is indicated when bladder cancer is invasive into the muscle wall of the bladder or when patients with superficial tumours have frequent recurrences that are not responsive to intravesical therapy (Chevallier 1994). A Cystectomy is either partial or complete surgical removal of the bladder and occasionally neighbouring organs, such as the prostate (Oosterlinck, Lobel *et al.* 2002). The benefits of surgically removing the bladder are disease control, eradication of symptoms associated with bladder cancer, and long-term survival. When the bladder is surgically removed, a replacement for the bladder needs to be constructed. The types of bladder reconstruction currently available for patients are ileal conduit, catheterizable pouch and neobladder (van der Meijden, Sylvester *et al.* 2005).

For advanced bladder cancer that has extended beyond the bladder wall, radiation and chemotherapy are treatment options. Local lymph nodes are frequently radiated as part of the therapy to treat any cancer cells which may have spread to the nodes. Current treatment of advanced bladder cancer can involve a combination of radiation and chemotherapy. Chemotherapy before surgery nearly doubles survival rates in patients with locally advanced bladder cancer compared to surgery alone, according to a trial reported at the annual meeting of the American Society of Clinical Oncology in San Francisco on May 14, 2003 (Grossman, Natale *et al.* 2003). The clinical trial showed that in the group receiving chemotherapy before surgery, median survival, that is the point at which half of the patients were still alive, was 6.2 years. In contrast, median survival was 3.6 years in the patients who had surgery without chemotherapy. Although, despite aggressive surgical resection, radiotherapy, and/or chemotherapy, the overall cure of invasive TCC remains, dependent on stage and grade, in the range of 20% to 50% (Sanchez-Carbayo and Cordon-Cardo 2007).

1.7 Risk Factors

1.7.1 Environmental risk factors

There are many aetiological risk factors for the development of bladder cancer.

Aromatic amines were the first to be recognised in the 1970's. Cigarette smoking, which is related to socio-economic deprivation, is by far the most relevant risk factor, tripling the risk of developing bladder cancer and showing a clear dose-response relationship (Golka, Rettenmeier *et al.* 2006). Workers in printing, iron and aluminum processing, industrial painting, gas and tar manufacturing industries have also been shown to be at risk (Ohno and Aoki 1980; Golka, Seidel *et al.* 2005; Pelucchi, Bosetti *et al.* 2006). Being exposed to certain substances, such as soot from coal, or chemicals used to make rubber, certain dyes, or textiles have also been linked to bladder cancer risk. This industrial occupational exposure may account for the higher incidence of bladder cancer in men and the regional variation seen in bladder cancer rates.

Infections and stones in the urinary tract might cause chronic irritation of the bladder epithelium, and thus increase bladder cancer risk (Pelucchi, Bosetti *et al.* 2006).

Other possible risk factors may be (www.cancer.gov/cancertopics 2007):

- Working as a dry cleaner or in places where paper, rope, twine, or clothing is made
- Taking *A. fangchi*, a Chinese herb
- Drinking water that has high levels of arsenic
- Long-term use of urinary catheters
- Past treatment with certain anticancer drugs or radiation therapy to the pelvis
- Having a kidney transplant
- Having the inherited disorder hereditary nonpolyposis colon cancer (HNPCC; Lynch syndrome)

Pelucchi *et al.* suggest that other widely investigated lifestyle habits are probably not associated with risk of developing bladder cancer (e.g. coffee consumption, artificial sweetener use, hair dyes).

1.7.2 Genetic risk factors

There is no clear Mendelian inheritance pattern that can explain the observed two-fold increased risk of bladder cancer in first degree relatives of patients with the disease (Kiemenev 2008). In a recent review by Pelucchi *et al.*, first-degree relatives of bladder cancer patients were shown to have a 50-100% increased relative risk of developing the disease (Pelucchi, Bosetti *et al.* 2006). The association seen in these relatives could simply be caused by a combination of similar environmental exposure to exogenous carcinogens and a large number of susceptibility genes with modest effects (Kiemenev 2008).

A link between genes such as glutathione-S-transferase (GST) and bladder cancer have been made. GSTs are a family of isoenzymes which activate the detoxification of endogenous and exogenous agents and considering the environmental risks, are particularly relevant to bladder cancer risk. GSTs will be discussed in more depth in section 1.8.3. Epidemiological and meta-analysis studies have shown that glutathione-S-transferase mu 1 (GSTM1) null genotype increases the overall risk of bladder cancer (Garcia-Closas, Malats *et al.* 2005). The results of the pooled analysis and meta-analysis study by Engel and Taioli *et al.* suggest that persons with the GSTM1 null genotype are at increased risk of bladder cancer (Engel, Taioli *et al.* 2002). In a more recent study by Cengiz *et al.* it was also shown that the incidence of the GSTM1 null genotype was increased in bladder cancer patients compared to controls, however in this instance they showed no association between the

glutathione-S-transferase theta 1(GSTT1) polymorphism and bladder cancer incidence (Cengiz, Ozaydin *et al.* 2007). Meta-analysis of *N*-acetyltransferase 2 (NAT2) slow acetylation, GSTM1 null genotype and bladder cancer risk by Garcia-Closas *et al.* in 2005 provides compelling evidence for the role of common polymorphisms such as GSTM1 and NAT2 slow acetylators in the aetiology of cancer, figure 1.12. Showing that the GSTM1 null genotype increases the overall risk of bladder cancer and it was stated that although the relative risks are modest, these polymorphisms could account for up to 31% of bladder cancers because of their high prevalence. The overall association for GSTM1 was robust ($p < 0.0001$) and was not modified by smoking status (p for interaction 0.86), unlike the NAT2 which also increased overall risk of bladder cancer, but was stronger for cigarette smokers than for never smokers (p for interaction 0.008) (Garcia-Closas, Malats *et al.* 2005).

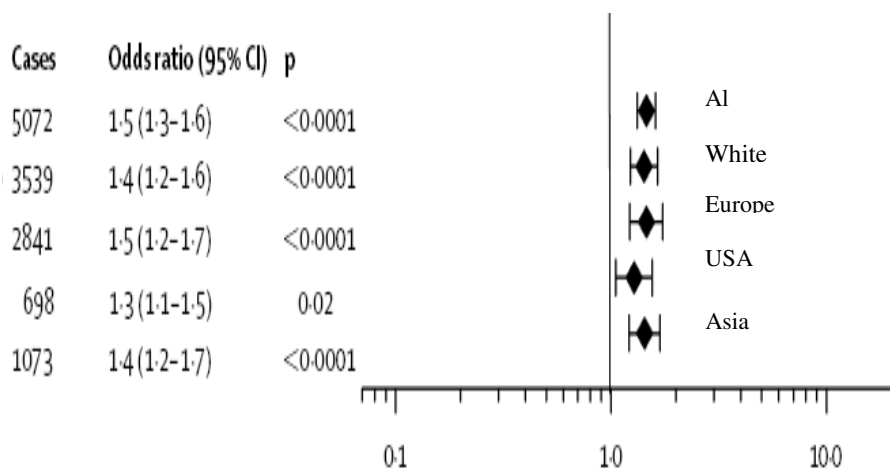


Figure 1.12 The GSTM1 null genotype increases the overall risk of bladder cancer and it was stated that although the relative risks are modest, these polymorphisms could account for up to 31% of bladder cancers because of their high prevalence. (Modified - Garcia-Closas *et al.* Lancet. 2005 Aug 20-26;366(9486):610-12) (Garcia-Closas, Malats *et al.* 2005).

1.8 Diet and Bladder Cancer

Epidemiological studies have shown significant decreases in the incidences of bladder cancer in individuals with a high intake of cruciferous vegetables. Zhao *et al.* epidemiological report showed that ITCs from cruciferous vegetable consumption protect against bladder cancer (Zhao, Lin *et al.* 2007). Data from Michaud *et al.* indicated that many cruciferous vegetables were inversely related to bladder cancer risk, however only the associations for broccoli and cabbage were statistically significant (Michaud, Spiegelman *et al.* 1999). In particular, it is thought that the naturally high intake of cruciferous vegetables in such countries as Japan and Singapore contribute to the low incidence of bladder cancer seen in these parts of the world (Ohno and Aoki 1980; Nakata, Sato *et al.* 1996).

Many studies have highlighted the relationship between a decreased cancer risk and/or cancer progression and dietary intake of various foods such as cruciferous vegetable (Donaldson 2004), soy products (Takahashi, Hursting *et al.* 2006), green tea (Park and Surh 2004; Patel, Hotston *et al.* 2005) and red wine phenolics (Lopez-Velez, Martinez-Martinez *et al.* 2003; Kaur, Roberti *et al.* 2007). Diet may have a greater impact on bladder carcinogenesis compared to other cancers, as many compounds contained in foods and their metabolites are ultimately stored in the bladder for periods of time at high concentrations before excreting through the urinary tract. Cruciferous vegetables such as broccoli, cabbage and Brussels sprouts contain glucosinolates (GLS) (Mithen 2001), a class of organic compounds which are responsible for the bitter or sharp taste of many common crucifers such as mustard, horseradish, and cabbage. GLS often attain millimolar levels inside cells, which makes them one of the most highly concentrated class of intracellular antioxidant (LC 1998). These GLS compounds are hydrolyzed to form bio- active isothiocyanates (ITCs), figure 1.13. Glucoraphanin

accounts for 34%-60% of glucosinolates in broccoli and is converted to the ITC sulforaphane (Joseph, Moysich *et al.* 2004) and glucoiberin is converted to the ITC iberin, figure 1.13 & table 1.4.

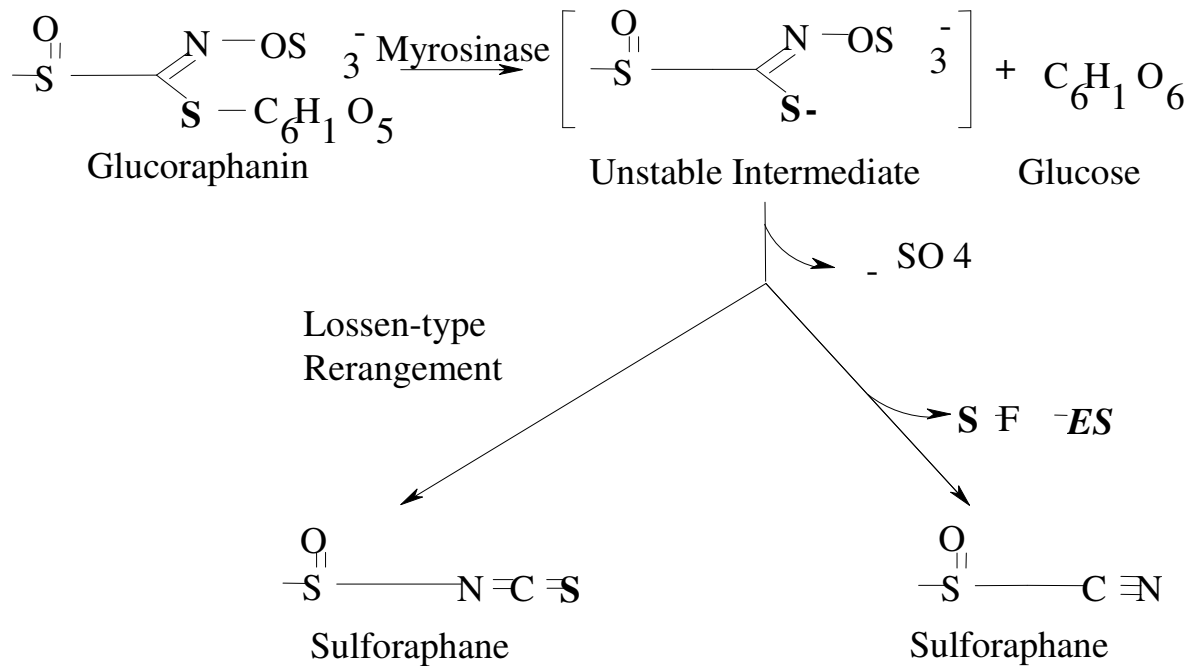


Figure 1.13 Biochemical pathway of glucoraphanin hydrolysis to bio-active sulforaphane.

The primary route of *in-vivo* metabolism of ITCs is by the mercapturic pathway, a major pathway for elimination of many xenobiotics. The glucosinolate, glucoraphanin is sequestered within the intact vegetable tissue, figure 1.13. When the plant tissue is disrupted by physical damage e.g chewing, myrosinase (thioglucoside glucohydrolase) cleaves the thio-glucose bond giving rise to the unstable intermediate; thiohydroximate O-sulfonate. This unstable intermediate undergoes further re-arrangement to form isothiocyanates (figure 1.13 showing sulforaphane). Most frequently, the unstable intermediate undergoes a lossen-type rearrangement to produce an isothiocyanate (Wittstock and Halkier 2002). During cooking of brassica

the glucosinolate-myrosinase system may be modified as a result of inactivation of plant myrosinase, loss of enzymic cofactors such as epithiospecifier protein, thermal breakdown and/or leaching of glucosinolates and their metabolites or volatilisation of metabolites.

Cooking of brassica affects the site of release of breakdown products of glucosinolates, which appear to be in the upper gastrointestinal tract following raw brassica consumption containing active myrosinase (Rungapamestry, Duncan *et al.* 2007). After consumption of cooked Brassica, devoid of plant myrosinase, glucosinolates are hydrolysed in the colon under the action of the resident microflora (Rungapamestry, Duncan *et al.* 2006). Cooking by steaming, microwaving and stir-fry has been shown not to result in a significant loss of glucosinolates and myrosinase activity is effectively lost after 2 min of microwave cooking and after 7 min of steaming (Rungapamestry, Duncan *et al.* 2006). Whereas boiling showed significant losses by leaching into cooking water (Song and Thornalley 2007). Most of the loss of the glucosinolates (~90%) can be detected in the cooking water. Therefore increased bioavailability of dietary isothiocyanates may be achieved by avoiding boiling of vegetables (Song and Thornalley 2007).

Glucosinolates	Hydrolyzed products	Trivial names
Glucoiberin	3(methylsulfinyl)-propane	Iberin (IB)
Glucoraphanin	4-(methylsulfinyl)-butane	Sulforaphane (SF)

Table 1.4 Major aliphatic glucosinolates and their hydrolysis products in broccoli.

Results from some case control studies involving cruciferous vegetable intake and cancers such as prostate, and ovarian have reported confounded results. Keum *et*

al. suggest this may be due to the remote location of the organs in the body, resulting in concentration of ITCs within these organs being at a very low level (Keum, Jeong *et al.* 2004). This may not be the case with bladder cancer, where the ITCs and ITC metabolites will be at comparably higher concentration and thus may exerting greater effects.

1.8.1 Isothiocyanates and the Bladder

Cruciferous vegetables high in isothiocyanates such as sulforaphane (4-methylsulphinyl-butylisothiocyanate) and iberin (3-methylsulphinylpropyl isothiocyanate) have been demonstrated to inhibit carcinogenesis by epidemiological studies and in a variety of rodent organs and cell models (Zhang Y 1994; Zhang, Munday *et al.* 2006), including rat bladder (Zhang, Munday *et al.* 2006). The effects of the ITC sulforaphane (SF) and its metabolite *N*-acetylcysteine conjugate have been extensively studied. Iberin (IB) has been far less studied and I am unaware of any work looking at its effects on bladder cancer.

Cruciferous vegetables such as broccoli contain high concentrations of the glucosinolate glucoraphanin, which yield SF when hydrolyzed by the plant enzyme myrosinase. The effect of SF, which occurs as the precursor glucosinolate in broccoli and other cruciferous vegetables, and its metabolite *N*-acetylcysteine conjugate has been extensively studied. The effect of IB which occurs as the precursor glucosinolate glucoiberin and its metabolite *N*-acetylcysteine conjugate has been far less studied. Table 1.4 lists these major aliphatic glucosinolates and their hydrolysis products found in broccoli. The anticancer activity of IB, SF and other ITCs lies partly in the induction of phase II enzymes of xenobiotic transformation, such as quinone reductase and glutathione *S*-transferase (GST). These ITCs are substrates for phase II enzymes and

can also induce these enzymes, which may afford protection against certain carcinogens and other toxic electrophiles, including reactive oxygen species. Therefore it has been suggested that subjects with GST null genotypes (ie lacking enzyme activity), in particular GSTM1 null genotype, could benefit from greater fruit and vegetable consumption. However a recent large scale case control study indicated a greater significant inverse associations with bladder cancer risk and high intakes of berries, liliaceae vegetables and yellow-orange vegetables among GSTM1 positive individuals compared to GSTM1 null individuals (Garcia-Closas, Garcia-Closas *et al.* 2007). As Garcia-Closas points out the interaction maybe a false positive finding or could act through different mechanisms then previously suggested.

1.8.2 Phase II enzymes

Phase II enzymes are part of the body's defence system against environmental substances and carcinogenic compounds, which can alter these toxic compounds and facilitate their excretion (Tijhuis, Visker *et al.* 2006). Enzyme systems such as, UDP-glucuronosyltransferases (UGT), quinone reductase (NQO1), glutathione s-transferase mu, glutathione s-transferase theta and glutathione s-transferase pi may afford protection against certain carcinogens and other toxic electrophiles, including reactive oxygen species. Data from Munday and Munday also suggests that genetic and associated functional variation in these phase II enzymes, and also polymorphisms in receptors and transcription factors that interact with these compounds, may further contribute to variation in response to cruciferous vegetable intake (Munday and Munday 2004). Genetic polymorphisms of these phase II enzyme may increase susceptibility to oxidative stress, therefore leading to an increase risk of chronic disease, meaning that a higher intake of micronutrients and active plant compounds

may be required to maintain health (Reszka, Wasowicz *et al.* 2006). ITCs have the capacity to not only modulate these phase II enzymes but also to inhibit phase I enzymes which are responsible for the bioactivation of carcinogens (Zhang Y 1994). Munday and Munday studies indicated that various ITCs, especially benzyl, are potent inducers of phase II enzymes in rat bladder tissue (Munday and Munday 2004).

1.8.3 Glutathione S-transferase

Glutathione S-transferases (GST) are a family of related isoenzymes that catalyse the conjugation of reduced GSH to a wide range of electrophilic substances (Mannervik 1985). There are seven distinct classes of soluble and membrane GSTs in humans - alpha, kappa, mu, omega, pi, sigma and zeta. GSTs activate the detoxification of endogenous and exogenous agents. Genetic polymorphism in these genes may change the response of individuals to environmental toxicants.

The genetic polymorphisms of GSTT1, GSTM1 and GSTP1 have been studied extensively in the determination of cancer risks. Some studies have shown a strong relationship between polymorphism of GSTs and cancer risk. Zhang *et al.* recently showed that a broccoli sprout extract rich in several ITCs, particularly SF, was a potent inducer of GST in both rat bladder tissue *in-vivo* and cultured bladder cells *in-vitro* (Zhang, Munday *et al.* 2006). Although the molecular mechanisms of action were not explored in this study, it is thought that GST enzymes are transcriptionally activated through Nrf2-dependent signalling pathway (Keum, Jeong *et al.* 2004; Keum, Jeong *et al.* 2005; Zhang, Munday *et al.* 2006). It was shown that knockdown of Nrf2 in mouse embryonic fibroblasts completely abolished GST induction and markedly attenuated NQO1 induction (Zhang, Munday *et al.* 2006). ITCs have been shown to activate this Nrf2/antioxidant response element (ARE) pathway (Thimmulappa, Mai *et al.* 2002),

figure 1.14, emphasised by Romos-Gomez *et al.* studies which showed that Nrf2 null mice eliminated induction of GST(Ramos-Gomez, Kwak *et al.* 2001).

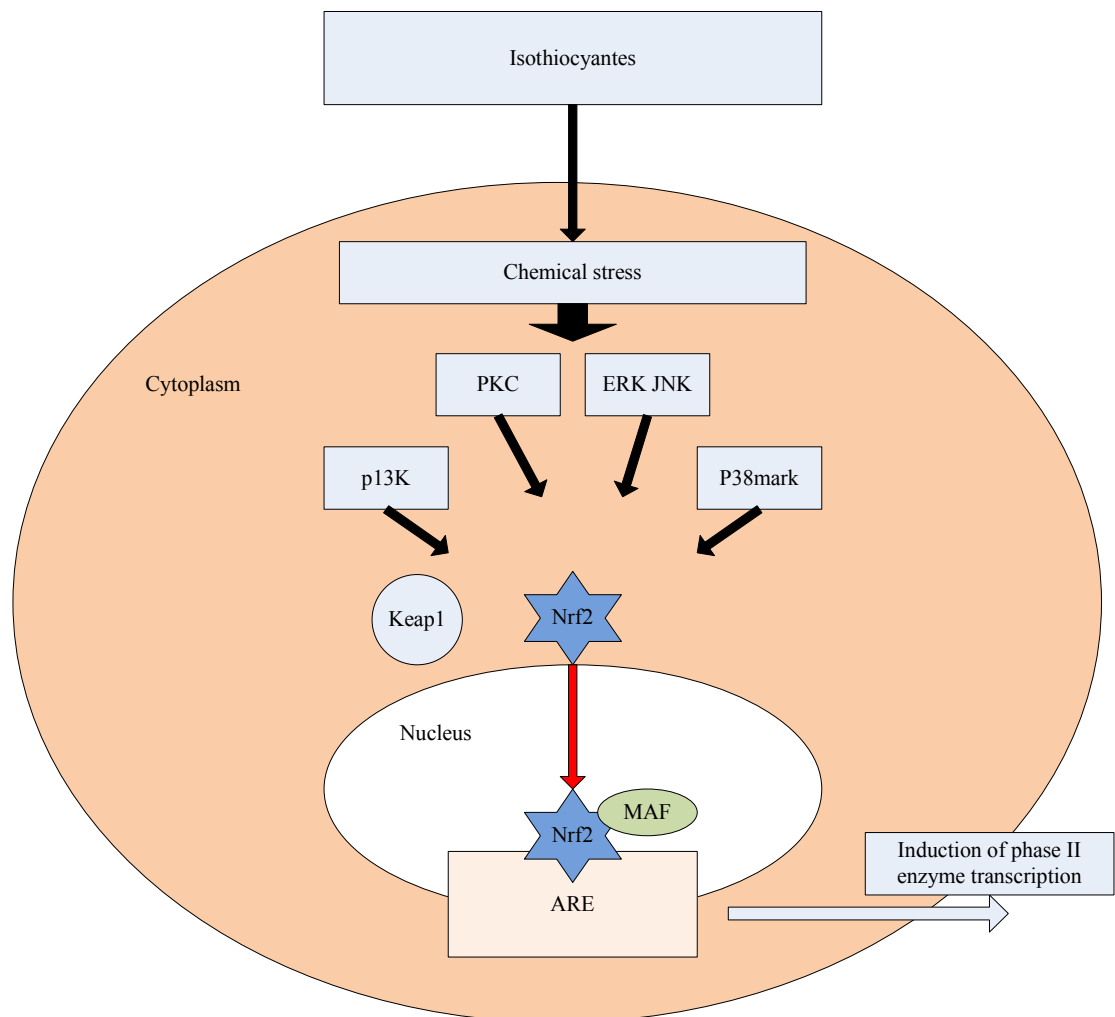


Figure 1.14 Nrf2-dependent signalling pathway shows a model of the signalling pathways involved in activation of Nrf2. Nrf2 is tethered to Keap1 in the cytosol. Upon activation of upstream protein kinases (MAPKs, PI3K, PKC and PERK), and/or direct effect on Keap1, Nrf2 is released from Keap1 and translocates into the nucleus, where Nrf2 binds to the ARE with association of small Maf proteins inside the nucleus leading to induction of phase II enzymes. Isothiocyanates also directly cause the cleavage of disulfide bond between Nrf2 and Keap, adapted from *Keum et al.* (Keum, Jeong *et al.* 2004).

The mu family *GST* (*GSTM*), on chromosome 1p13.3, comprises *GSTM* from 1 to 6. Deletion of the *GSTM1* gene is found in approximately 50% of Caucasians and Asians resulting in a lack of the *GSTM1* enzyme activity and is associated with a risk of cancer and other diseases (Engel, Taioli *et al.* 2002). However it was shown that patients with head and neck carcinoma have a suppressed total GST activity, but this did not correlate with *GSTM1* genotype (Konig-Greger, Riechelmann *et al.* 2004),

therefore susceptibility to cancer maybe counterbalanced by overlapping substrate affinities within the GST family. Also it maybe possible that susceptibility to cancer is affected by a variation in the combination of GST deletions and mutations and thus the resulting enzymes formed.

The most extensively studied GSTs, in relation to bladder cancer, are GSTM1 and GSTT1 null genotypes, the GSTpi (Ile105Val polymorphism/ Ala114Val polymorphism), and the GSTM3 3 base pair deletion genotype. In tumours and adjacent mucosa of patients with TCC, three major cytosolic GST classes, Mu, Pi, and Theta, are expressed (Simic, Mimic-Oka *et al.* 2005). Although the GST enzyme pattern in TCC was similar to that of the corresponding adjacent mucosa, during cancer progression a clear increase in all the GST subtypes expressed was noted (Simic, Mimic-Oka *et al.* 2005). GSTP1-1 and GSTT1-1 activities were demonstrated in all bladder epithelial and TCC samples (24 patients), and GST Mu activity was detectable in 11 of 24 patients. In the tumour specimens, significant upregulation of all expressed GST subtypes was observed.

A previous human intervention study investigating how broccoli consumption interacts with GSTM1 genotype was conducted in a healthy population (Gasper, Al-Janobi *et al.* 2005), but there are no existing studies in populations with bladder cancer.

GSTP1 is frequently over expressed in many cancers, including tumours of the brain, breast, ovary, colon, skin and kidney (Cengiz, Ozaydin *et al.* 2007). However GSTP1 has also been reported to be down regulated in particular cancers such as prostate, pituitary adenomas and breast (Beer, Evans *et al.* 2002) (Yuan, Qian *et al.* 2008), to the extent that GSTP1 hypermethylation and thus the down regulation of GSTP1 in breast cancer is said to be a statistically significant prognostic factor (Arai, Miyoshi *et al.* 2006). In TCC bladder tumour tissue significant up regulation of

GSTP1 expression was detected; showing the mean level of GSTP1 increasing by 2.3-fold compared to the mean level in normal adjacent mucosa from 28 of 30 patients with TCC. Moreover GSTP1 expression in TCC gradually increases with tumour grade and the difference in GSTP1 expression between superficial and muscle-invasive tumours was also significant (Savic-Radojevic, Mimic-Oka *et al.* 2007). A recent study conducted to determine the effect of polymorphisms on the disease course and clinical outcomes of urinary bladder neoplasm showed a lower chance of disease-free survival over a 5 year period, in patients diagnosed with TCC ($\geq T2$) who genotyped with the variant allele than non-carriers of the GSTP1 (I105V) polymorphism (Sanyal, Ryk *et al.* 2007). In contrast, loss of GSTP1 expression by promoter hypermethylation is a signature of prostate cancer (Jeronimo, Varzim *et al.* 2002; Nakayama, Bennett *et al.* 2003; Nakayama, Gonzalgo *et al.* 2004; Rosenbaum, Hoque *et al.* 2005).

Distinct GST theta 1 activity has been demonstrated in normal human bladder epithelium, as well as its pronounced up regulation in TCC. GSTT1-1 activity was demonstrated in 24 patients with TCC, from both tumour tissue and normal adjacent mucosa. During cancer progression the mean GST theta 1 level in TCC was increased 3.6-fold, compared with the mean level in the adjacent mucosa (Simic, Mimic-Oka *et al.* 2005). A study investigating polymorphisms in DNA repair and metabolic genes in bladder cancer reported an association of increased bladder cancer risk with the GSTT1 null genotype (OR 2.54, 95% CI, 1.32-4.98, P 0.003) (Sanyal, Festa *et al.* 2004).

The extent to which any one GST gene contributes to the actual GST activity in mammalian urinary bladders is less clear. Whilst the mouse bladder shows high GSTM1 activity, which occurs at its sharpest increase during early development,

studies suggest that the human bladder GSTM1 subunit even when present, represents a very minor fraction of the GST protein in human bladder (Chico and Listowsky 2005). However the GSTP1 gene has been detected in human bladder post-mortem tissue specimens and comprised at least 80% of the total cytosolic GST subunit pool. The human GSTM2 and GSTM3 subunits were also detected at much lower levels than GSTP1.

Although associations indicate that GST expression may have important protective functions in the bladder, an overexpression of both GSTP1 and GSTM1 in patients with TCC exhibit increased resistance to chemotherapeutic drugs (Hung, Boffetta *et al.* 2004).

1.8.4 Apoptosis and Isothiocyanates

Tang *et al.* showed that an aqueous extract of broccoli sprout inhibited the growth of human bladder carcinoma cells *in-vitro*, showing potent anticancer activity at 7.5 to 30 μ mol/L ITC concentration. Also another recent study showed that sulforaphane suppressed the growth of T24 bladder cancer cells *in-vitro* in a dose-dependent manner (Tang and Zhang 2004).

The activation of the Nrf2/antioxidant response element (ARE) pathway is not the only potential chemopreventative mechanism elicited by ITCs, for example induction of apoptosis and cell cycle perturbations have also been extensively studied in bladder cancer. Sulforaphane has been shown to suppress proliferation of bladder cell growth in culture as well as *in-vivo* by inducing apoptosis (Tang and Zhang 2004). Bax and Bak have been shown to play a critical role in apoptosis induction by sulforaphane, as well as irreversible arrest in the G (2)/M phase of the cell cycle, but the sequence of events leading to cell death appears to be poorly defined, refer to figure 1.15.

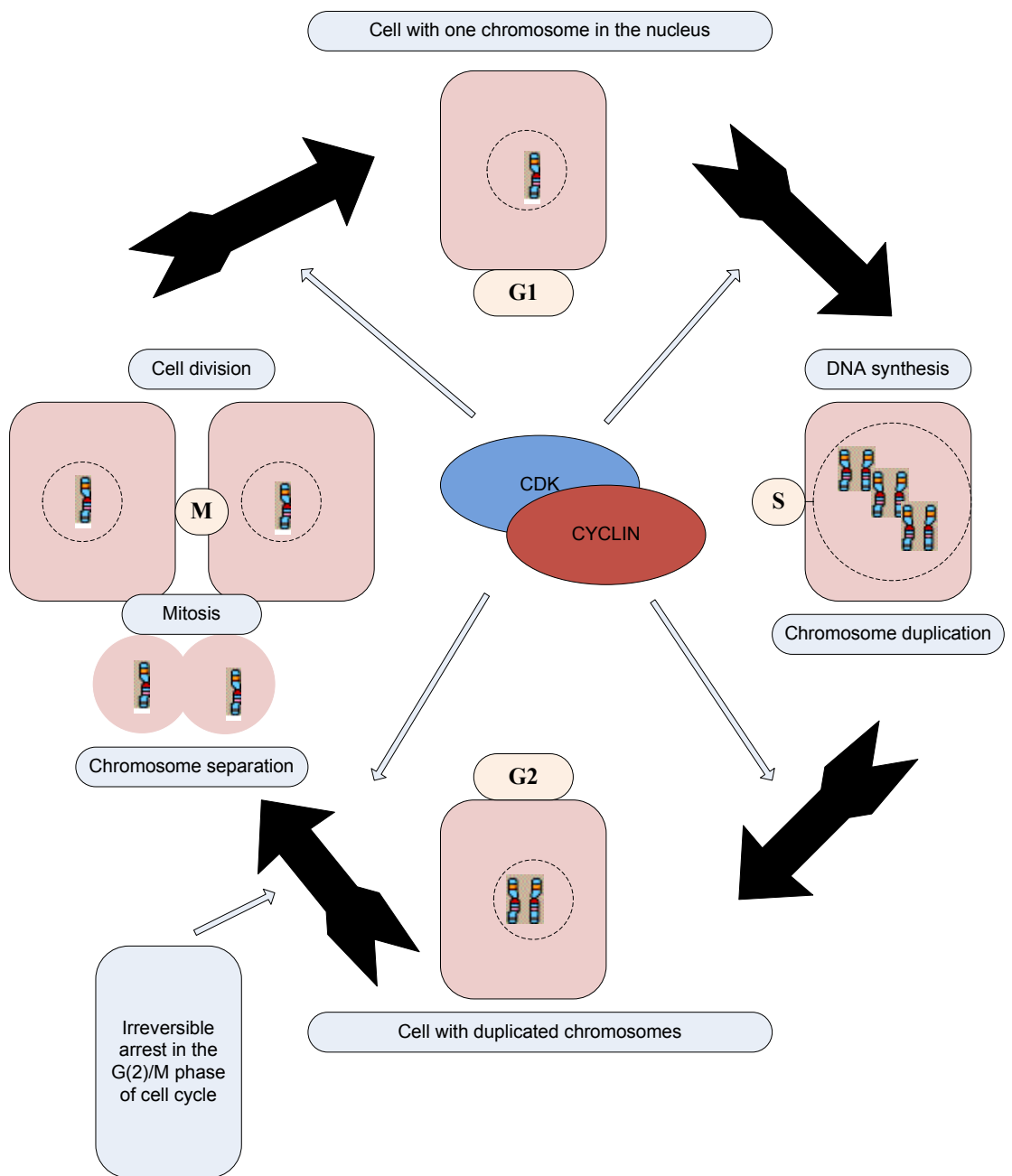


Figure 1.15 The cell cycle, adapted from Leland H. Hartwell, R. Timothy Hunt and Paul M. Nurse diagram.

1.9 Nutritional cell culture studies and the rationale for studying the role of diet and nutrition in relation to bladder carcinogenesis

Identifying bioactive compounds, such as Isothiocyanates, and establishing their health effects are active areas of scientific inquiry. There are exciting prospects that select bioactive nutritional compounds will reduce the risk and/or progression of many diseases, including chronic diseases such as bladder cancer. However many different environmental factors may affect the bladder *in-vivo*; these include diet, medication, stress, age and general life style choices, such as smoking and exercise. Due to the potential number of bioactive compounds consumed in our diet the diversity of biological effects of diet alone on the bladder are vast and as such, cells in culture may behave differently from cells *in-vivo* in many ways . A further problem is the possibility that the cell culture media can catalyse the oxidation of compounds added to them, resulting in apparent artefactual cellular effects which would not be observed *in-vivo*. Furthermore it should be remembered that in this study, cell cultures are treated with concentrations of the pure compound, whereas *in-vivo* exposure to this compound would be accompanied by a diverse mixture of compounds. As such caution must be taken when extrapolating findings from cell culture studies to the possible effects of a single compound *in-vivo*.

It is fair to say that little work has been carried on bladder cancer in relation to diet in comparison to other cancers such as breast and prostate. The underlying rationale for studying the role of diet and nutrition in relation to bladder carcinogenesis is for two main reasons. Firstly, because the impact of nutrition on bladder carcinogenesis is entirely plausible since most substances or metabolites, including carcinogens, are excreted through the urinary tract and stored in the bladder. Previous studies have shown that isothiocyanates are primarily metabolised through the mercapturic acid

pathway, and their metabolites are bioactive and almost exclusively excreted and stored in the bladder adjacent to the epithelial tissue where most bladder cancer arises (Zhang 2004). Secondly, although bladder cancer has a lower incidence than other cancers such as breast and prostate, the rate of recurrence is significantly higher and as such makes bladder cancer the most expensive of all cancers to treat from diagnosis to death (Botteman, Pashos et al. 2003). A systematic review of available literature on the economic burden of bladder cancer in developing countries states that individual patient management is more costly for bladder cancer than for prostate cancer (mean cost per patient - £8349 vs.£7294, respectively) but less is invested in research than for prostate cancer (Botteman, Pashos et al. 2003; Sangar, Ragavan et al. 2005). The annual research fund allocated to bladder and prostate cancer in the year 2001-2002 were 20.56 million UK pounds and 4.62 million pounds respectively (Sangar, Ragavan et al. 2005). The report suggests a need to re-evaluate future strategies for cost effectiveness in cancer research and more interventions aimed at reducing the economic burden of bladder cancer.

1.10 Aims

Transitional cell carcinoma is a common malignancy characterized by a poor clinical outcome when tumours progress from superficial disease into invasive disease. The majority of bladder cancer mortality is associated with invasion into the bladder wall leading to metastatic disease. Diet may have a greater impact on bladder carcinogenesis compared to other cancers, as many compounds contained in foods and their metabolites are ultimately stored in the bladder for periods of time at high concentrations. I sought to define genes or gene profiles which are modulated in invasive disease and to assess the potential effects of selected isothiocyanates on the invasive potential of bladder cancer.

The following aims were set at appropriate stages through out the project:

- 1) To characterise superficial and invasive bladder tissue. To compare global gene expression profiles between superficial and invasive transitional cell carcinoma and define gene expression profiles or selective genes that are characteristic of aggressive clinical behaviour in invasive bladder tumours for further studies *in-vitro*.
- 2) To characterise human bladder cell cultures and assess the effects of sulforaphane and iberin and their *N*-acetylcysteine conjugate on phase II gene expression.
- 3) To use whole genome array analysis to enable the identification of genes which are modulated by a short term dietary intervention in bladder tissue from patients with recurring bladder cancer, and to select genes for further studies *in-vitro*.
- 4) To investigate the effects of sulforaphane, iberin and their conjugates on the cellular motility of the characterised UM-UC-3 cell line and their potential capability to reduce invasion into extracellular matrix.
- 5) To evaluate the differential expression of Tn-C splicing domains in human transitional cell carcinoma of the bladder with respect to the invasive behaviour.

Chapter Two

General Methods and Materials

2.1 Cell Culture

Human bladder cancer UM-UC-3 and RT4 cells were purchased from the European Collection of Cell Cultures (ECACC) and grown in EMEM and McCoy's (Sigma-Aldrich UK) medium respectively, supplemented with 2mM L-glutamine (Sigma-Aldrich UK), 5mg/ml penicillin/streptomycin antibiotics and 1X non essential amino acids (Invitrogen). UM-UC-3 and RT4 cells were also supplemented with 10% (v/v) foetal bovine serum. All cells were maintained in an incubator at 37°C with 5% CO₂ and according to the experimental design cells were grown in 6 well plates, 12 well plates, 6 well transwell or 10cm dishes. See table 2.1 for the recommended volume of media for each culture plates. Iberin and Sulforaphane were purchased from LKT Laboratories. Cell treatments occurred in complete media with less than 0.1% dimethyl sulphoxide (DMSO, Sigma-Aldrich). Each treatment, including control treatment was carried out in three separate wells to produce three biological replicates.

Size of cluster plate/dish/flask	Growth area (cm²)	Relative area	Recommended volume of media
96 well	0.32	0.04 x	200µl
24 well	1.88	0.25 x	500 µl
12 well	3.83	0.50 x	1.0ml
6 well	9.4	1.20 x	2.0ml
24 transwell	0.33	0.04 x	100 µl (insert)
3.5 cm dish	8	1.0 x	2.0ml
6cm dish	21	2.6 x	5.0ml
10cm dish	55	7.0 x	10.0ml
T25 flask	25	3.0 x	5.0ml
T75 flask	75	9.0 x	20.0ml

Table 2.1 Culture plate conversions. Dependent on the experiments requirements the following culture plates were used and the recommended volume of media.

2.1.1 Cell viability Assay WST-1

The Roche WST-1 assay is a colourimetric assay for the quantification of cell proliferation and cell viability. The assay can be used to measure cell proliferation in response to nutrients and also to analysis the potential cytotoxic effects of such compounds at varying doses to find an optimal treatment concentration. The assay uses the principle that tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. Cell viability assays were performed for UM-UC-3 and RT4 cell lines. Cells were seeded in 96 well plates and treated at 70-80% confluence with 0 to 30 μ M of SF, IB or their conjugates in a total volume of media with less than 0.1% DMSO. Six biological replicates were performed. After 24 hours treatment, 10% of WST-1 reagent was added and further incubation at 37°C, 5% CO₂ until a colour reaction occurred. The absorbance was measured at a wavelength of 420-480 nm using a reference value of 600 nm against a blank on the Dynex 96 well microplate reader. The cell viability of treated cells was calculated as a percentage of the control cells. The value at which half the cells will die, the IC₅₀, was calculated using CalcuSyn Statistics package.

2.1.2 Cell counting using a counting Chamber

A counting chamber can determine the number of cells per unit volume of a suspension. In this particular instance a hemocytometer was used, so named as it was originally designed for performing blood cell counts.

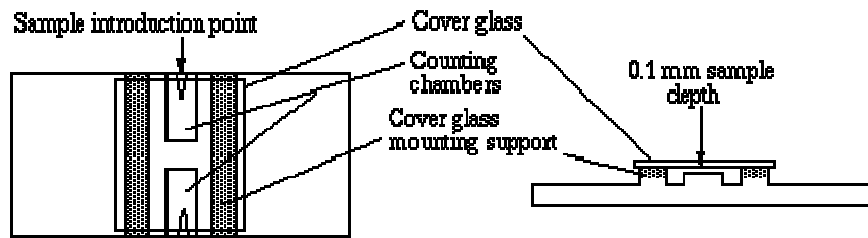


Figure 2.1 Diagram of counting chamber

A coverslip is placed over the counting surface prior to introducing the cell suspension. The suspension is introduced into one of the V-shaped wells with a pipette and the area under the coverslip filled by capillary action, see figure 2.1. The counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. One entire grid on standard hemacytometers with Neubauer rulings can be seen at X40 magnification, as seen in figure 2.2. The main divisions separate the grid into 9 large squares. Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm-cubed. Microscope magnification was set at X100 (10 X ocular and 10X objective) for UM-UC-3 cells. Cells were systematically counted in the four large corner squares so that the total count is approximately 100 cells. For cells that overlapped a ruling, a cell was counted as "in" if it overlapped the top or right ruling, and "out" if it overlapped the bottom or left ruling.

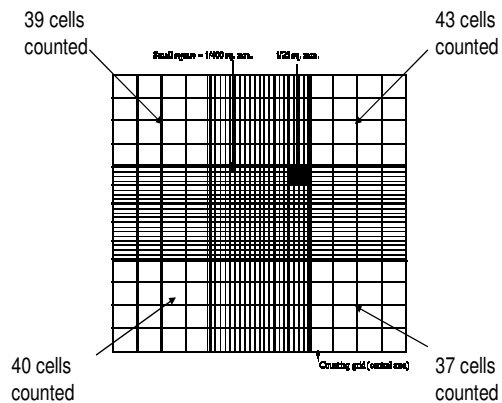


Figure 2.2 Counting grid with Neubauer rulings

Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 10^4 . Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations:

$$\text{Cells/ml} = \text{average cell count per square} \times 10^4;$$

Total cells = cells/ml x the original volume of fluid from which the cell sample was removed.

2.2 RNA stabilization in frozen tissue bank bladder tissue with RNALater-ICE™

RNALater™ ICE stabilizes and protects RNA and thus gene transcription in fresh and frozen tissue specimens, so that the RNA can be extracted at a later stage. The bladder tissue obtained from the NNUH tissue bank was stored at -20 °C in RNALater-ICE, to enable both DNA and RNA extraction.

The RNALater-ICE was chilled for 24 hours at -80 °C. Following this 24 hour period the RNALater-ICE was placed on dry ice. The tissue samples were removed from the -80 °C freezer and the weight of tissue was estimated (weighed precisely following RNALater-ICE incubation) to determine the approximate volume of RNALater-ICE needed to preserve the tissue sample (10 µl of reagent per 1mg of tissue). The appropriate volume of RNALater-ICE was dispensed directly into the tube containing the tissue, assuring the tissue was completely submerged and floated freely in tube. The lid was secured and the sample was placed in the -20 °C freezer. Biopsies were incubated at -20°C overnight before being analysed. Steps were performed as quickly as possible.

2.3 DNA extraction from human bladder cell lines

DNA was extracted using the QIAGEN DNeasy mini kit (Qiagen, UK), according the manufacturer's instructions. Bladder cells were grown in a monolayer to 80% confluency, media was removed and the layer was washed twice with warmed PBS. 200µl of warmed PBS was added to the dish and the cells were removed using a scraper into a 2ml centrifuge tube. 20µl of proteinase K was added to the lysate and a 200µl of AL buffer was added, mixed by vortexing and incubated at 56 °C for 10 min. 200µl of 96 % ethanol was added to the sample and mixed thoroughly by vortexing. The mixture was added to the DNeasy mini spin and centrifuged at 13000g for 1 min.

The flow through was discarded and the column was placed into a new collection tube. 500µl of AW1 buffer was added to the spin column and centrifuged at 13000g for 1 min. The flow through and collection tube was discarded. 500 µl of AW2 buffer was added to the column and once again centrifuged at 13000g for 3 min to dry the membrane. The spin column was placed into a new 1.5 ml microcentrifuge tube and 200 µl of AE buffer was added directly onto the membrane. The column was incubated at room temperature for 1min and then centrifuged at 13000g to elute DNA. DNA was quantified using a spectrophotometer and stored at -20 °C until analysis.

2.4 RNA extraction from human bladder cell lines

RNA was extracted using the QIAGEN RNeasy mini kit (Qiagen UK), according to the manufacturer's instructions. Bladder cells were grown in a monolayer to 70-80 % confluency, media was removed and the monolayer was washed twice with warmed PBS. 350µl of RLT/ β-Mecaptoethanol (10µl β-ME per 1 ml of RLT buffer) was added to the dish and cells were collected with a cell scraper. The lysate was dispensed into a microcentrifuge tube, vortexed and dispensed directly into a QIAGEN QIAshredder and centrifuged for two minutes at full speed. The lysate was removed and transferred into new microcentrifuge tube. 350µl of 70 % ethanol was added to the lysate and mixed immediately by pipetting. The 700µl sample was transferred to an RNeasy MiniElute spin column which was placed in a 2 ml collection tube and centrifuged for 15s at 13000g. The flow through was discarded and the 2 ml collection tube reused. 700µl of RW1 buffer was added to the column and centrifuged for 15 s at 13000 g. The flow through was discarded and the column was placed in a new collection tube 500µl of RPE buffer was added to the column and once again centrifuged for 15 s at 13000g. The flow through was discarded and 500µl

of 80 % ethanol was added to the column and centrifuged for 2 min at 13000g to dry the silica-gel membrane. The spin column was transferred to a new collection tube; the cap was opened and centrifuged at 13000 g for 5 min. Any flow through was discarded and the spin column was placed into a 1.5 ml tube. To elute the RNA, 14µl of RNase-free water was added directly to the centre of the silica membrane and the column was centrifuged for 1 min. All RNA samples were stored at -80 °C until analysis.

2.5. DNA extraction from human bladder tissue

DNA was extracted using the QIAGEN DNeasy blood & tissue kit (Qiagen, UK), according to the manufacturer's instructions. Two heating blocks were set at 56 °C and the other to 70°C prior to the experiment. The tissue samples and buffer AE were allowed to equilibrate to room temperature. The tissue was removed with sterile forceps and blotted to remove excess RNA later solution. On the sterile glass slide, 10-25 mg of tissue was washed with PBS, blotted then cut into small pieces and placed in a 1.5 ml microcentrifuge tube. 180µl of buffer ATL and 20 µl of proteinase K was added to the tissue and mixed by vortexing. The sample was incubated at 56 °C for 1-3 hours, vortexing occasionally to disperse the sample. Following incubation the sample was briefly centrifuged to remove droplets from inside the lid. 4µl of RNase A was added, pulse vortexed for 15 sec and incubated for 2 min at room temperature. The sample was briefly centrifuged before adding 200µl of buffer and mixed again by pulse vortexing for 15 sec followed by an incubation at 70 °C for 10 min. The sample was briefly centrifuged again prior to adding 200µl of 100% ethanol, mixed by pulse vortexing for 15 sec and briefly centrifuged. The mixture was carefully applied to the QIAamp Spin column. The cap was closed and spun at

6000 g for 1min. The column was placed in a clean 2 ml collection tube and the filtrate was discarded. 500 μ l of buffer AW1 was added and column was centrifuged at 6000 g for 1min, placing the column in a clean 2 ml collection tube and the filtrate was discarded. 500 μ l of buffer AW2 was added and the column was centrifuged at 13000 g for 3 min. The column was placed in a clean 2ml collection tube and the filtrate discarded. The column and filter was centrifuged again at 13000 g for a further 1 min to eliminate carryover. The column was placed in a clean 2 ml collection tube and any filtrate discarded. 200 μ l of buffer AE was added to the column and incubated at room temperature for 5 min. The column was centrifuged at 6000 g for 1min. This collection tube was capped and put to one side. The column was placed in a clean 2 ml collection tube and a further 200 μ l of buffer AE was added and incubated at room temperature for 5 min. The column was centrifuged at 6000 g for 1 min and the filtrate was mixed with the filtrate in the tube which had been set aside. The DNA was eluted in a total of 400 μ l of AE buffer.

2.6 RNA extraction from human bladder tissue

Total RNA was extracted from the biopsies following an overnight incubation at -20 °C in RNAlater-ICE™. Total RNA isolation was performed using the QIAGEN RNeasy micro or mini kit, protocol for fibrous tissue, according to the manufacturer's instructions. RNeasy micro or mini kits for RNA extraction were used according to tissue biopsy size. (Micro < 5 mg – Mini 17 mg – 20 mg)

All surfaces were wiped with RNA ZAP to remove all RNase contamination from surfaces. β -Mecaptoethanol (β -ME) was added to the RLT buffer prior to use (10 μ l β -ME per 1ml of RLT buffer) and 80 % ethanol was prepared with the RNase-free water. The tissue was removed with sterile forceps and blotted to remove excess

RNA later solution. On a sterile glass slide, 5-15 mg of tissue was cut into small pieces and placed in a 1.5ml microcentrifuge tube. 350 μ l of buffer ATL/ β -ME mix was added to the tissue and the microcentrifuge tube was placed on ice. The tissue was disrupted and homogenized using a rotor-homogenizer until it was uniformly homogeneous (15-30 sec) and this homogenised sample was centrifuged for 3 minutes at full speed, the supernatant was carefully removed and transferred into new microcentrifuge tube. 350 μ l of 70% ethanol was added to the lysate and mixed immediately by pipetting. The 700 μ l sample was transferred to an RNeasy MiniElute spin column which was placed in a 2ml collection tube and centrifuged for 15 sec at ≥ 8000 g. The flow through was discarded. 350 μ l of RW1 buffer was added to the spin column and centrifuged for 15 sec at ≥ 8000 g to wash the spin column. 10 μ l of DNase 1 stock solution was added to 70 μ l RDD and mixed by inverting to form the DNase 1 mix. This DNase 1 mix was pipetted onto the RNeasy MiniElute silica gel membrane and incubated at room temperature for 15 minutes. 350 μ l of RW1 buffer was added to the spin column and centrifuged for 15 sec at ≥ 8000 g to wash the spin column. The RNeasy MiniElute spin column was transferred into a new microcentrifuge collection tube and 500 μ l of RPE buffer was added to the spin column and centrifuged for 15 sec at ≥ 8000 g. 500 μ l of pre-prepared 80% ethanol was added to the spin column and centrifuged for 2 minutes for 15 sec at ≥ 8000 g to dry the silica gel membrane and the RNeasy MiniElute spin column was transferred to a new 2ml collection tube and centrifuged at full speed for 5 minutes with the lid open to further dry the silica gel membrane. The RNeasy MiniElute spin column was transferred to a new 1.5 ml collection tube and 14 μ l of RNase-free water was pipette directly onto the centre of the RNeasy MiniElute spin column silica gel. The lid was closed and centrifuged for 1 min at maximum speed. The concentration and the purity

of the RNA eluted was determined initially, using a spectrometer, as with the DNA extractions. Concentrations were determined at an absorbance of 260 nm and the purity of DNA elute was determined by the A_{260}/A_{280} ratio.

RNA profiling techniques such as real time RT-PCR and microarray analysis require generation of a cDNA copy of the RNA sample through a reverse transcription reaction. Therefore, good RNA quality is essential for the success of these applications because degraded RNA or RNA that contains impurities will perform poorly in most enzymatic applications.

2.7 Measurement of DNA and RNA concentration and quality

DNA concentration and purity was assessed using a spectrometer. The concentrations were determined at an absorbance of 260nm and the purity of DNA elute was determined by the A_{260}/A_{280} ratio. Samples were stored at -20°C for later use.

2.7.1 Using the Nanodrop Spectrophotometer

'Nucleic Acid Measurements' was selected from the Nanodrop software menu. 1 µl of RNase free water was added and 'Blank' was pressed. The blanking was repeated until there was a stable baseline, close to zero. The baseline was confirmed by measuring 1 µl of water, as if it were a first sample. 1 µl of the RNA was added and the instrument initialised. After each and all subsequent measurements the pedestal was cleaned by wiping with a dry lint-free tissue. The baseline was reconfirmed to be correct after the last sample by measuring 1 µl of RNase free water.

2.8 RNA Integrity – Agilent Bioanalyser

Although mRNA is often the RNA species of interest, the fact that mRNA only comprises 1-3% of total RNA sample makes assessing the integrity of mRNA difficult in a total RNA sample. Good quality RNA will have an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater. This is because nucleic acid is detected at 260 nm, whereas protein, salt and solvents are detected at 230 and 280 nm. A high OD 260/280 and OD 260/230 ratio therefore indicates that you have extracted RNA devoid of any of these contaminants. Degraded RNA should not be hybridised to GeneChips for array analysis, therefore it is important to assess the quality of RNA.

One can use northern analysis or RT PCR to study only the integrity of an individual transcript in a RNA sample. Since the ribosomal RNA (rRNA) makes up more than 80% of total RNA samples, the integrity of the major rRNA species (18S and 28S for mammalian rRNA) is used to assess the RNA sample integrity. This can be done by analyzing total RNA sample by denaturing agarose gel electrophoresis. The ethidium bromide-staining pattern of intact total RNA will have clearly defined 28S and 18S rRNA bands. The ratio of 28S:18S band intensities should be close to 2. Partially degraded RNA appears as smeared rRNA bands with increasing lower molecular weight smear. However the Agilent 2100 Bioanalyser (Agilent Technologies) is an alternative tool that provides better and more consistent assessment of total RNA quality. The instrument uses a combination of a nucleic acid intercalating dye and capillary electrophoresis to analyze both RNA concentration and integrity simultaneously. Agilent RNA kits contain chips and reagents designed for analysis of RNA fragments. Each RNA chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. The sample passes through these

microchannels into the separation channel, the RNA fragments are separated according to their size by means of molecular sieving and the fragments are detected by fluorescence peak that is greater in area compared with the 18S peak. An example of an electropherogram is shown in figure 2.3

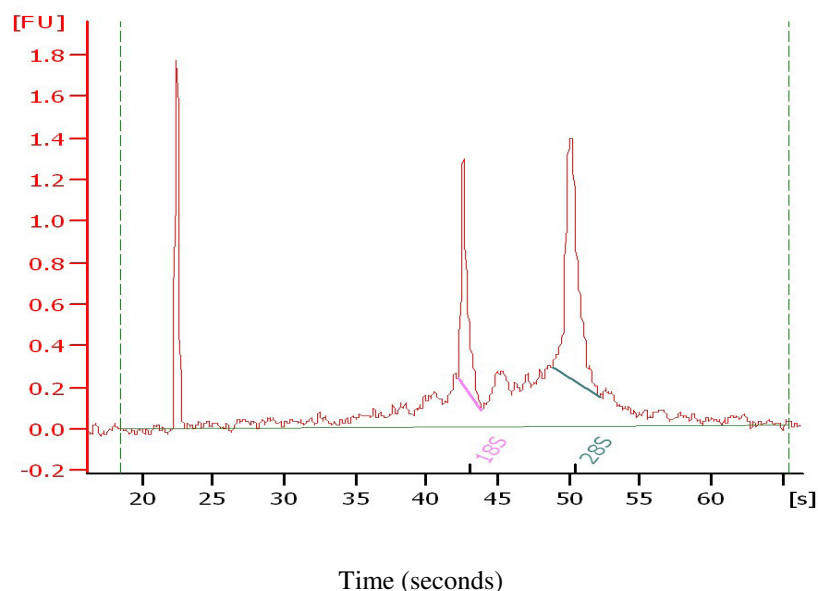


Figure 2.3 Example of an electropherogram showing good quality RNA generated by the Agilent 2100 Bioanalyzer.

2.8.1 Preparing sample for the 6000 Nano Chip

All reagents were allowed to equilibrate to room temperature for 30 minutes. Dye and dye mixtures were protected from light at all time by wrapping in tin foil. The RNA 6000 Nano gel matrix gel was prepared by pipette 550 μl of gel into a spin filter and centrifuging at $1500\text{ g} \pm 20\%$ for 10 minutes at room temperature. 65 μl the filtered gel was pipetted into 0.5 ml RNase-free microcentrifuge tubes. The RNA 6000 Nano dye concentrate was vortexed for 10 seconds, spin down and 1 μl of the dye was added into a 65 μl aliquot of filtered gel. The gel-dye mix was vortexed well and centrifuged at 13000 g for 10 min at room temperature. All RNA samples and RNA ladder were denatured before use by heating to 70°C for 2 minutes. The RNase decontamination procedure for the electrodes was performed before running the assay.

A new RNA 6000 Nano chip was put on the chip priming station and 9 μ l of the gel-dye mix was pipetted in the appropriate well. See figure 2.4 for graphical representation.

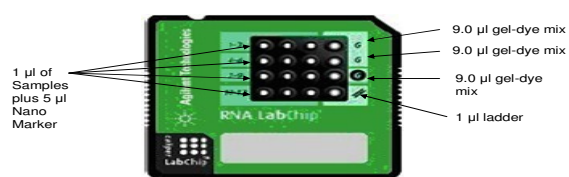


Figure 2.4 RNA 6000 Nano chip

2.8.2 Using the Agilent 2100 bioanalyzer

The chip priming station was closed and plunger was pressed down until it was held by the clip. The release clip was released after exactly 30 seconds. A further 9.0 μ l of the gel-dye mix was pipetted into appropriately marked wells and the remaining gel-dye mix was discarded. 5 μ l of the Agilent RNA 6000 Nano Marker was loaded in all 12 sample wells and in the well marked 'ladder'. 1 μ l of the prepared ladder was also loaded into the well marked 'ladder'. 1 μ l of sample was loaded in each of the 12 sample wells and 1 μ l of RNA 6000 Nano Marker was loaded into each unused sample well. The chip was put horizontally in the adapter and vortex for 1 min at 2400 rpm and run on the Agilent 2100 bioanalyzer within 5 min.

2.8.3 Concentration and clean-up of RNA samples for array hybridisation and analysis

The RNA extracted from the biopsy samples was concentrated using QIAgen RNeasy MiniElute Cleanup Kit according to the manufactures instructions (Qiagen, UK).

2.9 Array hybridisation and Dchip analysis

The RNA extracted from the biopsy samples was concentrated, and its quality assessed using the Agilent 2100 Bioanalyser. If there was insufficient quantity the RNA was amplified via PCR (array 2, intervention samples chapter 7). Microarrays are used to detect the transcript levels of thousands of genes simultaneously; the working principle of the technique is based on duplex (hybridisation) between target RNA extracted from cell lines or tissues and complementary short DNA-nuclotides oligomers grafted to thre solid surface of the chip (Zhu 2003)). Targets bound to the probe-ologomers are detected using florescence labels. RNA of sufficient quantity and quality was hybridised onto human Affymetrix U133 Plus 2.0 probe arrays at the Nottingham Arabidopsis stock centre, University of Nottingham. The arrays carries >47,000 transcripts, expression profiling was performed for each transcript and the data produced was analysed using DNA-Chip analyser 2006 software (dChip) (Li and Wong 2001). Double stranded synthesis and generation of biotin-labelled cRNA was performed according to the manufactures protocol (Affymetrix Inc). The fluorescence intensity for each chip was captured with an Affymetrix HP GeneArray laser confocal scanner (Agilent, UK).

The raw files generated containing the summary intensities for each probe was loaded into the dChip 2006 software, for normalisation, generation of expression values and statistical analysis. Following normalisation, probe expression levels was

calculated MM (perfect match-miss match subtraction) model and the PM-only model showed that applying the later model consistently produces less variable results and the PM only model is more suited to this type of study (Li and Hung Wong 2001). The PM probe binds cDNA exactly, forming exclusively Watson-Crick pairing, whereas the MM probe has a single base difference in the middle of the probe giving rise to self-complementary pairing. Therefore, the the PM pairing produces a higher/brighter signal than that of the MM pairing, as the stability of the Watson-Crick pairing is always stronger than that of the self-complementary pairing. Probe intensity values need to be normalised to adjust the brightness of the arrays to a comparable level as different arrays produce different image brightness and so are generally not comparable. dChip chooses an array with a median overall intensity, as measured by the median CEL intensity in an array, as the baseline array against which other arrays are normalised at the probe intensity level. The dChip software uses the Invariant Set Normalization method, which chooses a subset of PM probes with small within-subset rank difference in the two arrays (baseline and target), to serve as the basis for fitting a normalization curve. The normalized curve is used to generate new normalized values for every probe on the chip.

After normalization the expression levels were calculated using the PM model. Three comparisons criteria were applied to the data to detect differentially expressed genes by model based expression: 1) the fold change between the group means was chosen to exceed a specific threshold 2) absolute difference between the two groups means was set to > 100 to eliminate the low expressing genes that have intensity close to the background levels and 3) a chosen p – value for Welch modified 2-sample t-test, adjusted to compensate for multiple testing using False discovery rate (FDR). False discovery rate testing is a method used in multiple hypothesis testing as a means

to correct for multiple comparisons to gain more confidence in the statistical analysis of the data. The FDR is the expected false positive rate, in dChip the FDR was estimated by a 1000 permutations. This assess how many probes would be obtained by the same comparison criteria when permuting group labels randomly by 1000 times, that is 1000 observations were experimentally predicted to be different, and a maximum FDR for these observations was 0.10, then 100 of these observations would be expected to be false positives. After 1000 permutations, there will be 1000 values generated. The median of these 1000 values is reported as the median false discovery rate (MFDR) and the 90th percentile (90th largest value) of these 1000 values is reported as 90% FDR. Although many comparison criterions were applied to the data to detect differentially expressed genes by model based expression, only the more stringent criteria is shown in this thesis (fold change of 2, p-value of 0.001 and an absolute difference between the two groups means was set to > 100) so as to provide a manageable data lists

After obtaining model-based expression values, unsupervised hierarchical clustering was performed using a list of genes obtained by filtering probes using the criteria of standard deviation divided by the mean between 0 and 1000 across the samples. Unsupervised clustering analysis is used to identify novel sample clusters and their associated “signature genes” and to see if replicate samples or samples under similar conditions are clustered together.

2.10 Primer and Probe design

Forward and reverse primers, and the fluorogenic TaqMan probes for target genes, were designed using Primer Express Software (Applied Biosystems). Sequence homology of selected oligomers was checked using a National Centre for Biotechnology Information BLAST search to ensure that sequences will be specific to target genes. Primer and probe sets were synthesised by SigmaGenosys and assays optimised. A 5 μM solution of probe and a 10 μM solution of primers were made with double autoclaved RNase free water for regular use.

	Forward primer Sequence (5'-3')	Reverse primer Sequence (5'-3')	Probe Sequence (5'-3')
CO6A1	TGCATAGACAAGAAGTGTCAGATT	GGAGCCGTCACAGCAGGAT	CTGCCCCATCACGTTCTCCTCCC
Moesin	GCTACCTGGCCGGAGACA	CCACTGGTCCTTGTTGAGTTG	TTGCTCCCGCAGAGAGTCCTG
P4HA1	ATTATGGAGTTGGAGGACAGTATGAA	CTTTGAAAGCATCTGGCTCATCT	CCCATTTTGACTTTGCACGGA
GSTP1			
GSTM1	GGAGACAGAAGAGGAGAAGATTCTG	TGCCCAGCTGCATATGGTT	TCCATGGTCTGGTTCTCCAAAATGTCCA
GSTM1 A*/B*	TTCACGTGTATGGAGGTTCCA	GGGAGATGAAGTCCTCAGATTTG	AAGCACTGGGGCTCA
GSTT1	CCTGGCAGAGTTGGATGTGA	GGAGATGTGAGGACCAGTAAGGA	CCTGCAGTTGCTCGAGGACAAGTTCC
GSTM3 A*/B*	GCCGGGGAAAAGGTAGGAA	AGGAGTAGTAGCCTGCAGAGATAGAGA	AGGGAAAAGAAGAGGAT
18S	GGCTCATTAAATCAGTTATGGTTCCT	GTATTAGCTCTAGAATTACCACAGTTATCCA	TGGTCGCTCGCTCCTCTCCCAC
BRCA1	GTCTGCTTTTACATCTGAACCTCTGT	AGCCCTGAGCAGTCTTCAGAGA	ACTCTCACACCCAGATGCTGCTTCACCT

Table 2.2 Primer and probe sequences used the real time RT-PCR and PCR assays. Primers and probes were designed to be intron spanning and human specific.

2.11 TaqMan Real Time polymerase chain reaction (PCR)

DNA extracted from either bladder biopsy tissue or cell lines were amplified using real-time PCR. Target gene levels were determined by real-time PCR using the 7500 or 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR reactions were carried out in a microamp optical 96-well plate in a total volume of 25 μ l per well containing TaqMan one-step PCR master mix reagent (Applied Biosystems). The TaqMan real time PCR chemistry allows detection of PCR products via the generation of a fluorescent signal. TaqMan probes depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. TaqMan probes depend on the 5'-nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'-nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. The threshold cycle (Ct) is defined as when the fluorescence signal reaches ten-times the noise signal level (baseline).

2.11.1 GSTM1, GSTM3 and GSTP1 Allelic Discrimination Assay

An allelic discrimination assay is a multiplexed, end-point assay that detects variants of a single nucleic acid sequence. A multiplexed assay is one that uses more than one primer/probe pair per reaction. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. This is a qualitative assay as the actual quantity of target sequence is not determined. For each sample a unique pair of fluorescent detectors is used. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2). The assay measures the changes in fluorescence of dyes associated with the probes. For example, a subsequent increase in the VIC dye fluorescence only would indicate homozygosity for allele 1, a subsequent increase in the FAM dye fluorescence only would indicate homozygosity for allele 2 and an increase in both signals would indicate heterozygosity (both allele 1 and 2). For GSTM3 the A* (wild type) allele was labelled with FAM and the B* was labelled with VIC. Similarly for GSTM1 the A* allele was labelled with FAM and the B* allele was labelled with VIC. Again for GSTP1 the A* allele was labelled with FAM and the G* allele was labelled with VIC.

2.12 TaqMan Real Time reverse transcriptase polymerase chain reaction

In real-time relative RT-PCR a standard curve is generated from a dilution series constructed from a "reference" sample. The identity of the reference sample is not important as long as the relevant PCR target is present. The units used to describe the dilution series are relative, not absolute values, are based on the dilution factor. In all experiments RT-PCR has been performed on both the experimental samples and reference standards and the relative values for target abundance in each experimental sample have been extrapolated from the standard curve generated from the reference standard. Although the absolute values calculated for the experimental samples are meaningless, the relative differences in mRNA abundance between samples are accurate. The efficiency (Eff) of the reaction can be calculated by the following equation: $\text{Eff} = 10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should be 90-110% ($3.6 \geq \text{slope} \geq 3.1$) and is shown in every RT-PCR/PCR graph shown. A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, secondary structure, and primer.

RNA extracted from either bladder biopsy tissue or cell lines was amplified using real-time RT-PCR. Gene expression was determined by real-time RT-PCR using the 7500 or 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The RT-PCR reactions were carried out in a microamp optical 96-well plate in a total volume of 25 μl per well containing TaqMan one-step RT-PCR master mix reagent (Applied Biosystems).

2.12.1 Normalizing the absolute quantification according to the 18S

housekeeping gene.

Quantitative real-time RT-PCR is a very useful technique for estimating gene expression at the mRNA level. The expression of a tested gene has to be compared with that of a control gene or housekeeping gene because the amount of assayed mRNA may fluctuate due to differences in tissue mass, cell number, experimental treatment or variations in the efficiency of RNA extraction. For instance the RNA content per cell or per gram of tissue may vary in different tissues *in vivo*, in cell culture *in vitro*, between individuals and under different experimental conditions and a control gene can also correct for sample to sample variations in RT-PCR efficiency and errors in sample quantification. Therefore, normalisation of target gene expression levels must be performed to compensate intra- and inter-kinetic RT-PCR variations (sample-to-sample and run-to-run variations). Data normalisation in real-time RT-PCR is a further major step in gene quantification analysis (Pfaffl 2001; Bustin 2002).

To normalize the absolute quantification according to a single housekeeping gene, a second set of kinetic PCR reactions has to be performed for the invariant endogenous control on all experimental samples and the relative abundance values are calculated for internal control as well as for the target gene. For each target gene sample, the relative abundance value obtained is divided by the value derived from the control sequence in the corresponding target gene. The normalized values for different samples can then directly be compared (Pfaffl 2001).

2.13 Total protein extraction from cultured cells and BCA assay

Cells were cultured on a monolayer to 90% confluency. The media was removed and the monolayer was washed three times with cold PBS. The dish was put on ice and 1ml of complete RIPA buffer (50 mol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% (v/v) Triton-X , 0.5% (w/v) Na deoxycholate, 0.2 % (w/v) SDS, 1 mmol/L Na₃VO₄ 1 50 mg tablet/10 mL complete protease inhibitor cocktail tablet (Roche Diagnostics, Lewes, UK)) was added (10cm dish). The plate was left on ice for two minutes until the cells began to detach from the surface of the plate. The detachment of cells was verified under the microscope. The cell suspension was dispensed into 2ml eppendorf tubes and left on ice for a further 15 minutes. The suspension was vortexed three times during this 15 minutes. The cell suspension was centrifuged at 13000 g at 4 °C for ten minutes. The supernatant was removed and the pellet was discarded. The protein concentration was determined using the bicinchoninic acid (BCA) method, with bovine serum albumin (BSA) as the standard. The protein was quantified by measuring absorbance at 562nm plate reader (Dynex, UK).

2.14 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE and western blotting was performed using the XCell *surelock*TM Mini-Cell NOVEX[®] protein electrophoresis system and NuPage[®] gels and buffers (Invitrogen).

Tubes were labelled and samples were prepared by adding the follow volumes of reagents.

Reagent	Reduced Sample
Sample	x μ l
NuPage [®] LDS Sample Buffer (4X)	2.5 μ l
NuPage [®] Reducing Agent (10X)	1 μ l
Deionised Water	to 6.5 μ l
Total Volume	10 μ l

Table 2.3 Preparation of samples for SDS-PAGE. Samples were prepared in accordance with the manufactures instructions, as above.

All samples were incubated at 70 °C for 10 minutes. The gel was removed from the packet and washed with deionised water. The comb was gently slipped out and the white strip removed. The gel was placed in the chamber with wells at the top, and writing facing forward so as to be readable. The chamber was set up with transparent plate and wedges. 1X concentration of SDS running buffer was prepared by adding 50 ml of 20 x NuPage[®] MOPS SDS running buffer to 950 ml of deionised water. 500 μ l of NuPage[®] antioxidant was added to the middle chamber then filled with 600mls of 1x NuPage[®] SDS running buffer. The front chamber was filled with 200ml of 1x NuPage[®] SDS running buffer. 30 μ l of each protein sample were loaded into the wells, 2 μ l each of MagicMarkTM XP (Invitrogen) and SeeBlue[®] (Invitrogen) was added as markers. Western protein MagicMarkTM XP standards allow direct visualization of protein size standards on membrane blots without the need for protein modification or special detection reagents. The standard proteins are derived from E.

Coli cells containing a construct with repetitive units of a fusion protein forming the size variation and an IgG binding site. The proteins can be visualized with the colorimetric, chemiluminescent, or fluorescent detection system of choice simply by processing the membrane for the specific protein. The IgG binding site will allow all the standard proteins to react with the specific primary and secondary antibodies (Sasse and Gallagher 2008). The SeeBlue[®] pre-stained protein standard allows monitoring of the progress of the gel run and is an excellent tool for judging western transfer efficiency. Polypeptides that resolve into sharp, tight blue bands in the range of 4 kDa to 250 kDa (depending upon buffer system). An excellent indicator for judging western transfer efficiency as well as protein size. The gel was run at 200 volts for 60 minutes.

2.14.1 Western Transfer

Proteins were transferred on to nitrocellulose membrane (BioRad, Hemel Hempstead, UK) as follows. 1X concentration of NuPage[®] transfer buffer was prepared by adding 50 ml 20X concentration of NuPage[®] transfer buffer and 100ml of methanol to 850ml of deionised water. 1ml of NuPage[®] antioxidant was added to the transfer buffer.

Six blotting pads, filter papers and membrane were soaked in 700 ml of 1X NuPage[®] transfer buffer. Three pieces of filter paper and one nitrocellulose membrane were cut 8cm X 7cm. The right hand corner was cut, so as to insure gel/membrane sandwich is not assembled in a reverse direction. The Gel casing was broken open leaving the gel on the larger side of its casing and the groove line at the bottom cut off. A piece of soaked filter paper was placed over the gel and air bubbles removed. Gel/filter paper was removed from casing and placed over the membrane, removing air bubbles. Two

soaked blotting pads were placed into the cathode core of the blot module. The gel/membrane assembly was placed on the blotting pad in the correct orientation, so the gel is closest to the cathode core. The remaining soaked blotting pads were added to rise to 0.5cm over the rim of cathode core. The anode core was placed on top of these pads. The blot module was held together firmly and slid into the guide rails on the lower buffer chamber. The gel tension wedges were inserted into the lower chamber and locked into position and the blot module was filled with the 1X concentration NuPage[®] buffer until gel/membrane sandwich was covered in buffer, careful not fill all the way to the top, as this will generate extra conductivity and heat. The outer buffer chamber was filled with 650ml of deionised water. The water level should reach approximately 2 cm from the top of the Lower Buffer Chamber. This serves to dissipate heat during the run. The lid was placed on and transferred using 30 volts constant for 1 hour.

2.14.2 Western Blot

Firstly 10mls of 5% blocking agent were made up using 0.5 g milk power in 10mls Tris buffered saline 0.1% Tween 20 (v/v) (TBST). The membrane was removed from the blot module and washed in the 5% blocking agent for 30 minutes at room temperature, with rocking. Following the 30 minute wash the membrane was then rocked in the above blocking solution with a dilution of the primary antibody. Dilution, temperature and time of exposure was dependent on each particular antibody. For example AP-2 α rabbit polyclonal IgG primary antibody: 10 mls blocking solution 1:1000 dilution. The membrane was left rocking/washing overnight at 4 °C. The following day the membrane was given 2 brief washes with TBST; then one wash with TBST for 15 minutes and a further wash with TBST for 5

minutes. The membrane was rocked for 60 minutes room temperature in the blocking solution and anti-rabbit IgG horseradish peroxidase (HRP) conjugate secondary antibody (Sigma Aldrich), 1:20, 0000 dilution. Finally the membrane was washed three times in TBST to remove surplus HRP.

2.14.3 Immunodetection

Chemiluminescence detection was performed using a Pierce West Pico Chemiluminescent kit (Thermo Scientific, UK). The membrane was washed and incubated with equal volumes of solutions A and B for 5 minutes, room temperature. After incubation the membrane exposed to Hyperfilm™ ECL (GE Healthcare, Amersham UK) and the film developed on a X-ray film developer. Or the chemiluminescence was captured using a Fluor-S™ Multimager (BioRad, UK). Time of exposure for either method was dependent on strength of signal.

2.15 Gelatin-sodium dodecyl sulfate polyacrylamide (SDS-PAGE) zymography

2.15.1 Treatment of UM-UC-3 cells

On day one the cells were split from a 10cm dish into the 12 well plates. The following day the cells were left undisturbed to ensure good adhesion of cells to the plate surface. On day three the monolayer was washed with warm PBS and the conditioned media was added to the appropriate wells.

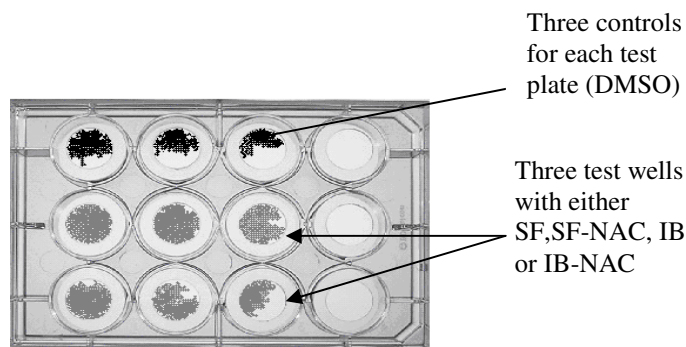


Figure 2.5 Twelve well culture plate

On day four the monolayer/cell morphology was examined under the microscope for any adverse effects of the treatments. If none were seen the plate was placed back into the incubator. On day five following the 48 hour treatment of the cells the conditioned media was removed and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on a 10 well, 1mm, 10% Tris-Glycine zymogram gel with 0.1% gelatin incorporated as substrate (Invitrogen). Electrophoresis was carried out at a constant voltage of 125 V for 90 minutes. The assay was normalised against the cell number in each well. Each well was seeded at the same density; however there was some well to well variation and therefore enzyme levels may fluctuate due to cell numbers in individual wells. Also enzyme/protein/cell levels may fluctuate due to the experimental treatment or variable efficiency of sample preparation. Therefore the monolayer was removed and the total protein concentration was determined using the BCA method (see total protein extraction from cultured cells and protein quantification using the bicinchoninic acid (BCA) method). Therefore enzymes can be expressed as enzyme activity per mg of protein to enable normalisation of the assay. Following collection of media the gel was removed from the packet and washed with deionised water. The comb was gently slipped out and the white strip removed. The sample wells were rinsed with 1X running buffer and the gel was inverted to remove buffer. This was repeated two

more times. The gel was placed in the chamber with wells at the top, and writing facing forward so as to be legible. The chamber was then set up in accordance with the mini-cell manual (Invitrogen IM-9003) with plastic buffer dam and locked into position.

Samples were prepared under non reduced conditions by adding 10 μ l of sample mixed with 10 μ l of sample buffer and loaded immediately on the gel without heating the sample. 20 μ l of SeeBlue[®] Plus2-stained standard (Invitrogen) was loaded in to well 1 and 20 μ l of each sample was loaded into each of the following wells 2-10. The 1X running buffer was prepared by adding 100 ml of (10X) running buffer with 900 ml of deionised water. A small amount of buffer was added to the upper chamber of the XCell *surelock*[™] Mini-Cell to check for tightness of seal. Once no leaks were observed between the upper and lower chamber the upper chamber was filled with 200 ml and the lower buffer chamber with 600ml of the (X1) Tris-Glycine running buffer.

After 90 minutes the XCell *surelock*[™] Mini-Cell power was turned off and electrodes disconnected and the gel within its cassette was removed. The cassette was carefully opened and the top plate removed. A piece of blotting paper was placed over the gel for ease of handling and the gel was removed and placed into a plastic dish. 100ml of both Novex[®] Zymogram 1X renaturing buffer and Novex[®] Zymogram 1X developing buffer were prepared by adding 10 ml of the 10X buffer to 90 ml of deionised water (100ml required per gel). The gel was incubated in the 1X renaturing buffer at room temperature for 30 minutes with gentle agitation. The 1X renaturing buffer was then decanted and 1X developing buffer was added to the dish containing the gel. The gel was equilibrated for 30 minutes at room temperature with gentle agitation. The 1X developing buffer was decanted and fresh 1X developing buffer

was added to the gel. The gel was incubated in the developing buffer at 37° C for varied times.

2.15.2 Staining of zymogram

The Invitrogen basic protocol for staining Novex® gels with SimplyBlue™ was followed. As the zymography gels do not need fixing, they were rinsed three times for 5 minutes with 100 ml deionised water to remove the SDS and buffer salts. The gels were stained for 1 hour at room temperature with gentle shaking with enough SimplyBlue™ stain to cover the gel (approx 20-100 ml, dependent on the area of the bottom of the dish that the gel was in). Following staining the gel was washed in 100 ml of deionised water for 2 hours.

2.16 UM-UC-3 bladder cell line migration using the wound healing assay

Passage 8 UM-UC-3 cells were split 1:4 and were grown as monolayer in four six well plates until they reached confluence; typically 5 day (refer to for general cell culture methods). On days two and four cells were treated with ITCs, either 12µM of SF, IB, SF-NAC, or IB-NAC. Each ITC was tested in triplicate and on day five a 1-mm scrape wound was made across the confluent layer monolayer with a sterile pipette tip and the displaced cells were removed by rinsing with PBS. The remaining cells were allowed to migrate in fresh medium. A microscopy imaging system (Olympus) was used to assess the wound at 0 hrs at x 200 magnification. The migration was assessed at specific time points (0, 24 and 36 hours) by measuring the leading edge of wound relative to the same position at 0 hours. The migration distances between the leading edge of the migrating cells and the edge of the original wound at 0 hours were compared. The migration rate of the treated cells was

expressed as a percentage and quantified as the following equation: migration rate = (migration distance of ITC treated cells/migration distance of untreated controls) x 100%.

2.17 BD BioCoat™ Matrigel™ Invasion Chamber to study cell invasion of treated and untreated UM-UC-3 cells.

BD BioCoat™ Matrigel™ 6 well plates (BD Biosciences, Oxford UK) were removed from -20°C storage and allowed to come to room temperature. Warmed (37°C), serum free culture medium was added to the interior of the inserts and the bottom wells (receiver well) of the companion plate. The plates were allowed to rehydrate for 2 hours in humidified tissue culture incubator, 37 °C, 5 % CO₂ atmosphere. After rehydration, the medium was removed without disturbing the layer of Matrigel™ matrix on the membrane. An equal number of control inserts were prepared by using sterile forceps to transfer them to empty wells of the BD Falcon™ TC Companion Plate (BD Biosciences, Oxford UK). Cell suspensions were prepared in serum free culture medium containing 1.25x10⁵ cells/ml for 6-well chambers. The media was conditioned with 12 μM of SF, IB, SF-NAC, IB-NAC or DMSO as a control all in triplicate. 2.5 ml of culture media containing serum was added to each well of the companion plate. 2ml of the cell suspension was added to each well chamber. BD BioCoat™ Matrigel™ Invasion Chambers were incubated in a incubator, at 37 °C, 5 % CO₂ atmosphere. After incubation, all the media was removed and the chambers were rinsed with warmed PBS. The non-invading cells were removed from the upper surface of the membrane by wiping with cotton tipped swab. To determine an optimal fixing and stain time for the UM-UC-3 cells a series of stain times from 3 to 5 minutes were completed following a series of fixing times of 2 and 3 minutes. Once an

optimal time was established, the cells were fixed using 1 ml of cold 100 % methanol and stained with 1 ml of Gill's No 1 Hematoxylin stain. The filter was carefully cut away from the transwell and mounted onto a microscope slide for cell counting.

Chapter Three

Characterisation Of Bladder Tissue

CHAPTER 3: Characterisation of bladder tissue

3.1 Introduction

Transitional cell carcinoma is a common malignancy characterized by a poor clinical outcome when tumours progress into invasive disease. The aim of this chapter is to compare global gene expression profiles between superficial and invasive transitional cell carcinoma of the bladder to enable identification of a subset of genes for subsequent cell experimental studies with cell cultures. I sought to define gene expression profiles or selective genes that are characteristic of aggressive clinical behaviour in invasive bladder tumours. Epidemiology studies have demonstrated that bladder cancer is strongly associated with cigarette smoking, and the risk of development of neoplasia may be modified by differences amongst individuals in carcinogen-metabolising enzymes, such as GSTs. I also sought to explore the frequency of genetic polymorphisms of GSTT1, GSTM1, GSTM3 and GSTP1 in superficial and invasive transitional cell carcinoma.

3.2 Specific materials and method

Following biopsy extraction, tissue was stored at the Norfolk and Norwich University Hospital (NNUH) Tissue Bank at -80 °C. Seventeen frozen bladder biopsies were transferred to the IFR laboratory from the Norfolk histopathology laboratory (see chapter 2 regarding the ethics). The samples were classified and graded by the NNUH histopathologist, Professor Richard Ball, prior to collection, in accordance with TNM classification World Health Organization system (Oosterlinck, Lobel *et al.* 2002; Oosterlinck, Lobel *et al.* 2002; Oosterlinck 2004) (section 1.9). RNA and DNA extraction, RNA quantification, quality assessment, Affymetrix U133 plus 2.0 GeneChip hybridisation and analysis were carried out according to section 2.

Prior to the DNA extraction the frozen tissue was weighed to determine the volume of RNALater™ ICE the biopsy should be incubated in to ensure optimal RNA extraction at a later date. Table 3.1 shows the tissue bank number, weight, time of commencement of incubation and volume of RNALater™ ICE that was used. Refer to chapter two for full explanation of RNALater™ ICE reagent and the detailed method of RNA stabilisation with RNALater™ ICE. Table 3.2 shows diagnosis, age, and gender of the individuals from which the biopsies were obtained. Refer to section 1.7 for an explanation of the tissue bank number and refer to section 1.9 for a more detailed explanation of grade. Biopsies with tissue bank numbers 02TB040, 02TB058, 02TB093, 04TB119 and 04TB125 had two biopsies taken from the bladder and therefore have the same tissue bank numbers but were distinguishable by their vial numbers. The first sample was seen to be TCC and graded accordingly, the second biopsy was taken from an adjacent histologically benign area of their bladder and therefore graded as 'normal'. Following a 48 hour soaking in the appropriate

volume of RNAlater ice, a weighed section of biopsy tissue was extracted for DNA isolation. All times, dates and volumes were recorded in table 3.1.

Tissue bank no	vial	WT of sample mg	volume of RNA later ice added ml	date/time soaking commenced	WT of tissue for DNA isolation mg	date of extraction of DNA
02TB040	1	105	2ml	05.10.06 at 15.00	10.6	6.10.06 at 10.45
02TB040	5	159.1	2ml	05.10.06 at 15.01	22.5	6.10.06 at 10.46
02TB058	1	90.1	2ml	08.10.06 at 10.15	12.7	10.10.06 at 10.00
02TB058	9	56.1	2ml	08.10.06 at 10.15		
02TB065	9	64.8	2ml	08.10.06 at 10.15	23.2	10.10.06 at 10.20
02TB067	10	60.6	2ml	08.10.06 at 10.15	11	10.10.06 at 10.30
02TB044	14	49.9	2ml	08.10.06 at 10.15	12.8	10.10.06 at 10.40
02TB093	1	195.1	2ml	08.10.06 at 10.15	13.5	10.10.06 at 13.15
02TB093	10	56.0	2ml	08.10.06 at 10.15		
03TB132	1	150.2	2ml	08.10.06 at 10.15	21.5	10.10.06 at 13.15
03TB146	1	124.5	2ml	08.10.06 at 10.15	13.2	10.10.06 at 13.15
03TB179	1	70	2ml	08.10.06 at 10.15	15.2	10.10.06 at 13.15
04TB021	1	75.1	2ml	08.10.06 at 10.15	11.3	10.10.06 at 13.15
04TB119	1	36.5	2ml	08.10.06 at 10.15	8.6	10.10.06 at 13.15
04TB119	4	36.0	2ml	08.10.06 at 10.15		
04TB125	1	19.3	2ml	08.10.06 at 10.15	5.4	10.10.06 at 13.15
04TB125	4	32.0	2ml	08.10.06 at 10.15		

Table 3.1 Tissue bank number of biopsy, weight of frozen biopsy received, volume of RNAlater ice the biopsy was soaked in and the weight of sample dissected for DNA extraction.

3.3 Results

3.3.1 Bladder tissue genotyping

Table 3.2 shows the genotype results of the 12 bladder biopsies obtained from the NNUH tissue bank, 17 tissue samples in total. Four (02TB040, 02TB093, 04TB119 and 04TB125) were samples taken from the cancerous bladder, but in an adjacent area to the actual tumour. These biopsies appeared histologically benign. GSTM1 and GSTT1 null genotypes, the GSTP1 (Ile105Val polymorphism/ Ala114Val polymorphism), and the GSTM3 3 base pair deletion genotype plus allele discrimination of GSTM1 positive individuals were investigated in all tissues obtained from the NNUH tissue bank. GSTM1 and GSTT1 null individuals have genetically deleted (non-functional alleles) and consequently do not produce the enzyme. The GSTM3* B affects the detoxification activity of the enzyme and the Valine residue at position 105 of the GSTP1 protein results in decreased enzyme activity.

Table 3.2 Histological results of bladder tissue and GSTM1, GSTM3, GSTT1 and GSTP1 genotypes.

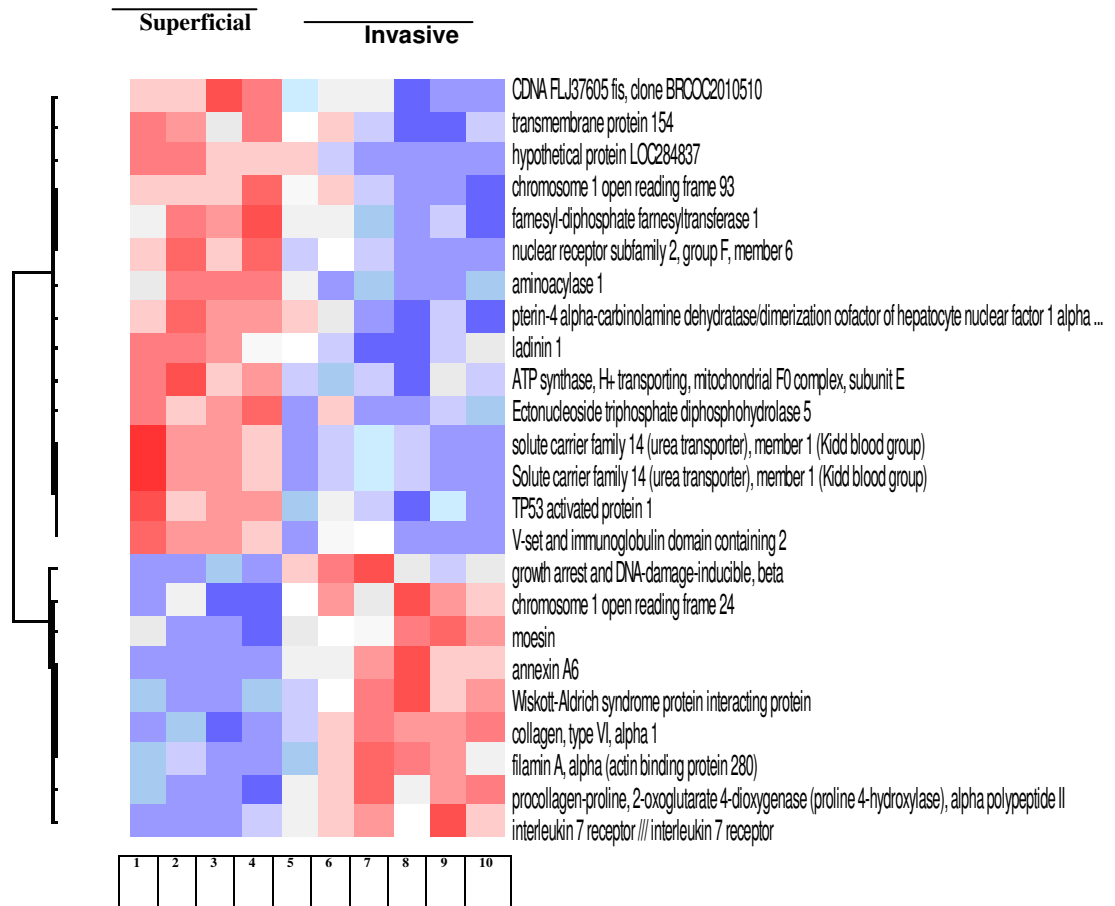
Tissue bank number	Age	Gender	Diagnosis	Grade	Interpretation of grade	Genotype			
						GSTM1	GSTM3	GSTT1	GSTP1
02TB040	66	M	TCC	G3,pT3b,pTis	Poorly differentiated/undifferentiated. Infiltrative beyond muscularis, extravesical mass. Also seen was a situ flat tumour.	Null	A/A	A/0	G/0
02TB040	66	M	normal						
02TB058	71	F	TCC	G3,pT3a	Poorly differentiated/Undifferentiated. Infiltrative beyond muscularis, microscopically.	B/0	A/A	A/A	A/0
02TB058	71	F	dysplasia		Pre-cancerous change in cellular structures.				
02TB065	82	M	TCC	G3,pT3a	Poorly differentiated/Undifferentiated. Infiltrative beyond muscularis, microscopically.	Null	A/A	A/0	G/0
02TB067	76	M	TCC	G2,pT1	Moderately differentiated. Superficial subepithelial connective tissue.	Null	A/A	A/0	G/A
02TB044	76	M	TCC	G3,pT3b	Poorly differentiated/undifferentiated. Infiltrative beyond muscularis, extravesical mass.	A/B	A/B	A/0	A/0
02TB093	76	F	TCC	G3,pT2b	poorly differentiated/undifferentiated. Infiltrative outer half of muscularis.	Null	A/B	A/A	A/0

Table 3.2 continued: Histological results of bladder tissue and GSTM1, GSTM3, GSTT1 and GSTP1 genotypes.

Tissue bank number	Age	Gender	Diagnosis	Grade	Interpretation of grade	Genotype			
						GSTM1	GSTM3	GSTT1	GSTP1
02TB093	76	F	normal						
03TB132	85	M	TCC	G2,pT1	Moderately differentiated. Superficial subepithelial connective tissue.	Null	A/B	A/A	G/A
03TB146	82	M	TCC	G3,pT1	Poorly differentiated/ undifferentiated. Superficial subepithelial connective tissue.	A/0	A/A	Null	G/A
03TB179	80	M	TCC	G2,pTa	Moderately differentiated. Non-invasive superficial papillary.	A/A	A/A	A/A	G/0
04TB021	78	M	TCC	G2,pTa	Same as above	Null	A/B	A/A	G/A
04TB119	83	F	TCC	G3, pT3b	Poorly differentiated/ Undifferentiated. Infiltrative beyond muscularis, extravesical mass.	A/A	A/B	A/A	G/0
04TB119	83	F	normal						
04TB125	66	F	TCC	G3, pT3b	Poorly differentiated/ undifferentiated. Infiltrative beyond muscularis, extravesical mass.	Null	A/A	A/A	G/A
04TB125	66	F	normal						

3.3.2 Bladder tissue array

Of the 17 bladder tumour biopsy samples received from the NNUH tissue, 12 were transitional cell carcinomas of varying grades, from superficial to muscle invasive, one was graded as dysplasia and four were graded as normal (see table 3.2). RNA was successfully extracted from 10 of these bladder tumour biopsies for microarray analysis. Normalisation of the signal intensities across all the arrays was performed to achieve signals that would be comparable between them. Following calculation of expression values for each probe set the data were submitted to higher-level analysis. Pair-wise comparisons between the superficial and invasive samples were performed to identify genes that were differentially expressed more than two-fold at a statistically significant level ($p < 0.005$). Sample and gene clustering was performed, the former to identify any similarity between the two groups and the latter to identify genes with similar patterns of expression across all samples (refer to section 2.9.2 for full explanation of array data analysis). The resulting dendrogram in figure 3.1, red illustrates an increase in probe expression, blue represents a decrease in probe expression and white represents no change in expression.



Number on dendrogram	Tissue Bank number	Grade
1	03TB179	G2pTa
2	03TB146	G3pT1
3	03TB132	G2Pt1
4	04TB021	G2pTa
5	02TB065	G3pT3a
6	02TB040	NORMAL
7	04TB125	G3pT3b
8	04TB119	G3pT3b
9	02TB044	G3pT3b
10	02TB099	G3pT2b

Figure 3.1 Hierarchical clustering dendrogram of 21 genes (P value of 0.005). Comparing superficial bladder cancer (Ta-T1) with invasive bladder cancer (\geq T2).

Table 3.3 A list of probes altered in bladder tissue when comparing superficial bladder cancer with invasive bladder cancer (21 probes)

<i>Accession No</i>	<i>Gene title</i>	<i>Fold change</i> ¹	<i>P-value</i> ²
AA931476	CDNA FL37605 fis, clone BRCO201510	-2.91	0.004
AA806282	Transmembrane protein 154	-2.04	0.004
BC034348	Hypothetical protein LOC284837	-2.8	0.003
AL35975	Chromosome 1 open reading frame 93	-2.01	0.003
AA872727	Farnesyl-diphosphate farnesyltransferase 1	-2.5	0.005
BC002669	Nuclear receptor subfamily 2, group F, member 6	-2.54	0.002
NM_000666	Aminoacylase 1	-2.25	0.002
NM_000281	Pterin-4 alpha-carbinolamine dehydratase/demerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)	-2.1	0.004
NM_005558	Ladinin 1	-2.49	0.004
NM_007100	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit E	-0.207	0.004
	Ectonucleoside triphosphate diphosphohydrolase 5	-2.78	0.001
BE673587	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	-7.85	0.004

Table 3.3 continued A list of probes altered in bladder tissue when comparing superficial bladder cancer with invasive bladder cancer (21 probes)

<i>Accession No</i>	<i>Gene title</i>	<i>Fold change</i> ¹	<i>P-value</i> ²
NM_015865	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	-7.44	0.003
B007457	TP53 activated protein 1	-2.23	0.002
NM_014312	V-set and immunoglobulin domain containing 2	-3.99	0
NM_015675	Growth arrest and DNA-damage-inducible, beta	3.21	0.005
AL359575	chromosome 1 open reading frame 24	3.71	0.003
NM_002444	Moesin	2.73	0.004
NM_001155	Annexin A6	3.75	0.001
AW058622	Wiskott-Aldrich syndrome protein interacting protein	3.83	0.003
AA292373	Collagen, type VI, alpha 1	5.7	0.001

Table 3.3 A list of probes altered in bladder tissue when comparing superficial bladder cancer with invasive bladder cancer (21 probes). ¹The fold change shown represents the mean gene expression ratio calculated using the PM-only model on normalised arrays in dChip between the superficial bladder cancer tissue and the invasive bladder cancer tissue. The positive values represent an up-regulation and the negative values represent a down-regulation and as such correspond to the red and blue of the heat map in figure 3.1
²Genes that were differentially expressed more than two-fold at a statistical significant level (p<0.005)

3.3.3 GeneOntology

The biological role of genes that were differentially expressed more than two-fold at a statistical significant level ($p < 0.005$), their relationship with cancer and bladder cancer, if any, are discussed below:

Farnesyl-diphosphate farnesyltransferase 1

This gene encodes a membrane-associated enzyme located at a branch point in the mevalonate pathway. The encoded protein is the first specific enzyme in cholesterol biosynthesis, catalyzing the dimerization of two molecules of farnesyl diphosphate in a two-step reaction to form squalene (RefSeq) Inhibition of squalene synthase maybe a method of lowering cholesterol levels in the prevention of cardiovascular disease (Do, Kiss *et al.* 2009).

Nuclear receptor subfamily 2, group F, member 6

The protein transcribed by this gene has many functions such as metal ion binding, sequence-specific DNA binding, steroid hormone receptor activity, transcription factor activity and zinc ion binding, however there is no association with cancer in the literature (RefSeq)

Aminoacylase 1

Aminoacylase-1 (Acy1) is a cytosolic, homodimeric, zinc-binding enzyme that catalyzes the hydrolysis of acylated L-amino acids to L-amino acids and acyl group(RefSeq). Its expression has been reported to be reduced or undetectable in small-cell lung cancer cell lines and tumours (Miller, Minna *et al.* 1989). The Acy1 gene which is highly expressed in the kidney, displays a significantly decreased level of expression in rat renal cell carcinoma and an increased number of apoptotic cells were observed following Acy1 transfection

(Hwa, Park *et al.* 2005). The array results also indicate a decrease of 2.25 fold in the invasive tissue.

Pterin-4 alpha-carbinolamine dehydratase/demerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)

This gene encodes pterin-4 alpha-carbinolamine dehydratase, an enzyme involved in phenylalanine hydroxylation. A deficiency of this enzyme leads to hyperphenylalaninemia. The enzyme regulates the homodimerization of the transcription factor hepatocyte nuclear factor 1 (HNF1) (RefSeq).

Ladinin 1

The protein encoded by the Ladinin 1 gene may be an anchoring filament that is a component of basement membranes (RefSeq). It may contribute to the stability of the association of the epithelial layers with the underlying mesenchyme (RefSeq). A decrease of 2.49 fold was seen in the invasive bladder tissue, perhaps indicating destruction of the ECM and basement membrane as the tumour invades. However gene expression profile analysis of ovarian serous papillary carcinomas (OSPCs) and normal ovarian (NOVA) epithelium showed an over expression of the Ladinin 1 gene in OSPC compared to NOVA (Santin, Zhan *et al.* 2004).

ATP synthase, H⁺ transporting, mitochondrial F₀ complex, subunit E (ATP5I)

Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. A study has shown that antisense of ATP5I inhibits the growth of human hepatocellular carcinoma cells. The authors suggested that antisense of ATP5I can inhibit cell proliferation through the MAP kinase pathway and also that ATP5I may become a new target in gene therapy (Ying, Yu *et al.* 2001).

Ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5/ PCPH)

The protein which is encoded by this gene may play an important role in mediating catabolism of extracellular nucleotides (RefSeq). Reports have shown that ENTPD5 is mutated or deregulated in some human tumours, suggesting its participation in malignant progression. A recent immunohistochemical analyses study has shown that ENTPD5 is not expressed in normal prostate, but its expression increases along cancer progression stages, being detectable in benign prostatic hyperplasia, highly expressed in prostatic intraepithelial neoplasia, and remaining at high levels in prostate carcinoma (Villar, Arenas *et al.* 2007). However in this study ENTPD5 shows a decrease in expression in the invasive tissue.

Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)

The protein encoded by this gene is a membrane transporter that mediates urea transport in erythrocytes. This gene forms the basis for the Kidd blood group system and the literature does not associate the gene with cancer.

TP53

p53, is a transcription factor which in humans is encoded by the *TP53* gene. p53 is important in multicellular organisms, responding to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism and thus functions as a tumor suppressor (RefSeq). p53 protein is a DNA-binding protein expressed at low level in normal cells and at a high level in a many transformed cell lines. If the *TP53* gene is damaged, tumour suppression is severely reduced and more than 50 percent of human tumours contain a mutation or deletion of the

TP53 gene. The array data indicates a down regulation of this tumour suppressor gene in the more invasive TCC.

Growth arrest and DNA-damage-inducible, beta

Growth arrest and DNA-damage-inducible, beta responds to environmental stresses by mediating activation of the p38/JNK pathway(RefSeq). This activation is mediated via their proteins binding and activating MTK1/MEKK4 kinase, which is an upstream activator of both p38 and JNK MAPKs(RefSeq). The gene has been verified to be specific in hepatocellular carcinoma and consistent with the degree of malignancy (Qiu, Zhou *et al.* 2007). A recent study showed the induction of growth arrest DNA damage-inducible gene in human hepatoma cell lines by S-adenosylmethionine (Yamamoto and Negishi 2008) Also, a DNA microarray analysis study investigating gastroenteropancreatic neuroendocrine tumours, showed an up-regulation of this gene in the malignant group (Qiu, Zhou *et al.* 2007). In this study an up-regulation of 3.21 was observed in the invasive bladder tissue.

Moesin

Moesin functions as a cross-linker between plasma membranes and actin-based cytoskeletons. Moesin is localized to filopodia and other membranous protrusions that are important for cell-cell recognition and signalling and or cell movement. An increase in Moesin protein expression is a marker of the basal subtype breast tumours (Charafe-Jauffret, Ginestier *et al.* 2006). In a recent study increased Moesin expression was shown to be nearly an independent prognostic marker of poor outcome in these patients (Charafe-Jauffret, Monville *et al.* 2007). From the bladder array study an increase of 2.73 fold in gene expression can be seen in the invasive TCC biopsy samples.

Annexin A6

Annexin A6 has been shown to inhibit Ras signalling in A431 breast cancer cells (RefSeq). Over expression of epidermal growth factor receptor (EGFR) is associated with enhanced activation of wild-type (hyperactive) Ras in breast cancer and the association of AnxA6 with H-Ras-containing protein complexes may contribute to regulate p120GAP/Ras assembly in EGFR-overexpressing breast cancer cells (Vila de Muga, Timpson *et al.* 2009). Annexin A6 has also been identified as a possible new marker of minimal residual disease (MRD) in B-lineage acute lymphoblastic leukemia (ALL) (Chen JS 2001). Using cDNA array analysis, Annexin A6 was shown to be expressed in B-lineage ALL cells at higher levels than in normal. In this study Annexin A6 was shown to have a increased fold change of 3.75 in the more invasive bladder biopsies.

Wiskott-Aldrich syndrome protein interacting protein (WASP)

This protein is involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton and plays a role in the regulation of actin-based motility (RefSeq). WASP is predominantly expressed in hematopoietic cells and regulates the reorganisation of the actin cytoskeleton in response to various cell stimuli, including T cell receptor signalling (RefSeq). Mutations in this gene encoding the WASP protein causes Wiskott-Aldrich syndrome (WAS) which is an X-linked primary immunodeficiency (Ramesh and Geha 2008). The WASP family proteins initiate directed actin assembly in the leading edge of the migrating cell (RefSeq). It has been shown that IQ motif containing GTPase activating protein 1 (IQGAP1) stimulates actin assembly through the N-WASP-Arp2/3 pathway. IQGAP1 is a conserved modular protein over expressed in many cancers and is involved in organising actin and microtubules in motile processes such as adhesion,

migration, and cytokinesis (Le Clainche C 2007). In this study the gene showed an increase of expression.

There is very little information in the literature regarding the following genes and no associations with cancer.

- **V-set and immunoglobulin domain containing 2**
- **Chromosome 1 open reading frame 24**
- **CDNA FL37605 fis, clone BRCO201510**
- **Transmembrane protein 154**
- **Chromosome 1 open reading frame 93**

There is very little literature regarding the following gene and its function is unknown.

- **Hypothetical protein LOC284837**

Collagen, type VI, alpha 1

Will be discussed later in this chapter (section 3.4.5)

3.3.4 Confirmation of Affymetrix Array results

For publication purposes, microarray results require verification and validation by an alternative and complementary gene expression profiling method. RT-PCR was used to validate the microarray data, analyzing transcript levels for two genes which significantly changed. Time permitting it would have been helpful to analyzing transcript levels for genes significantly changed in each direction and also transcript levels of genes whose expression was unchanged between the two sample populations. In this instance the selection of the genes for follow-up analysis was influenced by their possible biological function within the ECM in relation to cancer invasion . As such, perhaps does not validate/confirm the large data sets that was generated by the microarray but does confirm the up-regulation of these two genes in invasive tissue in independent sample. To confirm the large data set more conclusively a combination of analysis would be required. Independent confirmation of results such as *in silico* analysis and further laboratory-based analysis would be required. Furthermore to investigate whether the expression profiles are a universal feature of the biological phenomenon under study—in other words, are the data an essential descriptor of the biological state? This question could be addressed by evaluating a critical gene set in a larger and more extensive study group, which can be done either *in silico* or in the laboratory (Chuaqui, Bonner et al. 2002).

To validate the changes in these two chosen genes primers and probes were designed and optimised for Moesin and Collagen, type VI, alpha 1 (section 2). The invasive bladder tissue showed a significant ($p < 0.005$) increase of COL6A1 and Moesin expression in the array data of 5.7 and 2.73 fold respectively (Table 3.3). This was further confirmed with real time RT-PCR, and the extent of the increase of Moesin was approximately five fold greater than observed in the array data (data not shown). The extent of COL6A1 mRNA expression was much greater in the real time RT-PCR data, showing approximately a 40 fold increase of mRNA expression when comparing the superficial with the most invasive

tissue (Figure 3.4). This phenomenon of Affymetrix arrays underestimating the actual mRNA level expression has previously been reported to occur in Caco2 cells (Traka, Gasper *et al.* 2005). RT-PCR is more sensitive than microarray analysis and as such microarray results can rather underestimate actual changes in gene expression. From the seven remaining tissue samples I was able to select one sample with an invasive classification and one sample with a superficial classification for real time RT-PCR analysis (figure 3.2)

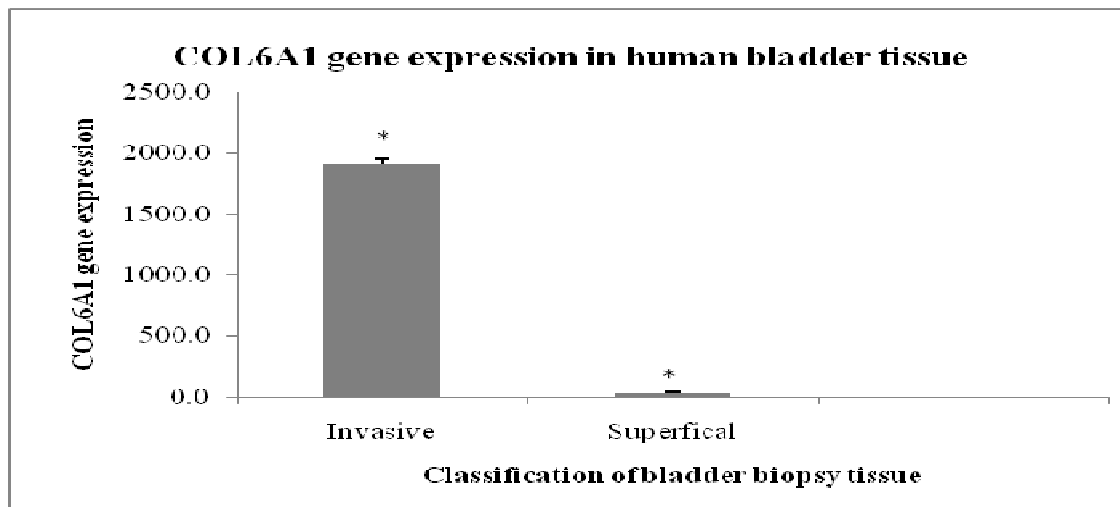


Figure 3.2 The relationship of COL6A1 gene expression and grade of bladder cancer. Real time RT-PCR confirmation of COL6A1 expression in human bladder tissue. Data is shown from three biological replicates plotted with SD (* $p < 0.005$). It can be seen from the graph that COL6A1 significantly increases with the grade of bladder cancer.

3.4 Discussion

3.4.1 Glutathione-S-transferase

There are three alleles at the *GSTM1* locus: *GSTM1 0*, *GSTM1 A*, and *GSTM1 B*. *GSTM1 0* is a deletion and homozygotes (*GSTM1 0/0* or *GSTM1* null genotype) express neither mRNA nor *GSTM1* protein/enzyme. Alleles *GSTM1 A* and *GSTM1 B* encode monomers that form the homo- and heterodimeric enzymes. *GSTM1 A* and *GSTM1 B* differ by a C→G substitution at base position 534, resulting in Lys→Asn substitution at amino acid 172. In

many studies *GSTM1* 0/A, 0/B, A/A, A/B, and B/B are typically categorized together as a single functional *GSTM1* positive phenotype as there is said to be little evidence of functional difference between *GSTM1* A and *GSTM1* B. The enzyme activity of *GSTM1* and *GSTT1* is absent in approximately 50 % and 15 % of the population, respectively, due to deletions of both chromosomal copies of the genes. A trimodal phenotype pattern exists in which individuals with two, one, or no functional genes are fast, intermediate, or slow "conjugators" respectively (Buchard, Sanchez *et al.* 2007). Most studies investigating the effect of the *GSTM1* and *GSTT1* deletions do not distinguish between fast and intermediate conjugators. Here the three alleles were distinguished although there are too few samples to draw any real conclusions.

From the 12 tissue biopsy samples 58 % were *GSTM1* null, which would result in a lack of *GSTM1* enzyme activity in these individual's bladder tissue. This corresponds to the observed homozygous deletion of this gene in approximately 51 % for those of European descent. The prevalence of *GSTT1* null individuals is 10% to 20% among Caucasians. In this data set only one individual was *GSTT1* null (8%), which is also consistent; however once again there are too few samples to draw any real conclusions. The *GSTM3* gene has a three-base-pair deletion in intron 6, and the two alleles are referred to as *GSTM3*^{*}A and *GSTM3*^{*}B. This deletion creates a recognition motif (-aagata-) for the YY1 transcription factor which could potentially affect detoxification activity by *GSTM3* B allele. That is, genotypes *GSTM3*^{*}AB and *GSTM3*^{*}BB potentially affect detoxification activity of *GSTM3* enzyme. There is little work in relation to bladder cancer and the *GSTM3*^{*}B allele, however risk of developing prostate cancer has been associated with *GSTM3* AB, and BB was 2.5-fold as compared to AA genotype (Kesarwani, Singh *et al.* 2008). The *GSTP1* gene displays a polymorphism at codon 105

(*Ile105Val*) resulting in an enzyme with altered substrate affinity (Ali-Osman, Brunner *et al.* 1997). Approximately 10% of Caucasians are homozygous for this mutation and 40% are heterozygous (Voho, Impivaara *et al.* 2006). A Valine residue at position 105 of the GSTP1 protein results in decreased enzyme activity. None of the samples were homozygous for the mutation and 33% were heterozygous G/O for the mutation and 42% were G/A heterozygous for the mutation and the remaining 25% did not have the mutation and were heterozygous A/O.

I sort to explore the frequency of the GSTT1, GSTM1, GSTM3 and GSTP1 genetic polymorphisms between the superficial and invasive tissue types however the small number of cases makes it impossible to reach substantial conclusions. As discussed above, many of the results correspond to published data and a difference in genetic polymorphism frequency between superficial and invasive tissue was not observed. In order to achieve this, a much larger sample population would be needed.

3.4.2 Specific bladder tumour grades have distinct gene expression profiles

The fold changes in table 3.3 correspond to the colour intensities in Figure 3.1. The fold changes and the colour intensities are the amount of florescence omitted, and is a reflection on the expression of the corresponding gene. When comparing superficial with invasive bladder cancer, a mean decrease in expression of a particular gene is indicated by blue and a mean increase of expression is indicated by red. Pair-wise comparisons between the superficial (Ta-T1) and invasive (\geq T2) samples identified 21 genes that were differentially expressed more than two-fold at a statistical significant level ($p < 0.005$). It can be observed that this microarray analysis identified patients with two clinically distinct phenotypes. That is clinically relevant subclasses of bladder TCC have been identified. Using unsupervised

sample clustering, genes clustered into two clinically distinct groups; the superficial transitional cell carcinoma of the bladder (Ta- T1) into one group and the muscle invasive transitional cell carcinoma of the bladder (\geq T2) into another. These findings suggest the possibility of not only distinguishing invasive from superficial lesions, but also separating carcinoma *in-situ* from superficial lesions and subgroups within early-stage and invasive tumours displaying different overall patient survival. Additionally it may be a valuable tool to recognize early-stage tumours showing gene profiles similar to invasive disease and as such gene profiling may be used to successfully classify bladder tumours based on their progression and clinical outcome. Further studies to identify molecular biomarkers of potential clinical significance and identification of genes differentially expressed between invasive bladder cancer and non-invasive bladder cancer progression are needed.

3.4.3 The field effect theory of urothelial epithelial cells carcinogenesis

The concept of "field cancerization" (field effect theory) was first introduced by Slaughter *et al.* (Slaughter, Southwick *et al.* 1953) in 1953 when studying the presence of histologically abnormal tissue surrounding oral squamous cell carcinoma. It was proposed to explain the development of multiple primary tumours and locally recurrent cancer in oral squamous cell carcinoma. Recent molecular findings support the carcinogenesis model in which the development of a field with genetically altered cells plays a central role in the reoccurrence of cancer. In this study an invasive-like pattern was observed in the bladder tissue taken from the 'normal' area of the cancerous bladder of patient OTB040 (Sample 6 in the dendrogram, figure 3.1), indicating molecular changes in the tissue which histologically presented as benign to the histologist. This supports the "field effect" theory of urothelial epithelial cells carcinogenesis (Jones, Wang *et al.* 2005). It has been shown

that multiple coexisting tumours often arise before clinical symptoms become apparent and that separate tumours may or may not share a similar histology. Two theories have been proposed which suggest a monoclonal or an oligoclonal origin of multifocal urothelial carcinomas (Cheng, Jones *et al.* 2005). The field effect theory suggests that carcinogenic changes have altered the epithelium rendering it more susceptible than "healthy" tissue to developing malignant tumours. Another possible cause for this apparent breakdown of the urothelium is the implantation theory which proposes that multiple tumours are the result of tumour cells that have been shed from an original tumour higher in the urinary tract. The tumour cells then attach to the urothelium in a different site and begin to grow. Recurrences occur in more than 60% of patients and are found at sites remote from the primary tumour and therefore both these theories may not only account for the frequent development of genetically unrelated tumours within the bladder, but also the high rate of reoccurrence of bladder TCC. Obviously it is of clinically important to develop genetic detection for recurrent or residual tumour cells and these results provide evidence to suggest that diagnosis and treatment of bladder cancer should not only be focused on the tumour but also on the field from which it developed.

3.4.4 Rationale for gene selection from microarray results of tissue to investigate in bladder cell culture models.

Bladder cancers represent a spectrum of diseases that can be grouped into three general categories: superficial, invasive and metastatic. Each differs in clinical behaviour, prognosis and primary management. The choice of genes to study was based upon the sample population of the tissue array data. As the array data was comparing superficial with more invasive grades of bladder cancer and there was neither a benign nor a metastatic group to

compare with, the molecular changes between the two groups may imply changes which would occur during progression of disease to an invasive state. Tumour invasion is defined as the penetration of tissue barriers, such as the basement membrane, by migrating cancerous cells (Pirila, Ramamurthy *et al.* 2003; Pirila, Sharabi *et al.* 2003; Matias-Roman, Galvez *et al.* 2005; Hwang and Lee 2006). Muscle invasive bladder cancer ($\geq T2$) and high risk superficial bladder cancer carry a significant metastatic potential despite radical surgical treatment (Hart and Saini 1992; Cheng, Weaver *et al.* 2000; Dalbagni, Genega *et al.* 2001; Gontero, Banisadr *et al.* 2004). Since the majority of bladder cancer mortality is associated with invasion into the bladder muscle wall and the progression to metastatic disease, the ability to inhibit cell segregation from the primary tumour and invasion of surrounding tissues would significantly reduce bladder cancer related morbidity and mortality. Early detection of molecular changes that enable or facilitate cancer cells to metastasize may help to identify low risk superficial from high risk superficial and more invasive bladder cancer that deserve more aggressive treatment (Gontero, Casetta *et al.* 2004). These molecular changes are said to be over and above those required for tumorigenesis, such as changes in cell cycle related proteins. Cell cycle related proteins and oncogenes have been shown to be able to confer unrestricted growth potential to cells but not necessarily have invasive and metastasis properties (Gontero, Banisadr *et al.* 2004). Tumour cells frequently invade surrounding tissues as the tumour begins to grow. Next, capillary endothelial cells invade the tumour and create tumour blood vessels. Thereafter, some tumour cells intravasate into the blood circulation for metastasis, where host immune cells invade the tumour. Finally, tumour cells arrest in distant organs, extravasate and migrate into new metastatic sites and then start the invasion cycle again (Matias-Roman, Galvez *et al.* 2005; Hwang and Lee 2006). Current models of carcinogenesis suggest that there are three major steps in the

molecular pathway that allow the cancer cells to acquire the metastatic phenotype: 1) breakdown in cell-cell and cell matrix adhesion, 2) modulation of the extracellular matrix (ECM), proteolysis and adhesion and 3) induction of angiogenesis. Therefore it was thought that the gene and protein expression patterns of ECM genes such as Collagen, type V1, alpha 1 maybe of more importance and more relevance to this particular study group. The microarray results show that the expression of COL6A1 was increased in the invasive group in comparison with the superficial group and therefore selected for further study with bladder tissue and in the bladder cell culture model, UM-UC-3.

Collagen VI has a ubiquitous distribution throughout connective tissues where it forms an extensive microfibrillar network, linking cells and many matrix components (Baldock, Gilpin *et al.* 2002; Baldock, Sherratt *et al.* 2003; Ball, Bella *et al.* 2003). They are important in maintaining the integrity of the tissues. Collagen types VI are integral to the formation of collagen fibres which are a major component of the ECM. The ECM supports most tissues and gives cells structure from the outside and is essential for cell differentiation and associated gene expression

Collagen VI is a large collagenous glycoprotein composed of three different α -chains, the $\alpha 1$ (VI), $\alpha 2$ (VI) and $\alpha 3$ (VI) (Chu, Mann *et al.* 1987; Baldock, Sherratt *et al.* 2003). Collagen, type V1, alpha 1, which has been shown to be unregulated in the more invasive tissue type by the microarray data, is an adhesive glycoprotein of the ECM and is evenly distributed throughout the stroma, but mainly associated with the basal lamina, collagen fibrils, and around the stromal cells.

Although there is no known work that I'm aware of on collagen V1 and bladder cancer, the potential importance of collagen VI protein has been studied in relation to other cancers such as prostate, colon and breast cancer. Increased stromal expression of collagen VI has

been correlated with various aspects of tumorigenesis and malignant progression and Lyengar *et al.* suggests collagen VI maybe an early diagnostic marker of mammary hyperplasia and a growth-promoting factor that maybe inhibited to reduce mammary tumour progression (Iyengar, Espina *et al.* 2005).

It has been shown that type VI collagen is upregulated during murine mammary tumour progression (Iyengar, Combs *et al.* 2003). Specifically, the $\alpha 3$ subunit of collagen VI is upregulated in the stroma surrounding colonic tumours compared with that surrounding normal tissue (Iyengar, Espina *et al.* 2005). Exposure of fibroblasts to collagen VI promotes proliferation and upregulation of cyclin D1 (Ruhl, Johannsen *et al.* 1999).

Furthermore, the protein inhibits apoptosis in a variety of cell types (Howell and Doane 1998). TGF- β -expressing melanoma cells induce collagen VI expression in mammary stroma, facilitating tumour progression and invasiveness (Berking, Takemoto *et al.* 2001).

Most recently, increased collagen VI, in particular COL6A3 expression in the ECM has been associated with the promotion of chemoresistance in ovarian cancer cells (Sherman-Baust, Weeraratna *et al.* 2003; Varma, Hector *et al.* 2005). In contrast

immunohistochemical investigation of primary biphasic synovial sarcomas showed low frequency of collagen type II and VI expression in the extracellular matrix of the tumours

(Osmanov Iu, Petrovichev *et al.* 2005). In prostate, collagen XXIII expression has been associated with prostate cancer recurrence and distant metastases. The levels of collagen

XXIII is a significant independent predictor of PSA-defined disease recurrence, suggesting a potential role as a molecular biomarker of prostate cancer progression and metastasis

(Banyard, Bao *et al.* 2007). Although in a recent experiment the gene expression of human collagen type VI was shown to be remarkably decreased in suspension-adapted PC-3 (saPC-3) cells (Patrikainen, Porvari *et al.* 2007). However Kopp *et al.* provide evidence that the

expression of collagen VI is completely inhibited in virally transformed fibroblasts and in many cell lines derived from spontaneous mesenchymal tumours and that DNA methylation plays an important role in this inhibition (Kopp, Winterhalter *et al.* 1997).

Collagen prolyl hydroxylases are required for proper collagen helix synthesis. Hypoxia is the microenvironment in which carcinomas thrive and results in the induction of a cluster of hypoxia related genes, shown in a recent cell culture study using hepatoma HepG2 cells (Cutroneo, White *et al.* 2007). The cellular response to hypoxia is characterized by an enhanced deposition of extracellular matrix (ECM) components, mainly collagens (Fahling, Perlewitz *et al.* 2004) and therefore may account for the increased expression of COL6A1 in the more advanced tumour tissue. The results from the Fahling *et al.* study indicate that hypoxia affects collagen homeostasis in a biphasic manner concerning basic mechanisms of gene expression. Proline 4 hydroxylase (P-4-H-alpha) subunits are said to be up-regulated at the transcriptional and translational level (Fahling, Perlewitz *et al.* 2004) and this is also observed in this microarray data. Collagen homeostasis is determined by the rate of synthesis and degradation. An earlier study using rat vascular smooth muscle cell line A7r5, found that an hypoxic atmosphere caused a characteristic time-dependent 5 to 12-fold up-regulation of the mRNAs of P-4-H, among other hypoxia related enzymes and strong evidence suggests that the expression of these enzymes during hypoxia is coordinated by HIF-1 (Hofbauer, Gess *et al.* 2003; Cutroneo, White *et al.* 2007; Cutroneo, White *et al.* 2007).

It has been speculated that in gastric carcinoma, activated fibroblasts participate in collagen biosynthesis at the tumour periphery rather than in the tumour centre and that increased collagen biosynthesis at the tumour periphery in gastric carcinoma may assist further invasion of tumour cell (Matsui, Kubochi *et al.* 1999). A quantitative RT-PCR assay was

designed and optimized for COL6A1 to validate array data and for further investigation in chapter five.

In summary, although the prevalence of the GST polymorphisms studied were in accordance with published data, it was very difficult to make any real conclusions regarding the results, due to the inadequate sample size. However the array analysis successfully identified two clinically distant phenotypes following unsupervised sample clustering. For the first time it has been shown that bladder tumours have distinct gene expression profiles in accordance to their grade. The array data also supported the field effect theory of urothelial cell carcinogenesis, by highlighting molecular changes in tissue which appeared histologically benign. In the following chapter the aim is to characterise the human bladder cancer cell lines UM-UC-3 and RT4 for the GST polymorphisms most extensively studied in bladder cancer and to explore the effects of two isothiocyanates, iberin and sulforaphane on phase 2 gene expression in these cell lines. Furthermore in chapter five these characterised cell lines will be used to explore the relationship of the COL6A1, highlighted in this chapter, on the grade of bladder cancer and explore the effects of iberin and sulforaphane on COL6A1 gene expression.

Chapter Four

*Characterisation Of Human Bladder
Cell Cultures And The Effects Of
Sulforaphane And Iberin On Phase 2 Gene
Expression.*

CHAPTER 4: Characterisation Of Human Bladder Cell Cultures And The Effects Of Sulforaphane And Iberin On Phase 2 Gene Expression.

4.1 Introduction

In the previous chapter I characterised the bladder tissue for the most extensively studied GST polymorphisms; GSTM1 and GSTT1 null genotypes plus allele discrimination of the GSTM1 positive samples, the GSTpi (Ile105Val polymorphism/ Ala114Val polymorphism), and the GSTM3 3 base pair deletion. In this chapter I describe the characterisation of UM-UC-3 and the RT4 human bladder cell lines, investigating genotype, gene expression, methylation status and enzyme activity of the GSTM1 gene.

Subsequently I explore the effects of sulforaphane and iberin and their *N*-acetyl-L-cysteine conjugate on the GST, and NQO1 expression in these human bladder cell lines. SF is highly reactive and has a very short half-life in the body, forming a glutathione conjugate that is further metabolized to the *N*-acetylcysteine conjugate (SF-NAC), which is the major excretory product found in the urine (Hwang and Jeffery 2005). This conjugate is a reversible complex, able to release free SF (Tang and Zhang 2004; Hwang and Jeffery 2005). As bladder cancer is being investigated in this project and the urine, with its potentially high concentration of SF and/or SF-NAC, collects and is stored in direct contact with the bladder epithelial tissue where the tumour originates, I also aim to investigate whether the SF-NAC and IB-NAC have a similar biological effect as their parent compounds.

4.2 Specific material and methods

Both DNA for genotyping and RNA for gene expression analysis were carried out in accordance with the manufactures recommendation (see section two). Total GST activity was determined in both bladder cell lines (see section two). A quantitative methylation specific PCR (QMSP) assay approach was used for the measurement of the methylation status of the GSTM1 gene in six bladder tissue biopsies and two bladder cell lines (see section two). UM-UC-3 and RT4 cell lines were obtained from the European collection of animal cell cultures (ECACC). UM-UC-3 (passage 18) and RT4 (passage 9) established cell cultures were seeded into a six well plate and treated with 12 μ M of SF, IB or SF-NAC for 48 hours (see section 2). Iberin and sulforaphane were purchased from LKT Laboratories and the cell treatments occurred in complete media with less than 0.1% dimethyl sulphoxide (DMSO, Sigma-Aldrich). The *N*-acetylcysteine conjugates were made by Dr Paul Needs, IFR chemist.

4.3 Results

4.3.1 Genotyping of UM-UC-3 and RT4 cell lines and base line gene expression.

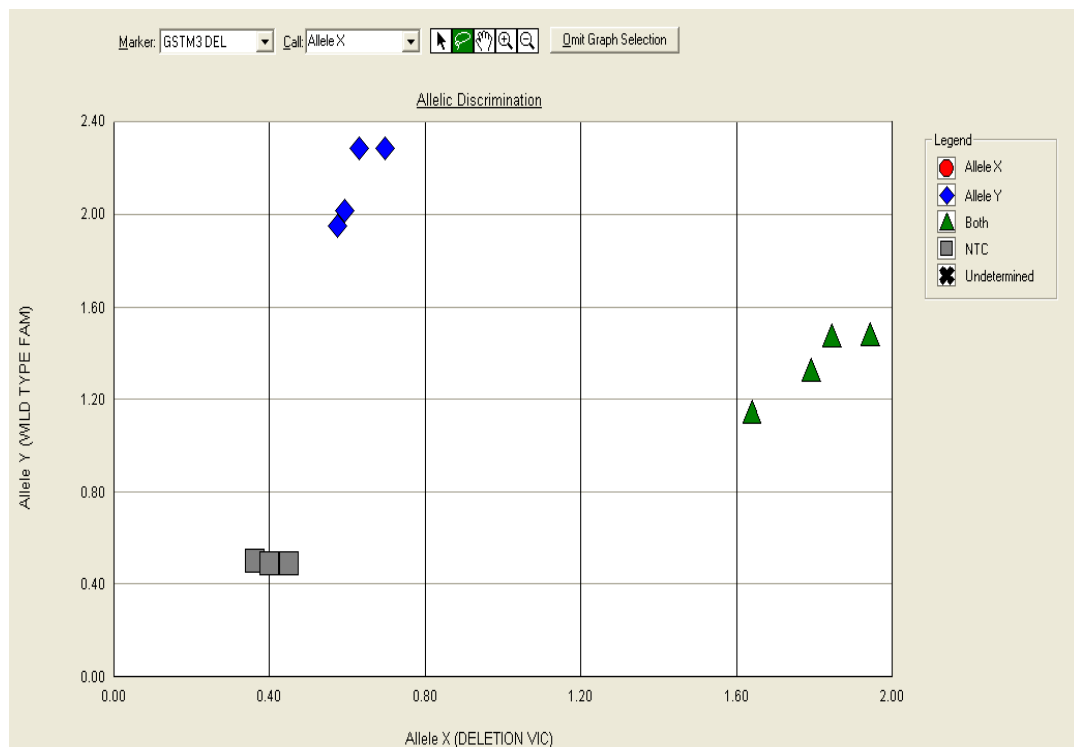


Figure 4.1 GSTM3 allelic discrimination of bladder cells.

▲ Indicate both A and B alleles resulting in A*B* genotype for the UM-UC-3 bladder cell line.

◆ Indicates two copies of the A allele resulting in A*A* genotype for the RT-4 cell line

■ Negative control

As can be seen in Figure 4.1, RT4 cells show a high reading on the Y axis with a low reading on the X axis which suggests two copies of the A allele and therefore corresponds with the wild type A*A* genotype. A high reading on the X axis with a low reading on the Y axis would suggest two copies of the B allele and as such, correspond with the B*B* genotype. Therefore, as the UM-UC-3 bladder cells show similar readings on both the X and Y axis which is roughly half the intensity of the A*A* genotype, then this would suggest one of each allele which corresponds with the A*B* genotype.

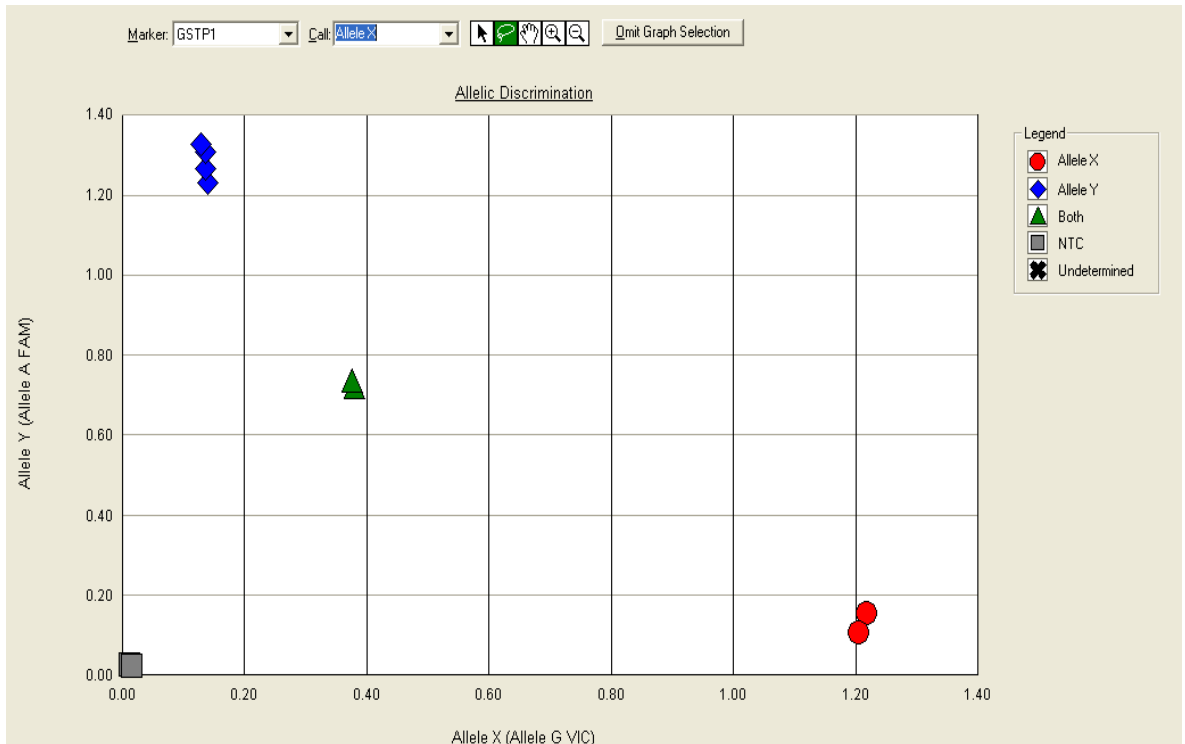


Figure 4.2 GSTP1 allelic discrimination of bladder cells

- ◆ Indicates two copies of the A allele resulting in A*A* genotype for the RT-4 cell line.
- Indicates two copies of the G allele resulting in G/G genotype for the UM-UC-3 cell line
- ▲ Positive control (known G/A genotype)
- Negative control

As seen in Figure 4.2, RT4 cells show a high reading on the Y axis with a low reading on the X axis suggesting two copies of the A allele and therefore corresponds with the A*A* genotype. UM-UC-3 cells show a high reading on the X axis with a low reading on the Y axis which suggests two copies of the G allele and as such corresponds with the G/G genotype.

The RT4 cell line is null for the GSTM1 gene and therefore no gene expression was observed. Although it was shown in table 4.1 that the UM-UC-3 cell line has 1 copy, there is no detectable expression of this gene. This may be due to methylation of the GSTM1 gene and this will be investigated later in this chapter.

Cell line	GSTM1	GSTM3	GSTT1	GSTP1
UM-UC-3	1 copy	A/B	A/A	G/G
RT4	Null	A/A	null	A/A

Table 4.1 Genotype characterisation of the two bladder cells lines

Cell line	GSTM1	GSTM3	GSTT1	GSTP1
UM-UC-3	No	yes	yes	Yes
RT4	N/A	yes	N/A	No

Table 4.2 Baseline gene expression of the two bladder cell lines

4.3.2 Total GST activity and the methylation of the GSTM1 gene.

Interestingly, it was observed that although the cell line UM-UC-3 has a positive GSTM1 genotype, there was no detectable gene product. The subsequent low expression of the GSTM1 enzyme appears to correlate with the relatively high percentage of methylation of the gene in this cell culture (64.1%, table 4.3), however further investigations are needed to be able to connect the two findings. Another interesting observation is that the UM-UC-3 cell line shows comparably high total GST activity; since the cell line is not expressing the GSTM1 gene and thus the protein/enzyme, this high total GST activity can not be as a result of the GSTM1 gene.

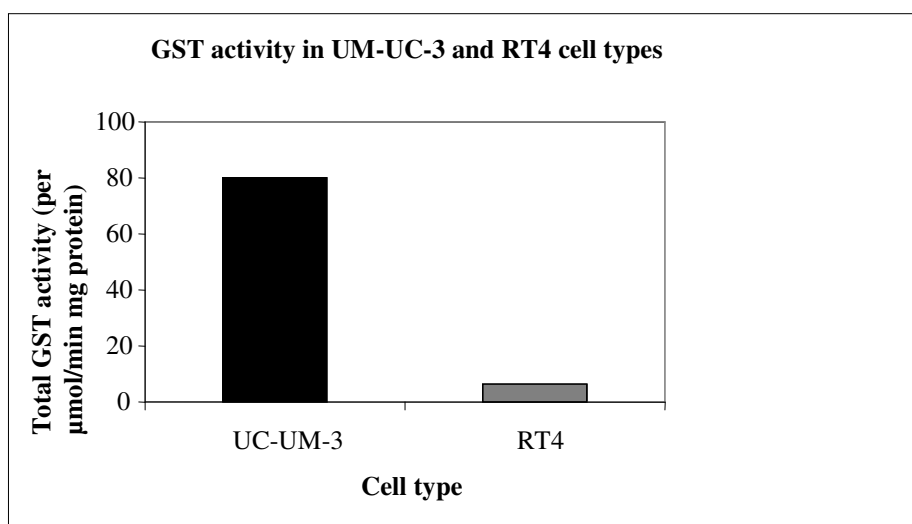


Figure 4.3 Total GST activity in the human cell lines.

The UM-UC-3 cell type shows a much greater degree of total GST activity than the RT4 cell type. As the RT4 cell type is null for both GSTM1 and GSTT1 and does not express GSTP1, and the UM-UC-3 cell type expresses all the GSTs studied except GSTM1, this may explain the low degree of total GST activity of the RT4 cell type in comparison to the UM-UC-3 cell type.

The results in Table 4.3 show the percentage of methylation detected by two methylation primer pairs in the GSTM1 gene and the standard deviation (Std. Dev) of the three replicates. Another bladder cell line was investigated (R24) as the RT4 cell line could not be used in this study as it is null for the GSTM1 gene.

Tissue bank number/cell type	Primer 1 ¹		Primer 2 ²	
	% methylation	Std. Dev	% methylation	Std. Dev
02TB058	9.99	0.91	10.79	0.49
02TB044	0.53	0.03	0.44	0.01
03TB146	0.62	0.09	0.49	0.01
03TB179	5.36	0.37	6.41	0.37
04TB119	3.32	0.39	2.87	0.10
04TB119	0.50	0.05	0.48	0.01
UM-UC-3	64.10	14.86	18.70	0.41
R24	0.34	0.03	0.34	0.01

Table 4.3 Methylation studies of the GSTM1 gene in UM-UC-3 and R24 bladder cell lines and selected bladder biopsy samples (in collaboration with Mr Jim Bacon)

¹ - Primer 1

² - Primer 2

4.3.3 Modulation of phase 2 gene expression following physiologically relevant treatments of SF, IB or SF-NAC in the UM-UC-3 and RT4 cell line.

In the UM-UC-3 human bladder cell line GSTP1 gene expression is significantly up-regulated following the 48hr treatments with 12 μ M of SF (up-regulation of 1.7, p0.008) and IB (up-regulation of 1.4, p0.3), but not following 48hr treatments with 12 μ M of SF-NAC. The RT-4 cell line showed no altered GSTP1 gene expression.

In the UM-UC-3 cell line GSTM3 gene expression is significantly up-regulated following 48hr treatments with 12 μ M of SF (up-regulation of 1.7, p0.01) and IB (up-regulation of 1.4, p0.02), but not following 48hr treatments with 12 μ M of SF-NAC. In the RT4 cell line GSTM3 gene expression is significantly up-regulated following 48hr treatments with 12 μ M of SF (up-regulation 12, p-0.001) and IB (up-regulation 7, p0.002) and also following the treatment of 12 μ M of SF-NAC (up-regulation 6, p0.002).

NQO1 gene expression is significantly up-regulated following 48hr treatments with 12 μ M of SF (up-regulation 7, p0.001), IB (up-regulation 5, p0.005) and also to a greater extent SF-NAC (up-regulation 8, p0.01) in the RT4 cell line. NQO1 gene expression is significantly up-regulated following 48hr treatments with 12 μ M of SF (up-regulation 3.5, p0.007) and IB (up-regulation 2.1, p0.03) and 12 μ M of SF-NAC (up-regulation 1.4, p0.03) in the UM-UC-3 cell line.

The RT-4 cell line was null for GSTM1 and GSTT1 and therefore showed no expression of these genes. The UM-UC-3 cell line is also null for the GSTT1 gene and as such showed no expression of the gene. The UM-UC-3 cell line showed no modulation of expression in the GSTM1 gene following the 48hr treatments.

Gene	12μM 48hr treatment					
	SF		IB		SF-NAC	
	Fold ¹	p-value	fold	p-value	fold	p-value
GSTP1	1.7↑ ²	0.008	1.4↑	0.3		ns ³
GSTM1		ns ³		ns ³		ns ³
GSTM3	1.7↑	0.01	1.4↑	0.02		ns ³
GSTT1		N/A ⁴		N/A ⁴		N/A ⁴
NQO1	3.5↑	0.007	2.1↑	0.03	1.4↑	0.03

Table 4.4 Changes in gene expressions following physiologically relevant treatments of SF, IB or SF-NAC in the UM-UC-3 cell line

¹- Mean gene fold change following 48 hour treatment

²- ↑/↓ Increase or decrease in mean gene folds change

³- No significant change in gene expression

⁴- Not applicable as the cell line is null for the gene.

Real time RT- PCR expression of GSTs in the UM-UC-3 cell line following a 48 hour treatment with 12μM of SF, IB or SF-NAC. The results are shown as mean expression of the three replicates.

gene	12μM 48hr treatment					
	SF		IB		SF-NAC	
	Fold ¹	p-value	fold	p-value	fold	p-value
GSTP1		ns ³		ns ³		ns ³
GSTM1		N/A ⁴		N/A ⁴		N/A ⁴
GSTM3	12↑ ²	0.001	7↑	0.007	6↑	0.002
GSTT1		N/A ⁴		N/A ⁴		N/A ⁴
NQO1	7↑	0.001	5↑	0.005	8↑	0.014

Table 4.5 Changes in gene expressions following physiologically relevant treatments of SF, IB or SF-NAC in the RT4 cell line.

¹- Mean gene fold change following 48 hour treatment

²- ↑/↓ Increase or decrease in mean gene fold change

³- No significant change in gene expression

⁴- Not applicable as the cell line is null for the gene

Real time RT- PCR expression of GSTs in the RT-4 cell line following a 48 hour treatment with 12μM of SF, IB or SF-NAC. The results are shown as mean expression of the three replicates.

4.4 Discussion

In this chapter I have characterised the two bladder cell lines by investigating GST genotype, gene expression and enzyme activity of the GSTM1 gene. The biological consequences of failure to express some GST proteins such as hGSTM1 or hGSTT1 can include susceptibility to bladder, colon, skin, and possibly lung cancer. A decrease in GST enzyme activity could result in inefficient detoxification of various carcinogens, which could lead to genetic damage and increased cancer risk. This decrease maybe a result of deletion polymorphisms, such as GSTM1 and GSTT1 or substitution A-G polymorphism as observed in the GSTP1 gene, resulting in an amino acid substitution (Ile105Val). This residue lies in the substrate binding site of the enzyme and the polymorphism has been shown to affect enzyme activity. Also where no expression of a gene is detected, such as the GSTp1 gene in the RT4 cell line would result in no enzyme activity. Furthermore it was demonstrated that SF, IB and their conjugates significantly augmented expression of the phase II enzyme genes GST and NQO1 in these cell lines, which may suggest that ITC treatment increases phase II activity.

4.4.1 Characterisation of UM-UC-3 and RT4 cell lines

It was observed that whilst the cell line UM-UC-3 had a positive GSTM1 genotype, it is not expressing this gene. The subsequent low expression of the GSTM1 enzyme appears to correlate with the relatively high percentage of methylation of the gene in this cell culture, however further investigations are required to be able to connect the two findings. The UM-UC-3 cell line showed a much greater degree of total GST activity compared with the RT4 cell line, this may be due to the nature of the cell line, that is, UM-UC-3 is derived from a much higher/invasive form of bladder cancer than the RT4 cell line. This is not surprising as over-expression of GSTs have been demonstrated in a

number of different human cancer cells. Another interesting observation is that the UM-UC-3 cell line shows comparably high total GST activity, as the cell line is not expressing the GSTM1 gene and thus the enzyme and the high total GST activity can not be due to the GSTM1 gene. Several sub-classes of GST exist (section 2) and strong induction of a particular subunit may be partially masked by the background activity of other subunits when measuring total GST activity (Juge, Mithen *et al.* 2007). If time permitted, investigating specific GST activity between the two cell lines would have been valuable.

Significant concordance between methylated genes and the high rate of bladder cancer recurrence have been observed (Friedrich, Weisenberger *et al.* 2004; Christoph, Hinz *et al.* 2007). GSTpi was shown to be methylated, along with a panel of genes, in disease recurrence in superficial bladder cancers (Tada, Wada *et al.* 2002). It has been suggested that GST-pi CpG island hypermethylation may account for EBV-associated gastric carcinoma, as the loss of GSTpi expression is clustered in a subset of gastric carcinomas with EBV incorporation, but not EBV-negative gastric carcinomas (Kim, Lee *et al.* 2005).

Epigenetic inactivation via methylation of members of the glutathione pathway has been studied in relation to Barrett's adenocarcinoma (BAC), a form of esophageal cancer. Seven GST genes were shown to have CpG islands around their transcription start sites in BAC samples; GSTM2-M5, GSTA4, GSTP1, GSTZ1, GSTT2, GSTO1 and GSTO2, demonstrating mRNA down regulation of GSTM2, GSTM3 and GSTM5 in more than half of the BAC samples (Peng, Razvi *et al.* 2009). Furthermore treatment of oesophageal cancer cell lines with 5-aza-2'-deoxycytidine and trichostatin-A led to reversal of the methylation pattern and re-expression of these genes (Peng, Razvi *et al.* 2009).

The prostate cancer cell lines LNCaP, PC3, and Du-145 have been used to identify candidate genes, which are inactivated by CpG methylation in prostate cancer, by microarray analysis (Lodygin, Epanchintsev *et al.* 2005). The cells were treated with 5-aza-2' deoxycytidine and trichostatin A, which leads to reversion of epigenetic silencing of many candidate genes, including GSTM1. However several hundred genes were found to be induced by 5-aza-2' deoxycytidine when compared with cells treated with trichostatin A, suggesting a much higher degree of epigenetic inactivation via methylation than via phosphorylation. Furthermore the study investigated the frequencies of CpG methylation in the promoter regions of primary prostate cancer samples derived from 41 patients and observed that in 58% of the 41 patients they detected methylation in the promoters of GSTM1 (Lodygin, Epanchintsev *et al.* 2005).

4.4.2 Effects of SF, IB and their conjugates on GST and NQO1 gene expression

Consumption of cruciferous vegetables, such as broccoli has long been associated with a reduced risk of disease especially cancer. The protective effect is most often attributed to SF, as SF has been shown to be the most potent naturally occurring inducer of phase 2 enzymes in *in-vitro* and *in-vivo* studies (Petri, Tannergren *et al.* 2003; Munday and Munday 2004). However it is important to highlight other phytochemicals such as dithiolethiones which are organosulfur compounds also present in cruciferous vegetables and have been shown to have similar anticarcinogenic effects as ITCs (Zhu, Jia *et al.* 2009). The induction of GSTs by ITC has been extensively explored in cell culture and animal models. Furthermore several epidemiological studies also indicate that the dietary consumption of ITCs or ITC-containing foods, inversely correlates with the risk of developing lung, breast, and colon cancers. The proposed methods of induction have been discussed in chapter one.

Briefly, the anticarcinogenic properties of ITCs have been attributed to their ability to alter detoxification pathways leading to decreased activation of procarcinogens and increased excretion of carcinogens. Mechanistically, ITCs are capable of inhibiting both the formation and development of a cancer cell through multiple pathways; i.e. the inhibition of carcinogen-activating cytochrome P450 mono-oxygenases, induction of carcinogen-detoxifying phase 2 enzymes, induction of apoptosis, and inhibition of cell cycle progression (Juge, Mithen *et al.* 2007). The biological interactions of ITCs are strongly related to modulation of cellular redox status, and a number of studies have documented their indirect antioxidant properties, particularly related to induction of phase-2 enzymes (Valgimigli and Iori 2009). SF induces antioxidant response element (ARE) expression through disruption of the Keap1–Nrf2 complex. SF can interact directly with sulfhydryl residues on Keap1, causing Nrf2 to be released. Alternatively, SF can activate the MAPK pathway, causing phosphorylation of Keap1 and release of Nrf2. Once released, Nrf2 enters the nucleus, where it transactivates ARE-responsive genes. A study showed that dietary isothiocyanates modulate phase I and phase II enzyme expression and increase the rate of detoxification of the dietary carcinogen PhIP in human HepG2 cells. SF decreased the number of PhIP-DNA adducts by approximately 40% in human HepG2 cells pre-treated with 1 μ M SFN followed by 10 ng PhIP. However, there was no decrease in DNA adduct formation with post-treatment of SF, suggesting that SF had no effect on PhIP DNA-adduct repair, but rather prevented PhIP interactions with DNA (Bacon, Williamson *et al.* 2003).

Recently it has been shown that many ITCs and their *N*-acetylcysteine conjugates are potent inducers of phase 2 enzymes in rats bladder (Munday and Munday 2002; Munday and Munday 2004; Munday, Zhang *et al.* 2006). It is clear from these *in vitro*

studies that SF and possibly SF-NAC have the capability to modulate some phase 2 gene expression. In concordance with these findings, a study investigating the effects of ITCs on the expression of both NQO1 and GST in rat tissues *in vivo* found the urinary bladder to be the most susceptible to the inductive activity of the ITCs. However, in contrast, this induction of GST and NQO1 *in vivo* did not correlate with that in bladder cells *in vitro* (Munday, Zhang *et al.* 2008).

It has been observed that GSTpi protects mitochondria against oxidative stress in human hepatic mitochondria (Gallagher, Gardner *et al.* 2006). The mitochondrial environment is rich in reactive oxygen species (ROS) that may ultimately peroxidize mitochondria membrane proteins and generate unsaturated aldehydes. These chemicals decrease cell viability and reduced mitochondrial membrane potential. However it was observed that over expression of GSTpi diminished these changes (Goto, Kawakatsu *et al.* 2009).

Overall NQO1 gene expression appears to be the most sensitive to induction by the ITC. This is not surprising, as this has been shown at a functional enzyme level in many studies (Jones and Brooks 2006; Zhang, Munday *et al.* 2006). NQO1 was also shown to be induced by SF-NAC in both cell lines. The RT4 showed the greatest induction of the gene by SF-NAC (8 fold). A similar study compared quinone reductase activity induced by SF and SF-NAC in murine hepatoma cells. This study showed a SF and SF-NAC dose-dependent induction of QR and a greater induction of the gene was seen in the SF-NAC-treated cells. These are important considerations, as the half life of free SF in plasma is very short, being approximately an hour (Ye, Dinkova-Kostova *et al.* 2002), whereas SF-NAC is several times longer. Therefore it is important to consider the possibility that ITC conjugates are as effective, if not more effective than the free ITC, especially in the

bladder, where the SF and its conjugates maybe in direct contact with the bladder epithelia for long periods of time.

GSTM3 showed a marked up-regulation in the RT4 cell line by both SF and IB and also a six fold increase in GSTM3 gene expression by SF-NAC. The effect of polymorphisms of GSTM3 on the influence of cigarette smoking on urinary bladder (Golka, Schmidt *et al.* 2008), larynx (Jourenkova-Mironova, Voho *et al.* 1999) and lung (Reszka, Wasowicz *et al.* 2007) carcinogenesis has been investigated with conflicting results. Genotypes GSTM3 A*B* and GSTM3 B*B* potentially affect detoxification activity of GSTM3 enzyme. The RT4 cells have been shown to be the A*A* wild type genotype (table 4.2) and thus this up-regulation of the GSTM3 would potentially be more beneficial than the moderate up regulation of the GSTM3 gene in the UM-UC-3 cell which have a genotype of GSTM3 A*B* (table 4.1). Note that it is the more invasive/high grade cell line (UM-UC-3) which exhibits the high risk genotype.

Although many cell lines respond to SF treatment, there appears to be some variability in the extent and type of Phase 2 enzyme induction, depending on cell type. For instance, in HT29 cells a decrease in GST activity was observed, whereas CaCo-2 cells demonstrated a dose- and time-dependent induction of GSTA1 and UGT1A1 upon SF administration (Svehlikova, Wang *et al.* 2004). These effects of SF in cell culture can be extrapolated to *in vivo* situations, as induction of GST and QR activities was observed in tissues of mice given SF orally, demonstrating that systemic uptake and bioavailability of SF and/or its metabolites was feasible (Zhang, Talalay *et al.* 1992). In concordance with these results, rats treated by gavage for 5 consecutive days demonstrated a dose-dependent (200–1000 μmol SF/kg per day) increase in QR and GST activity in the liver, colon and pancreas (Matusheski and Jeffery 2001); and 40 μmol SF/kg per day for 5 days by gavage

increased QR and GST activities in the rat forestomach, duodenum, and bladder (Munday and Munday 2004). Confirming these observations, that oral administration of SF induces phase 2 enzymes in bladder tissues, male F344 rats were gavage fed 50 mg/Kg/day of SF and the prostate, liver, kidney and bladder tissues were harvested on day six. Compared to control animals, SF significantly induced NQO1 or total GST enzyme activity in the liver, kidney and, most significantly, in the bladder tissues. A study investigating the antioxidant capacity in human TCC, observed a marked increase in the GSTP1 protein which correlated with the tumour grade. The GSTP1 isoenzyme was expressed in all normal and tumour TCC tissue samples. The study compared tumour samples with adjacent histological benign tissue biopsies (n=30). All normal TCC tissue samples expressed similar levels of GSTP1. In the tumour tissue significant up regulation of GSTP1 expression was observed (mean ng/mg cytosolic protein). The mean level of GSTP1 expression in TCC was increased 2.3 fold compared to the mean level in the normal adjacent mucosa. Furthermore, when comparing the mean level of GSTP1 expression of superficial and invasive tissue biopsies, it was observed that although the benign tissue expressed similar levels of GSTP1, the expression in the tumour samples were significantly up regulated in the invasive tumour tissue compared to the benign tissue, suggesting GSTP1 protein expression correlates with the grade of TCC (Pljesa-Ercegovac, Savic-Radojevic *et al.* 2009). This highlights the importance of further studies investigating the up regulation of the GSTP1 in invasive TCC in relation to chemotherapy resistance, as it's unknown whether GSTP1 is active towards the agents used in the chemotherapy of TCC (Berendsen, Peters *et al.* 1997).

Chapter Five

*The Relationship Of COL6A1 Expression
And The Grade Of Bladder Cancer And The
Modulation Of The Expression Of COL6A1
Gene In Human Cell Cultures.*

Chapter Five: The Relationship COL6A1 Expression And The Grade Of Bladder Cancer And The Modulation Of The Expression Of COL6A1 In Human Cell Cultures.

5.1 Introduction

The first aim of this chapter is to investigate the association between COL6A1 gene expression in human bladder tissue in relation to the severity of bladder cancer. A significant 5.7 fold change was observed in the array analysis in chapter three, showing an increase in this gene's expression in the invasive bladder cancer samples. To investigate these results further, subsequent samples were received for RT-PCR analysis. Although there is no known work on collagen type VI alpha 1 and bladder cancer, the potential importance of collagen VI protein has been studied in relation to other cancers such as prostate, colon and breast cancer. Increased stromal expression of collagen VI has been correlated with various aspects of tumorigenesis and malignant progression and it has been suggested that collagen VI maybe an early diagnostic marker of mammary hyperplasia and a growth-promoting factor that maybe inhibited to reduce tumour progression. An initial investigation into the effects of SF and IB was carried out in the last chapter; the second aim of this chapter is to examine these phenomena in more detail. The possibility of modulating the expression of the gene encoding COL6A1 and the COL6A1 protein by exposing the previously characterised human bladder cell cultures to nutritionally relevant doses of sulforaphane, Iberin and their congenates will be investigated.

5.2 Specific materials and methods

RNA for gene expression analysis was carried out in accordance with the manufactures recommendation (see section two). UM-UC-3 and RT4 cell lines were obtained from the European collection of animal cell cultures (ECACC). Briefly, UM-UC-3 (passage 20) and RT4 (passage 11) established cell cultures were seeded into a six well plate and treated with 12 μ M of SF, IB or SF-NAC for 48 hours (see section 2).

A preliminary study was carried out to assess the feasibility of a larger study investigating the relationship of COL6A1 expression and grade of bladder cancer. From the seven remaining tissue samples I was able to select four samples with increasing grades. Further samples were requested from the NNUH tissue bank. The samples were analysis blinded; that is, the histological results were unknown at the time of the RT-PCR analysis.

The protein expression of COL6A1 was endeavoured to be assessed using western blotting. UM-UC-3 (passage 22) and RT4 (passage 14) established cell cultures were seeded into a 10 cm dishes and treated with 12 μ M of SF, IB or SF-NAC for 48 hours (see section 2). Briefly, protein was extracted with RIPA buffer and separated by SDS-PAGE as outlined in section 2. Proteins were transferred onto nitrocellulose membrane and probed with human COL6A1 antibody.

5.3 Results

5.3.1 Correlation of COL6A1 gene expression with the grade of bladder cancer

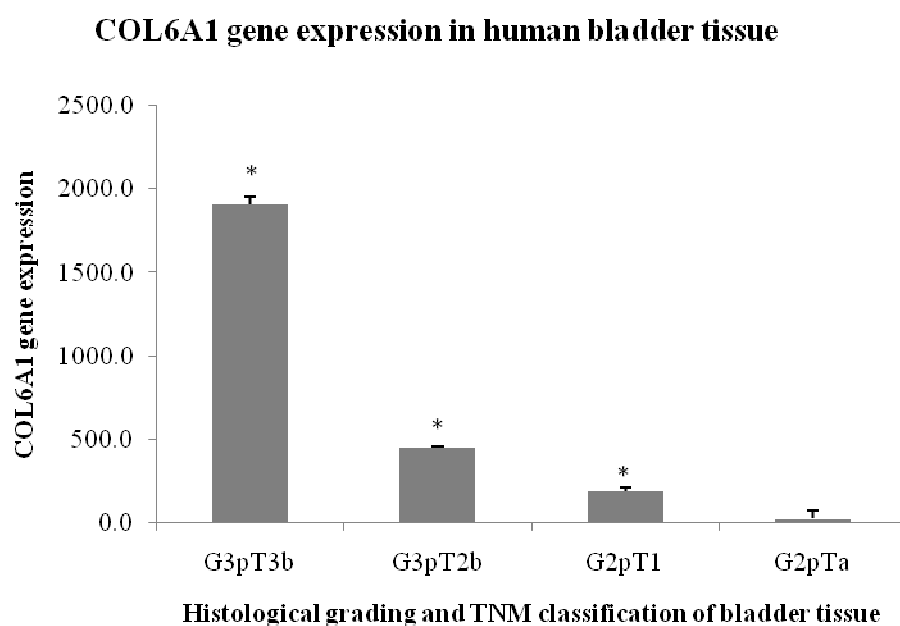


Figure 5.1 Real time RT-PCR analysis of COL6A1 expression in human bladder tissue with varying severity of grades. RNA was extracted from four biopsies taken from four individuals and data is shown from three biological replicates of each sample plotted with SD (* $p < 0.005$).

It can be seen from figure 5.1 that COL6A1 gene expression significantly increases with the grade of bladder cancer. This was a preliminary study to firstly confirm the results from chapter 3 (figure 3.4) and secondly to assess the feasibility of a larger study investigating the association of COL6A1 expression and grade of bladder cancer. Figure 5.1 shows a steady increase of COL6A1 gene expression from the lowest grade to the highest grade and a 40 fold increase in COL6A1 gene expression from the superficial (G2 pTa) to the invasive (G3 pT3b). Analysis of variance (ANOVA) was employed to test the hypothesis that the means of the four populations are equal. The null hypothesis stated that all population means are equal. The resulting overall P value (< 0.001) was small and

therefore it was unlikely that the differences observed are due to random sampling. The large F ($F=56038.96$) is evidence against the null hypothesis, since it indicates that there is more difference between groups than within groups. The Null hypothesis was rejected; not all the means are equal.

Grade*	Tissue bank sample number	Grade if known**	***COL6A1 gene expression
T _a -T ₁	03TB179	Ta	155.1
	03TB192		533.4
	03TB163	T1	1490.3
	04TB067		1606.3
	04TB023		2149.3
	04TB018		2365
	03TB135		2476.3
	03TB195		2634.4
T ₂	03TB165		3387.4
	04TB035		3514.9
	04TB269		3966.1
	04TB093	T2b	5691.1
T ₃	03TB180		7856.2
	04TB099		9087.2
	03TB189		9680.3
	02TB040	T3b	11943.5
T ₄	04TB280		17391.2

Table 5.1 COL6A1 gene expression in 17 bladder cancer tissue samples.

* refer to section 1 for a detailed explanation of grading system.

** Samples from the previous experiment (results shown in figure 5.1) are highlighted in red and as such the grade of cancer is known.

*** Real time RT-PCR result.

Table 5.1 gives the results of RT-PCR analysis of the 13 further biopsy samples received with unknown grades and also the four samples previously analysed with known grades

(total of 17 samples). All samples were analysed together in one RT-PCR experiment and the ‘unknown samples’* results were entered into table 5.1 in accordance to the COL6A1 gene expression result.

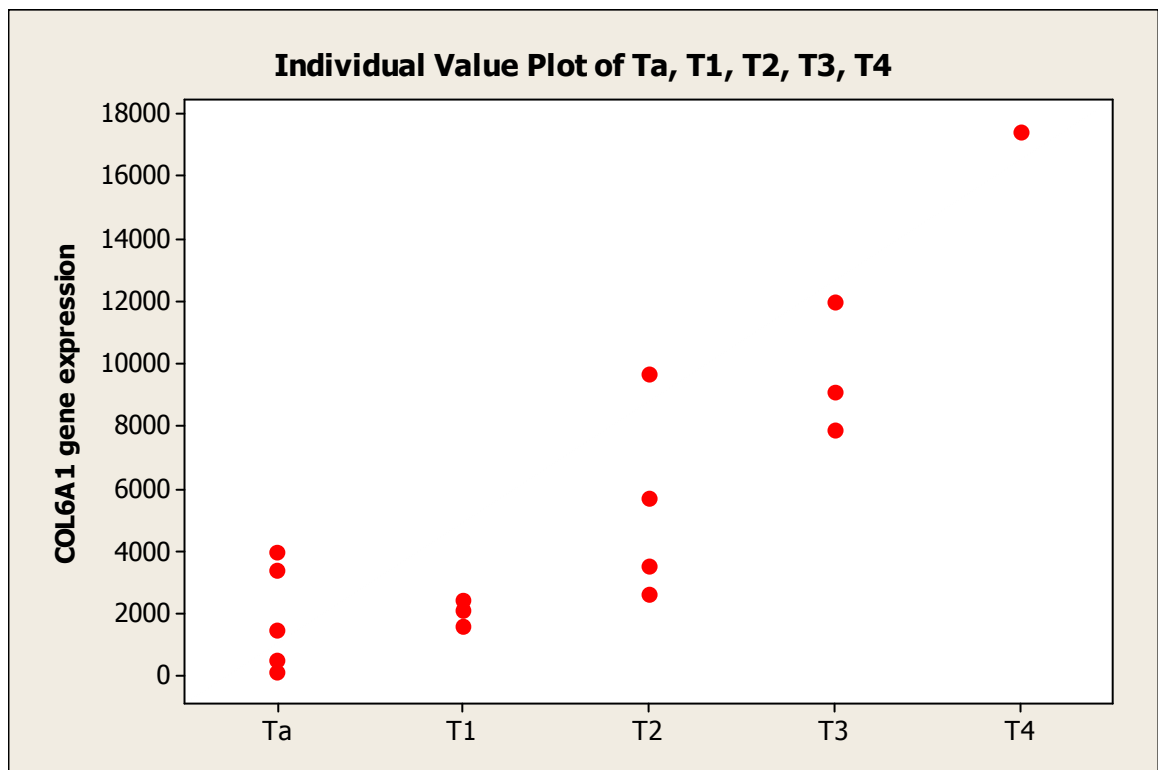


Figure 5.2 COL6A1 gene expression plotted against the histological results of the seventeen bladder biopsies.

Finally the unknown histological results were requested from the histopathologist and the COL6A1 gene expression was plotted against grade of bladder cancer, Ta being the lowest grade and T4 the highest grade. Although there is variation and overlap in the degree of COL6A1 gene expression, especially in the lower Ta grade, it can be seen that the degree of gene expression does follow a definite trend towards increasing with severity of grade, showing approximately an 18 fold increase in COL6A1 gene expression at the highest grade of T4 compared to the lowest, Ta. As for the preliminary analysis (Figure 5.1) ANOVA was employed to test the hypothesis that the means of the five populations (TA,

T1, T2, T3 & T4) are equal. The null hypothesis stated that all population means are equal. The resulting overall P value (<0.001) was small and therefore it was unlikely that the differences observed are due to random sampling. The large F ($F=147.90$) is evidence against the null hypothesis, since it indicates that there is more difference between groups than within groups. The Null hypothesis was rejected; not all the means are equal.

5.3.2 Modulation of COL6A1 gene expression in bladder cell lines by physiologically relevant treatments of sulforaphane, iberin and their *N*-acetylcysteine conjugates.

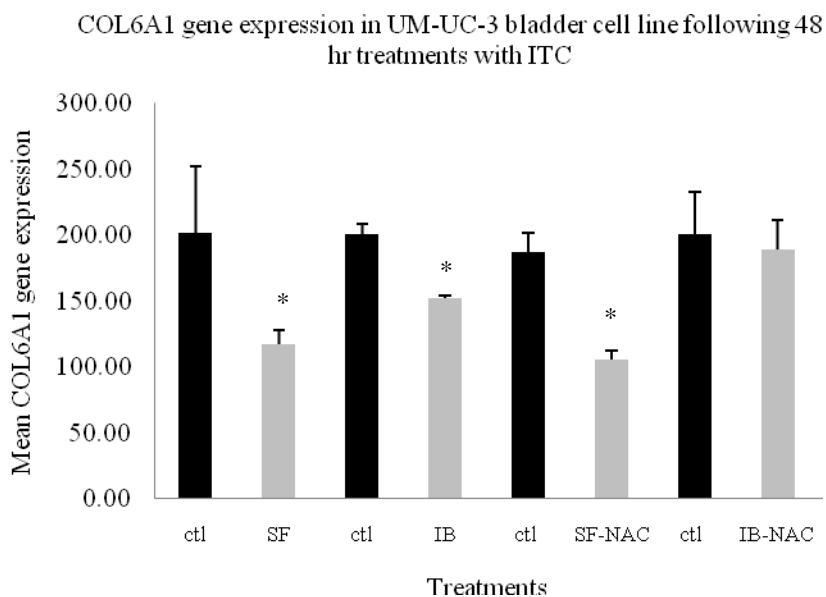


Figure 5.3 Real time RT-PCR of COL6A1 gene expression in human bladder UM-UC-3 cell cultures. The data is shown from three biological replicates plotted with SD.

Figure 5.3 shows that COL6A1 gene expression is significantly down regulated following 48hr treatments of 12 μ M of SF (fold change of 1.7, * p 0.005), IB (fold change of 1.3, * p 0.001) and SF-NAC (fold change of 1.3, * p 0.004) in the UM-UC-3 human bladder cell line. No significant decrease in gene expression was observed following the 48hr treatment of IB-NAC.

COL6A1 gene expression in RT4 bladder cell line following 48 hr treatments with ITC

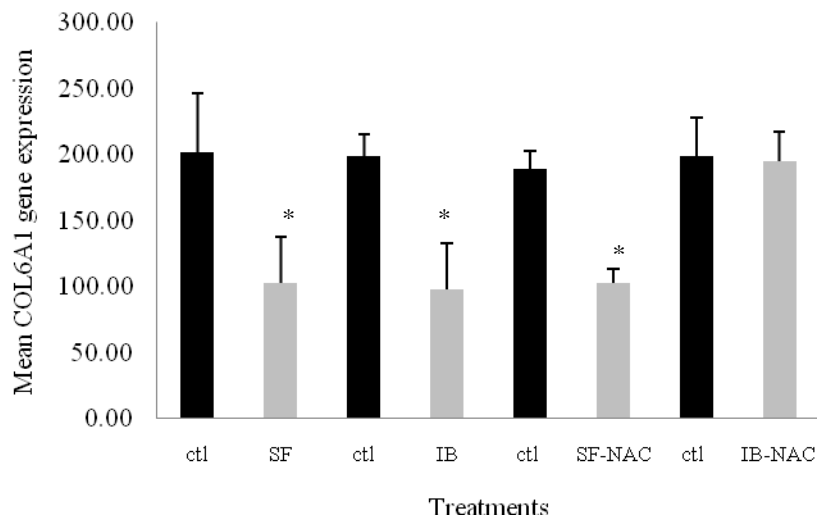


Figure 5.4 Real time RT-PCR of COL6A1 gene expression in human bladder RT4 cell cultures. The data is shown from three biological replicates plotted with SD.

COL6A1 gene expression is significantly down regulated following 48hr treatments of 12 μ M of SF (fold change of 2.1, *p0.03), IB (fold change of 2.3, *p0.02) and SF-NAC (fold change of 2.2, *p0.02) in the RT4 human bladder cell line. No significant decrease in gene expression was observed following the 48hr treatment of IB-NAC.

COL6A1 gene expression in UM-UC-3 bladder cell line following 48 hr treatments with SF-NAC or NAC

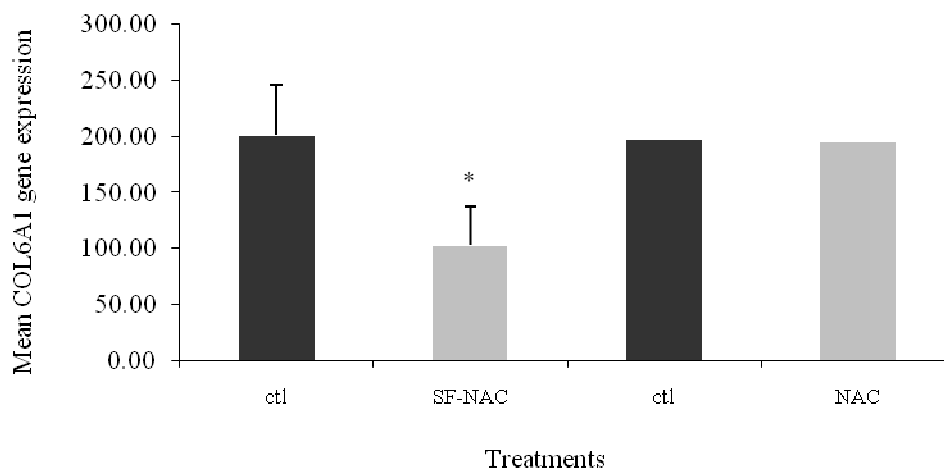


Figure 5.5 Real time RT-PCR analysis of COL6A1 gene expression in the UM-UC-3 human cell culture following treatment with 12 μ M of Sulforaphane *N*-acetylcysteine conjugate or *N*-acetylcysteine. Data is shown from three biological replicates plotted with SD.

Figure 5.5 shows a significant decrease in COL6A1 expression following a 48 hour treatment of SF-NAC (2 fold change, *p0.005). These results are in accordance with the previous experiment, figure 5.3, where a fold change of 1.3 was observed in the UM-UC-3 cell line. However no change in gene expression was observed following treatment with *N*-acetylcysteine alone.

5.3.3 COL6A1 protein expression in the UM-UC-3 cell line

The presence of the COL6A1 protein was not detected in protein lysate from either the cells, Figure 5.6, or the media in which the cells were grown, Figure 5.7. Modulation of the protein by treatment with SF, IB and their conjugates was not observed in either cell lysate or media protein lysate, results not shown.

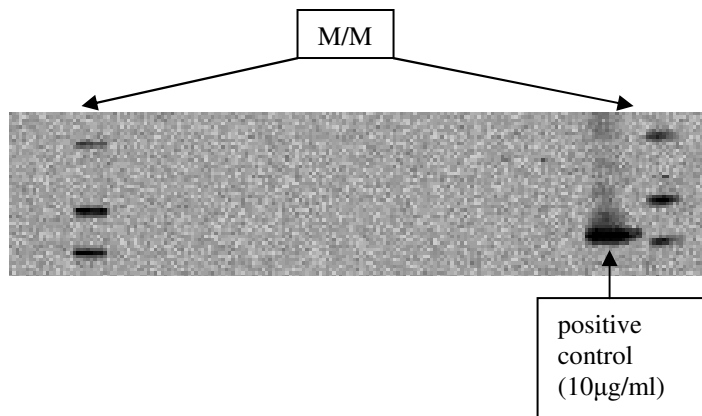


Figure 5.6 Western blot analysis of the protein lysate from treated UM-UC-3 cells. Western blot with different concentrations of cell lysate are in wells 2-6 (5, 10, 20, 40, 80 µg of protein was subjected to SDS-polyacrylamide gel electrophoresis), MagicMark™ XP (M/M) are in wells 1 and 8 and the positive control (10 µg) is in well 7.

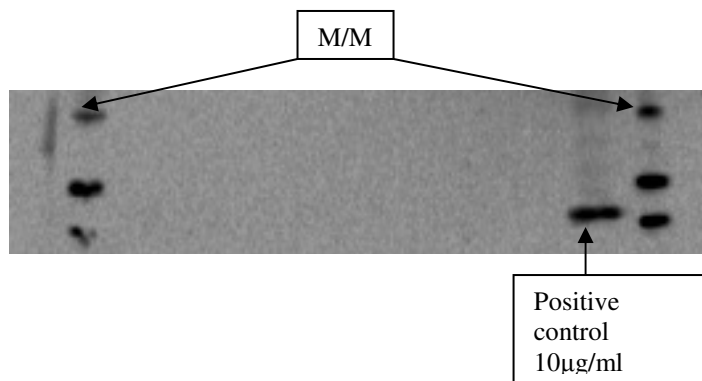


Figure 5.7 Western blot analysis of the protein lysate from culture media of treated UM-UC-3 cells. Western Blot with different media concentrations are in wells 2-6 (5, 10, 20, 40, 80 µg was subjected to SDS-polyacrylamide gel electrophoresis), MagicMark™ XP (M/M) are in wells 1 and 8 and the positive control (10 µg) is in well 7.

5.4 Discussion

COL6A1 is an extracellular matrix protein and like other collagen proteins plays an important role in maintaining the integrity of various tissues (see section 1). Mutations in the genes that code for the collagen VI subunits result in the autosomal dominant disorders known as Bethlem myopathy and the less severe, Ullrich congenital muscular dystrophy. As such the greatest area of research on this gene is concerned with these conditions. These conditions, once thought to be completely separate entities, are dominantly inherited disorders associated with mutations in the three COL6 genes encoding type VI collagen (Lampe and Bushby 2005). Ullrich congenital muscular dystrophy is characterized by congenital muscle weakness, proximal joint contractures and marked distal joint hyperextensibility (Baker, Morgelin *et al.* 2005). Bethlem myopathy is a relatively mild dominantly inherited disorder, characterised by proximal weakness and distal joint contractures. Ullrich congenital muscular dystrophy causes severe muscle weakness with proximal joint contractures and distal hyperlaxity (Bertini and Pepe 2002).

The COL6A1 gene first highlighted by the microarray analysis in chapter 3 also appears to be modulated following treatment with SF and IB. In this chapter the relationship of COL6A1 gene expression with the grade of bladder cancer and the modulation of this expression was explored in more detail. The results reveal for the first time that the expression of COL6A1 increases with the grade of TCC and that SF and IB can alter the mRNA expression of COL6A1 *in vitro* and thus potentially modulate the extracellular matrix and stroma *in vivo*.

5.4.1 The expression on COL6A1 is increased with the grade of Transitional Cell Carcinoma.

The COL6A1 gene was highlighted in the microarray analysis and an observed increase in the genes expression in the invasive tissue was a novel finding. It can be seen in figure 5.2 that the degree of gene expression does follow a definite trend towards increasing with severity of grade. An approximate 18 fold increase in COL6A1 gene expression was observed when comparing the lowest grade (Ta) with the highest grade (T4).

Identification of the COL6A1 gene being differentially expressed in human astrocytomas has recently been discovered (Fujita, Sato *et al.* 2008). The study showed very similar results to this bladder study. That is, the tumours are classified by the WHO according to their histopathological and clinical characteristics into four classes: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma multiforme). The expression of the gene had significantly different means when normal glia was compared with low-grade astrocytomas (grades I and II) and high-grade astrocytomas (grades III and IV), with a tendency to be greater in higher grade samples (Fujita, Sato *et al.* 2008). Once again this was a novel finding in this tissue type. These discoveries suggest COL6A1 as potential tumour marker of astrocytomas and bladder cancer progression. To the best of my knowledge the astrocytoma and this bladder study are the only studies to identify differential expression of COL6A1 with tumour grade.

5.4.2 The Modulation of the COL6A1 gene *in vitro*

In this chapter it can be seen that the COL6A1 gene can be modulated by treatment with physiological doses of SF and IB in both bladder cell lines. This down regulation of the gene is greater in the more invasive UM-UC-3 cell line. This may be due to the basal expression of this gene being higher in the UM-UC-3 cell line due to the fact it originated from an invasive form of transition cell carcinoma. As was first discovered in chapter three and confirmed in this chapter, the COLA1 gene increases with the severity/invasiveness of bladder cancer. In contrast to the observed effect of ITC on COL6A1 gene expression, Se-methylselenocysteine (MSC) has been shown to significantly increase the expression of COL6A1 in LNCaP and PNT1A prostate cell lines (Hurst, Elliott *et al.* 2008). Microarray analysis showed that the expression of several collagen genes were modulated by MSC treatment compared to the control. A significant decrease in the expression of collagen type I alpha 1 genes and significant increases in COL6A1 and COL4A5 gene expression were observed in response to the MSC treatment (Hurst, Elliott *et al.* 2008).

As discussed in chapter four, ITCs are unstable compound and readily dissociates into their *N*-acetylcysteine conjugates which are primarily concentrated in the urine and stored in the bladder. As human bladder cancers occur almost exclusively in the bladder epithelium, which is directly exposed to the urine stored in the bladder it is important to assess the activity of the conjugates of both sulforaphane and iberin. However due to the continuous reversible reaction from the parent ITC to their *N*-acetylcysteine conjugates it is questionable as to whether this activity is actually due to the conjugates themselves or because the ITC-NAC readily releases the parent compound that the observed down regulation of the COL6A1 is actually due to the SF. To test this theory the UM-UC-3

bladder cell line were treated with just *N*-acetylcysteine (NAC), at the same concentrations and time scale as the previous experiment using SF-NAC (12 μ M for 48 hours). In contrast to SF-NAC there is no significant effect of NAC alone on COL6A1 gene expression after a 48hr treatment. Therefore the observed down regulation of the COL6A1 gene expression (tables 5.3 and 5.4) may not be due to SF-NAC but in fact be caused by the SF, following the dissociation of the SF from the SF-NAC. However this does not explain why the SF-NAC appeared to have a greater effect on the COL6A1 gene down regulation than SF alone in the previous experiment. The initial results of the SF-NAC are in accordance with the findings by Tang *et al.* (Tang, Li *et al.* 2006), that is, ITC conjugates elicit similar effects as their parent compounds in bladder cell cultures, although Tang's experiments were investigating the apoptotic effects of ITC-NAC.

5.4.3 Functional modulation of the COL6A1 protein

Protein was extracted from UM-UC-3 cells to determine whether treatments with SF and IB have an effect on COL6A1 protein expression. The resulting membrane picture showed the COLA61 protein to be undetectable. To examine the possibility that the COL6A1 protein was being excreted into the media, the protein lysate from the media was collected for Western Blot analysis. It can be seen from figure 5.2 that the Western Blot with different protein concentrations from the media of the treated cells has led to the same membrane picture as the Western Blot with the UM-UC-3 cell lysate. Again no bands were visible. Neither isolations of protein from the UM-UC-3 cells, or from the media in which they were cultured, gave a positive result for the COL6A1 protein. Reasons for this could be that the 48h treatment was not long enough to produce a functional change, or perhaps a different cell line should have been used. The UC-UM-3 cell line is a human

bladder epithelial cell line, whereas COL6A1 is predominantly synthesized *in vivo* by fibroblasts. Although I am not aware of any bladder fibroblast cell lines, if time was permitting the use of a primary fibroblast cell line may have shown a functional change in protein expression following treatment.

In summary, although a functional change could not be established, the combination of the tissue gene expression results and the cell culture work is not only exciting, but also novel. For the first time it has been shown that COL6A1 gene expression increases with the severity of bladder cancer *in vivo* and the gene can be down regulated by physiologically relevant doses of SF and IB *in vitro*. These results suggest that both IB and SF may have important effects on the extracellular matrix (ECM), as collagen is a dominant component. Therefore the following chapter will be investigating the effects of SF and IB on markers of bladder cancer progression and invasion, using the previously characterised UM-UC-3 human bladder cell line.

Chapter Six

Anticancer Effect Of Sulforaphane And Iberin On Markers Of Progression/Invasion of Cultured Human Bladder Cells

Chapter 6.0 Anticancer Effects Of Sulforaphane And Iberin On Makers Of Cancer Progression/Invasion In Cultured Human Bladder Cells

6.1 Introduction

In chapter three the COL6A1 gene encoding for the extracellular protein was identified by the array analysis to increase with the grade of bladder cancer. These results were confirmed in chapter five by RT-PCR, showing that the degree of gene expression does follow a definite trend towards increasing with the severity of grade. Furthermore in chapter five it was shown that physiologically relevant doses of sulforaphane and iberin could down regulate the expression of the COL6A1 gene in high grade human bladder cell culture. These results suggest that isothiocyanates may have an important effect on the extracellular matrix. Since the majority of bladder cancer mortality is associated with invasion of cancerous cells into the extracellular matrix and the bladder muscle wall, leading to metastatic disease, the ability to inhibit cell motility and cell segregation from the primary tumour and invasion of surrounding tissues may significantly reduce bladder cancer-related morbidity and mortality. When tumour cells invade extracellular matrix they produce proteases such as metalloproteinases (MMP), which degrade the extracellular matrix. The aim of this chapter is to investigate the effects of SF, IB and their conjugates on the cellular motility of the UM-UC-3 cell line and their capability to reduce invasion into extracellular matrix by assessing their effects on the activity of both matrix metalloproteinases 2 and 9.

6.2 Specific Materials and Methods

6.2.1 Semi-quantitative cell motility for *in-vitro* wound healing in the UM-UC-3 bladder cell line.

The wound-healing assay I employed provides semi-quantitative data on cell motility/migration. The assay is simple, inexpensive, and one of the earliest developed methods to study cell motility/migration *in-vitro* (Rodriguez, Wu et al. 2005). The basic steps involved displacing a group of cells by scratching a line through the monolayer with a pipette tip. The open gap was inspected microscopically over time as the cells migrated to fill the damaged area. The images were captured by photographing the cell monolayer at the beginning and at regular time points during cell migration. Cell migration was quantified by measuring the distance between two standard points on either side of the gap to quantify the migration rate of the cells (chapter 2.6). It has been suggested that this assay is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration (Rodriguez, Wu *et al.* 2005).

6.2.2 The use of a BD Matrigel™ Matrix basement membrane to measure UM-UC-3 cell invasiveness.

BD Matrigel™ Matrix provides a biologically active basement membrane model for *in-vitro* invasion studies. BD Matrigel™ Matrix is a soluble basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin(<http://www.bdbeurope.com> 2009). Cells were grown in 10cm dishes to approximately 70% confluency and starved (serum free culture media) for a 24 hour period. Cells were harvested following the 24 hour period and re-suspended in

conditioned serum free media. The cell suspension was seeded (3×10^5 cells per well) onto the BD Matrigel™ Matrix in the top transwell insert (1.5 ml) and 2 ml of complete media was placed in the reservoir (bottom well). The cultures were incubated for 24 hours. The media was aspirated from the top transwell and the bottom receiver well and the Matrigel™ Matrix was washed away with warmed PBS solution. The cells that penetrated to the lower surface of the transwell filter was fixed, stained, detached from the transwell and mounted on to a microscope slide. The cells were 6 randomized fields at a X200 magnification. The cell counts were performed in at least four independent experiments.

6.2.3 Investigation of the influence of physiologically relevant doses of sulforaphane, Iberin and their conjugates on metalloproteinases 2 & 9 production in the UM-UC-3 cell line using the gelatin zymography assay.

The extracellular proteinases MMP 2 and MMP 9 are secreted into the cell culture media which was collected after 48 hour. The Invitrogen Novex® polyacrylamide zymogram gels were used for the gelatin zymography assay to enable the detection of matrix metalloproteinases 2 & 9. Zymography is an electrophoretic technique, in which the substrate gelatin is copolymerized with the gel for the detection of the enzyme activity. Samples were prepared without denaturing the active enzymes (section 2.7) and, following electrophoresis, the gel was placed in an enzyme activation buffer which allows the enzymes present in the sample to become active and digest the substrates copolymerized in the gel. The zymogram was subsequently stained and the areas of enzyme activity and digestion that became visible identified (chapter 2.7). The Invasion Index (I %) is calculated to determine the invasive potential of the cell line:

$$\text{percentage of invasive cells} = \frac{\text{no. of invaded cells}}{\text{no of migrated cells}} \times 100.$$

6.3 Results

6.3.1 Assessing the effects of SF, IB and their *N*-Acetylcysteine conjugates on UM-UC-3 bladder cell line migration using the wound healing assay.

The wound healing assay enables the motility of a particular cell line to be qualitatively assessed and thus enable investigation of the influence of SF, IB and their *N*-acetylcysteine conjugates. Figures 6.1 and 6.2 show the capability of SF, IB and SF-NAC to reduce the mobility of the UM-UC-3 cell line following a 24 hour period of incubation. IB-NAC also shows this capability, but to a much lesser extent. Following a further 12 hour incubation SF and IB still reduce the cell lines mobility further by approximately 50%, however there conjugates do not.

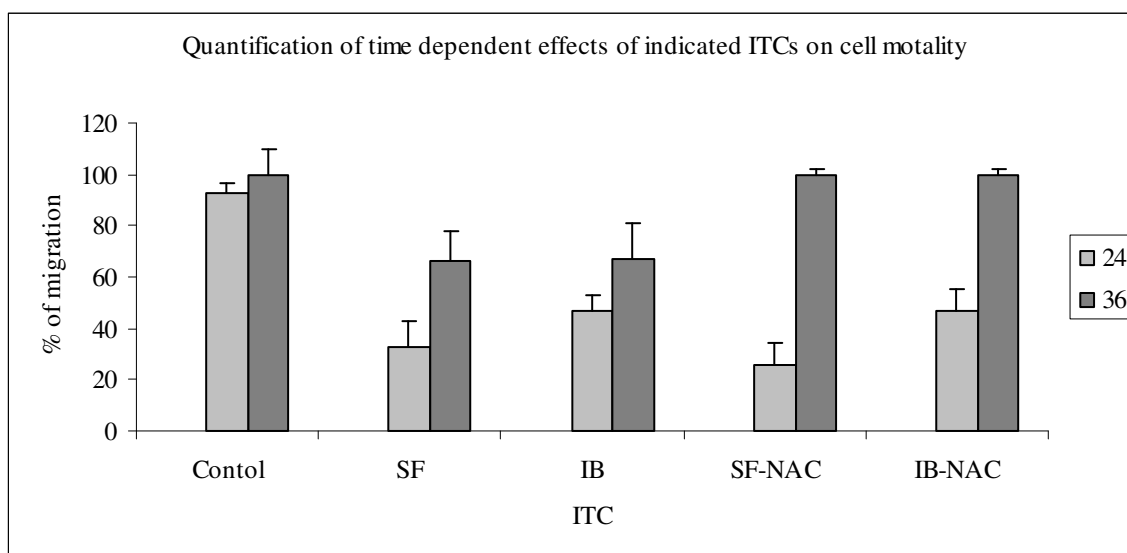


Figure 6.1 Quantification of the time dependent effects of the indicated ITCs on cell motility. Measurement of the percentage of migration as the cells move to close the wound.

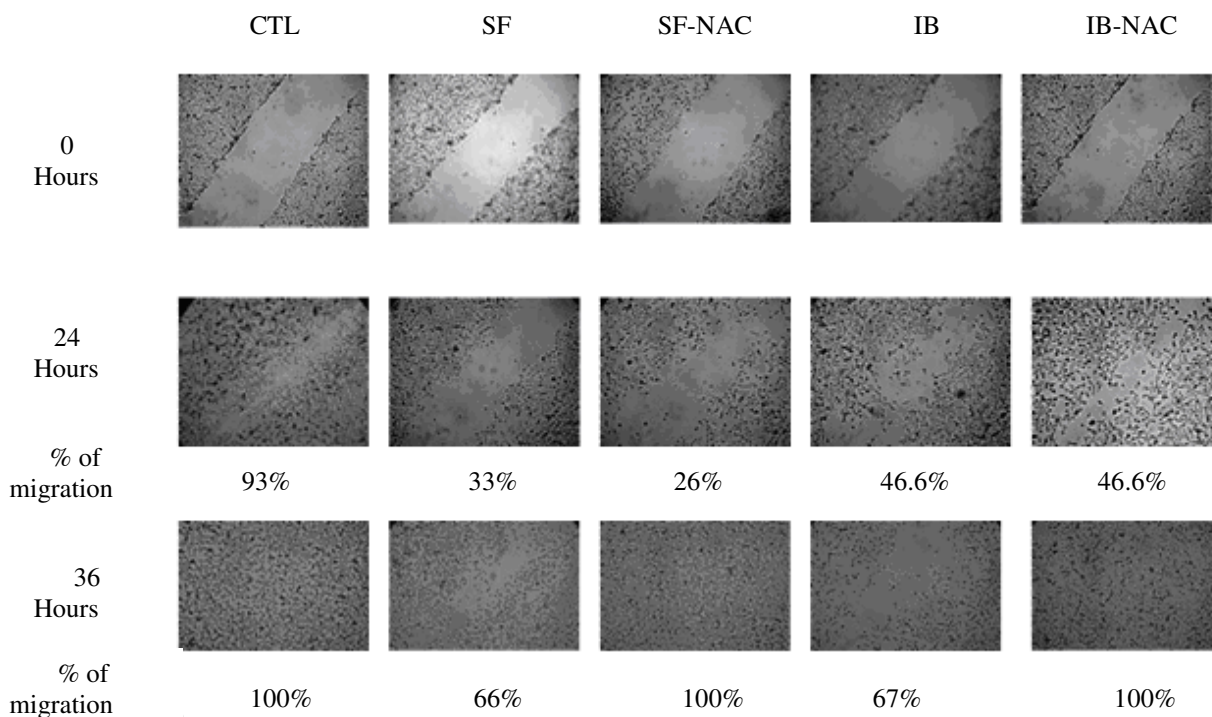


Figure 6.2 Graphical representation of the wound healing assay. The cellular motility was controlled by 24 hour, 12 μ M treatments of SF, IB and to a lesser extent SF-NAC and IB-NAC. Note SF-NAC, and to a lesser extent IB-NAC, exhibit an inhibitory pattern similar to that of their parent compounds treatment.

SF, IB and their conjugates affected wound healing migration of UM-UC-3 cells. The leading edge of the closing wound was compared between ITC treated, ITC-NAC treated and control. As shown in figure 6.1, cellular migration was reduced by 12 μ M treatment of the SF, inhibited by 67% and 34% after 24 and 36 hours of incubation, respectively. Treatment with 12 μ M of IB also reduced cellular migration, inhibited by 53.4% and 33% after 24 hours and 36 hour of incubation, respectively. SF-NAC at 12 μ M showed a similar inhibitory pattern on cell migration, but with a slightly stronger effect than its parent compound and IB at the same molar concentration following 12 hours of incubation. SF-NAC inhibited the migration rate of UM-UC-3 cells by 74% after 24 hour incubation; however no inhibitory effects were seen following 36 hour incubation. IB-NAC appeared to have the same inhibitory effects on the cell migration of the UM-UC-3 cells as its parent compound following 24 hour incubation; IB-NAC inhibited the migration rate of the UM-

UC-3 cells by 53.4% after the 24 hour incubation. However no inhibitory effects were seen following 36 hour incubation with IB-NAC.

6.3.2 Assessing the effects of SF, IB and their *N*-Acetylcysteine conjugates on UM-UC-3 bladder cell line ability to invade extracellular matrix using matrigel invasion studies

To assess the invasive ability of tumour cells *in vitro* a large variety of systems have been developed that permit the assessment of their capacity to invade through basement membranes. Matrigel is widely accepted as a biologically active basement membrane mimic and numerous publications have proved its usefulness for *in vitro* invasion assays (Amar, DeArmond *et al.* 1994; Holting, Zielke *et al.* 1994; Okamura, Mori *et al.* 1996; Zielke, Hoffmann *et al.* 1999).

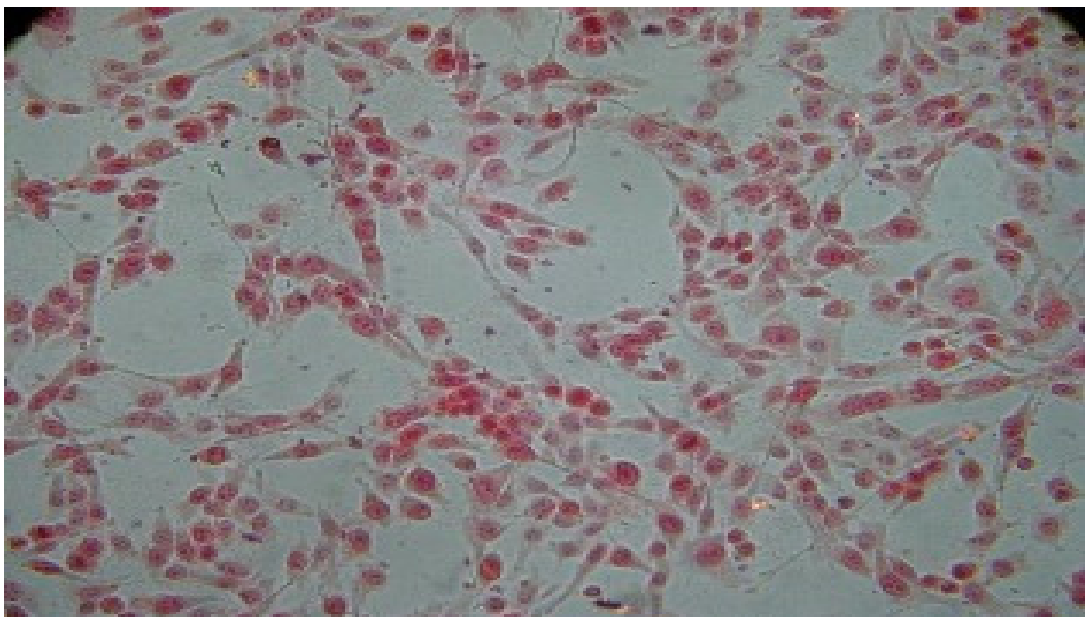


Figure 6.3 UM-UC-3 bladder cells following staining with Gill's No 1 Hematoxylin stain. The picture was taken at x200 magnification. The cells have an appearance intermediate between that of stratified cuboidal epithelium and stratified squamous epithelium, hence "transitional". The Morphological characteristics of the UM-UC-3cell lines in tissue culture show large elongated cell morphology with a large prominent nucleus.

In order to visualise the cells under the light microscope the cell required fixing to the underside of the filter and staining. It was determined that a fixing time of 3 minutes and a staining time of 4 minutes with Gill's No 1 Hematoxylin stain gave the optimum results for this cell type. Figure 6.3 represents the result of fixing and staining UM-UC-3 cells at x200 magnification and clearly shows the large prominent nucleus which enabled ease of cell counting. The filters were fixed, stained, detached from the transwell casing and mounted on to a microscope, shown in figure 6.4.

Figure 6.4 are photographic examples of the appearance of the filters following cell migration and cell invasion. The cells were stained a pink/purple colour and a much greater degree of cell migration can be observed in comparison to cell invasion. It may also be observed that the migrating cells have moved through the filter in a comparably even manner, giving an appearance of a relatively even distribution of cells on the underside of the filter. Whereas the cells which invaded the ECM appear to have done so through the centre of the filter, giving the appearance of a dense congregation of cells just in the centre of the underside of the filter.

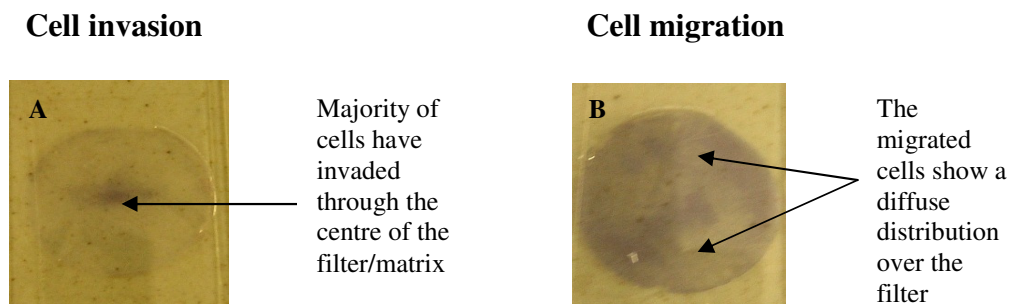


Figure 6.4 Appearance of the transwell filters following fixing and staining.
A – Representation of a filter following cell invasion through the filter and the Matrigel™ ECM
B – Representation of a filter following cell migration through the filter only.

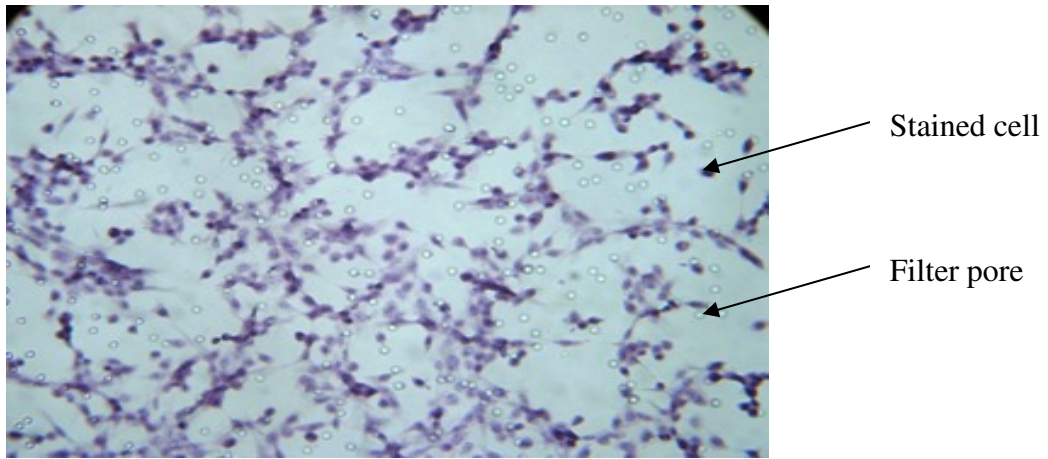


Figure 6.5 Cells migrated through a filter with no extracellular matrix. Cell migration following 48hr treatment with SF, picture taken at x100 magnification. The cells were counted under the microscope in 13 randomized fields at x 200 magnification.



Figure 6.6 Cells invaded through the BD Matrigel™ Matrix. Cell invasion through the BD Matrigel™ Matrix following 48hr treatment with SF, picture taken at x100 magnification. The cells were counted under the microscope in 13 randomized fields at x 200 magnification.

Figure 6.4 and 6.5 are photographic examples of the appearance of the filters under the microscope following cell migration and cell invasion. The cells stained a pink/purple colour were clearly visible for cell counting at x 200 magnification.

	No Treatment			SF Treatment		
	Number of cells counted. (counts in six different fields on each plate)					
	Plate 1	Plate 2	Plate3	Plate 1	Plate 2	Plate 3
Cell migration (x 3) ctl plate	210	178	185	176	149	173
Mean cell count	191			166		

	No Treatment			SF Treatment		
	Number of cells counted. (counts in six different fields on each plate)					
	Plate 1	Plate 2	Plate3	Plate 1	Plate 2	Plate 3
Cell invasion (x 3) test plate	40	45	43	39	35	40
Mean cell count	42.67			38		

% invasion	$42.67/191 \times 100 = 22.34$	$38/166 \times 100 = 21.68$
invasion index	$22.34/21.68 = 1.03$	

Table 6.1 Determination of the invasive potential of the UM-UC-3 cell line by calculating the invasion index. To determine the invasive properties of the studied cell lines, the invasion index (I %) was calculated. Percentage of invasive cells = no. of invaded cells/no of migrated cells x 100.

Table 6.1 represents the calculation of the invasion index of SF treated and untreated UM-UC-3. No reduction in invasiveness was observed when the cells were treated for 24hrs with SF. Likewise no significant reduction in invasiveness was observed when the cells were treated for 24hrs with IB or the ITC conjugates, results not shown. The invasion

index of the UM-UC-3 cell line is very low (1.03) indicating a low metastatic potential of this cell line.

6.3.3 Assessing MMP-2 and MMP-3 activity in UM-UC-3 bladder cell line following treatment with physiological relevant concentrations of SF, IB and their N-Acetylcysteine conjugates using Gelatin Zymography.

The substrate gelatin is copolymerized with the gel and upon enzyme activation by the buffer the enzymes present in the sample become active and digest this substrate. When the zymogram is stained, the areas of enzyme activity/digestion become visible because there is no or less staining due to the digestion of the gelatin by the proteinases. The original zymogram appears as follows:

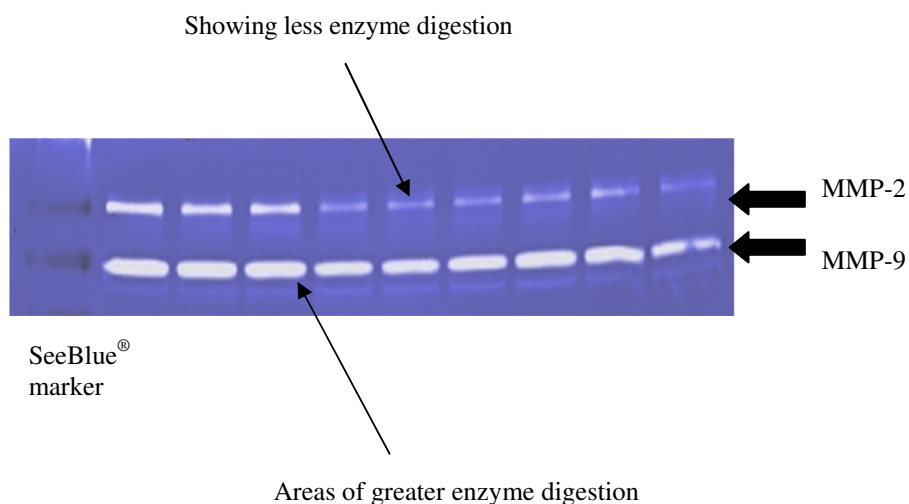
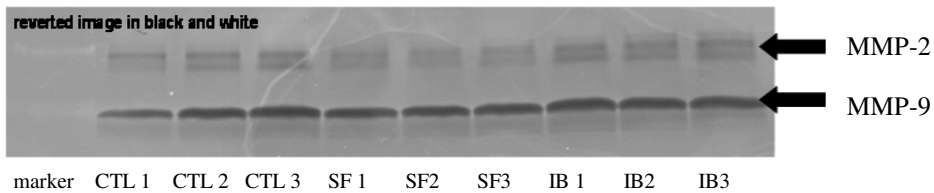


Figure 6.7 An example of Gelatin-SDS-PAGE zymography for semi quantitative study of MMP-9 and MMP-2 activity in treated and non-treated UM-UC-3 cell lines. This photograph of an electrophoresis gel is representative of three independent experiments that essentially gave the same result.

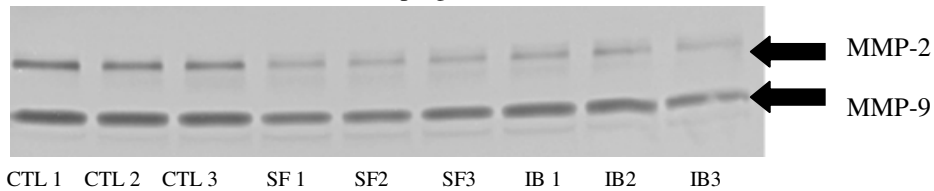
Unlike western blotting the SeeBlue® marker is not being used to judge protein size, only the efficiency of electrophoresis. The enzymes are not visible following electrophoresis, whereas the SeeBlue® marker is visible. Therefore if the marker can be seen to have

electrophoresed through the gel, it confirms efficient electrophoresis of the target. The zymogram was stained with Coomassie Brilliant Blue, and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme. However for the following results the membrane was reversed and changed to black and white for ease of digestion detection.

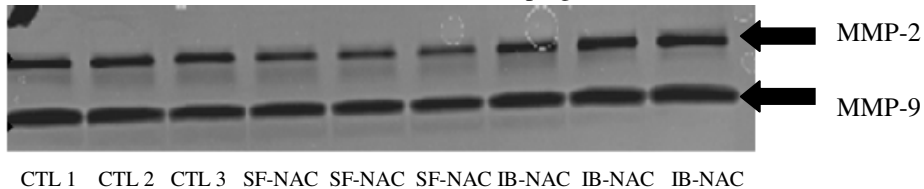
A) 5 μ l aliquot of media from SF treated cells and IB treated cells following a 24 hour incubation of developing buffer.



B) 10 μ l aliquot of media from SF treated cells and IB treated cells and a 24 hour incubation of developing buffer.



C) 10 μ l aliquot of media from SF-NAC treated cells and IB-NAC treated cells and a 24 hour incubation of developing buffer.



D) 5 μ l aliquot of media from SF treated cells and IB treated cells and a 12 hour incubation of developing buffer.

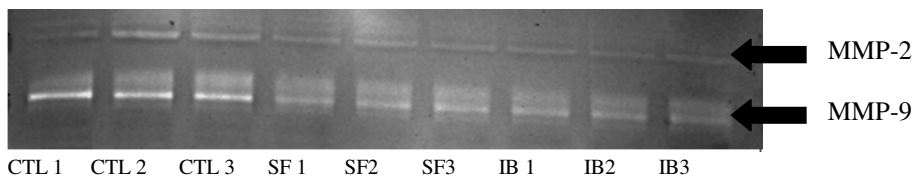


Figure 6.8 Reversed images of zymography gels following either 10 μ l or 5 μ l aliquots of media from ITC treated cells following either 12 or 24 hour incubation of developing buffer. Each electrophoresis gel photograph (A-D) is representative of three independent experiments that essentially gave the same result.

Zymography following a 5 μ l aliquot of media from SF treated cells and IB treated cells following a 24 hour incubation of developing buffer (figure 6.3.4 A) showed that the MMP-9 enzyme has fully saturated its substrate and therefore little difference can be seen between control and treated samples. There is a small decrease in MMP-2 enzyme activity from the SF conditioned media. Conjugates showed no difference at this volume and incubation time (picture not shown). As a small decrease in MMP-2 could be observed in figure 6.3.4 A, the quantity of media applied to the gel was increased to 10 μ l to increase the likelihood of digestion and incubated with the developing buffer for 24 hours. As expected the MMP-9 enzyme has almost fully saturated its substrate therefore there is little difference to be seen between the control and treated samples. But there does appear to be a small reduction in MMP-9 following treatment with SF compared to control. However, with this higher media loading, it can be seen that treatment with 12 μ M of SF and 12 μ M of IB can down regulate MMP-2 in UM-UC-3 human bladder cells (figure 6.3.4 B).

Zymography following 10 μ l aliquot of media from SF-NAC treated cells and IB-NAC treated cell following a 24 hour incubation of developing buffer (figure 6.3.4 C) showed that MMP-9 enzyme has fully saturated its substrate, therefore little difference can be seen between the control and treated samples. However at this media volume it can be seen that treatment with 12 μ M of SF-NAC can down regulate MMP-2 in UM-UC-3 human bladder cells. IB-NAC showed no difference at this volume and incubation time. To prevent the MMP-9 totally saturating the substrate a lower aliquot of 5 μ l of media was applied and the developing buffer time was reduced to 12 hours. Treatment with 12 μ M of SF or 12 μ M of IB down regulated both MMP-2 and MMP-9. It can be seen in figure 6.3.4 D that by reducing the media loading dose and incubation time to 12

hours prevents the MMP-9 saturating its substrate and therefore a reduction in MMP-9 enzyme by treatment with 12 μ M of SF or IB can be observed. Conjugates showed no difference at this load and incubation time (results not shown).

In conclusion treating with 12 μ M of SF, IB or SF-NAC has the capacity to down regulate the production of MMP-2 and 12 μ M of SF or 12 μ M of IB have the capability to down regulate the production of MMP-9 in the UM-UC-3 cell line.

6.4 Discussion

6.4.1 Reduction of UM-UC-3 cell migration/motility following treatment with SF, IB and their conjugates.

The wound healing assay mimics cell migration during wound healing *in vivo*. It is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration and the inhibitory effects of chemicals on the cell migration of a particular cell type. In this model the gap is mainly covered by cells that move to close it, rather than cells that proliferate, at least at the early time points, when cells do not have enough time to proliferate (Androulidaki, Dermitzaki *et al.* 2009). At the 36 hour time point the result is a combination of proliferation and motility of the UM-UC-3 cells. In this wound healing assay it was demonstrated that SF, IB and SF-NAC have the capability to reduce the mobility of the UM-UC-3 cell line following a 24 hour period of incubation. IB-NAC also shows this capability, but to a lesser extent. Following a further 12 hour incubation, SF and IB still reduce the cell lines mobility by about 50%, but their conjugates do not. Ultimately these results suggest that the ITCs can reduce the motility that resulted in slower closure of the gap. This wound healing model has been employed in many studies, such as the effect of corticotropin releasing factor on the breast cancer invasiveness and

demonstrating that activation of FAK/PI3K/Rac1 signalling inhibits cell motility in MCF7 cells and A375 human melanoma cells (Kallergi, Agelaki *et al.* 2007). Furthermore it has been shown that the migration of HMEC-1 cells in this wound healing assay was effectively prevented by SF. In addition, within 6 hours of incubation, SF also inhibited tube formation of HMEC-1 cells on basement membrane matrix (Bertl, Bartsch *et al.* 2006).

6.4.2 UM-UC-3 cell invasion through a BD BioCoat™ Matrigel™ invasion chamber.

The inhibitory effects of SF, IB, SF-NAC and IB-NAC on the ability of UM-UC-3 cells to invade through a reconstituted extracellular matrix were examined. Only invasive cells are capable of digesting the matrix and move through the insert membrane. The BD BioCoat™ Matrigel™ invasion chamber is useful to study cell invasion of malignant cells and therefore make an assessment of their metastatic potential. This matrix provides a barrier to non-invasive cells, while presenting an appropriate protein structure for invading cells to penetrate before passing through the membrane. *In vivo* basement membranes are specialized extracellular matrixes that are comprised of several biological components including collagens, laminins, and proteoglycans. They form thin continuous sheet like structures that separate epithelial tissues from the adjacent connective tissue stroma; thus, under normal conditions they form barriers, which block the passage of cells and other macromolecules. In this study the inhibitory effects of SF and IB on the ability of UM-UC-3 cells to invade through reconstituted ECM (Matrigel™) with or without treatment were investigated. The mean UM-UC-3 cells migration through the 8µm pore without reconstituted ECM was 191 for non treated cells and 166 for the treated cell. The mean UM-UC-3 cells invasion through 8µm pore with reconstituted ECM was 43 for the non treated cells and 38 for the treated cells. This resulted in 22% invasion potential for both the non treated and treated cell cultures, consequently an invasion index of 1.03 was calculated and therefore demonstrating that treatment with these ITCs do not modulate UM-UC-3 cell invasion. Furthermore, the low invasion index suggests that UM-UC-3 cells have a low-invasive phenotype, suggesting that the UM-UC-3 cell line has little metastatic potential. However, it has been demonstrated in HT-1080 fibrosarcoma, MCF7 breast carcinoma, and U251.3 glioma cell lines that tumour cell invasion through

Matrigel™ is regulated by activated MMP-2 (Deryugina, Luo *et al.* 1997) and as it was shown in 6.3.3 that the UM-UC-3 cell line appear to have low levels of MMP-2 secretion in comparison to MMP-9 and as such may contribute to a negligible metastatic potential. Therefore the cells have the capability to move and this capability is reduced by treatment with SF, IB and to a lesser extent by their conjugates as demonstrated in 6.3.1; however they have little capability to invade extracellular matrix.

The observation that migrating cells have moved through the filter in a comparably even manner, whereas the cells which invaded the ECM appeared to have done so through the centre of the filter, may be a direct result of the influence of Matrigel™ Matrix™ on cell differentiation. A study has shown that epithelial cells cultured on plastic continue to multiply and proliferate to the extent that they occupy the whole surface of a plastic dish and become confluent (Novin and Nouri 2007). While epithelial cells cultured on ECM undergo polarization and differentiation. Using electron microscopy, the morphology of the cells appeared different compared to the cells grown on plastic, such as an increase in mitochondria and glycogen was observed. It was also observed that the amount of total RNA in cultured cells on ECM was 2.5 times more than the amount of RNA isolated from cells grown on plastics. These results suggest that Matrigel™ ECM plays an important role in gene expression, polarization and differentiation of human epithelial cells and as such may account for the observed differences in cell movement (Novin and Nouri 2007).

In contrast to these results, an experiment with SK-Hep1 cells demonstrated the ability to invade through a similar reconstituted ECM and that this invasive potential of the cells were reduced by treatment with AITC and AITC-NAC in a dose dependent manner in the range 0.1-5µM. A significant reduction in the number of invasive cells was seen when the cells were treated with AITC and AITC-NAC for 18 hours, as compared to level

of invasion in the controls. The level of invasion being reduced to 60% and 53% of the control levels for 5 μ M of AITC and 5 μ M of AITC-NAC, respectively (Hwang and Lee 2006).

6.4.3 Modulation of Matrix metalloproteinases 2 and 9 following treatments with SF, IB or SF-NAC.

Metalloproteinases play a significant role in the control of cellular interactions and response to their environment in physiological conditions that promote tissue turnover, such as normal development; or pathological conditions, such as inflammation and cancer. Matrix MMP, notably the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are major contributors of stromal degradation and are vital to the process of cellular invasion (Nabeshima, Inoue *et al.* 2002).

MMP-2 and 9 have been correlated with the processes of tumour cell invasion and metastasis in many tissue and cell types. MMP-2 and MMP-9 have been found in large quantities in cancer tissues (Liotta, Tryggvason *et al.* 1980). Enhanced level of MMPs facilitate metastasis and cell invasion of tumour cells by removal of physical barriers, such as collagen, as well as modulation of biological activities of the proteins residing in the extracellular matrix (Yanagisawa, Geironson *et al.* 2005). For instance, many studies have shown that MMPs are implicated in the functional regulation of non-ECM molecules that include growth factors and their receptors, cytokines and chemokines, adhesion receptors and cell surface proteoglycans, and a variety of enzymes (Beaudeau, Giral *et al.* 2004).

To my knowledge there is little concrete evidence in published data regarding the extent of MMP down regulation that would be effective for anticancer effects. However it is known that decreased expression of MMP-2 and MMP-9 contributes to the inhibition of

lung metastasis of B16/F10 melanoma cells and have been correlated with the processes of tumour cell invasion and metastasis in many human cancers such as uterine neoplasms. Up-regulation of MMPs was associated with progression of cervical uterine neoplasms (Libra, Scalisi *et al.* 2009). More specific to bladder cancer, an examination was carried out to determine if a tumour-specific pattern of appearance existed among the MMPs detected in the urine of 189 patients with prostate or bladder cancer and controls. The frequency of detection of any MMPs was significantly higher in the urine from prostate and bladder cancer groups than controls. Approximately 140 >220 kDa, and approximately 190 high molecular weight gelatinase species found in the urine were identified as MMP-9/tissue inhibitor of metalloproteinase 1 complex, MMP-9 dimer, and ADAMTS-7. The study suggests identification of a tumour-specific urinary MMP fingerprint that may noninvasively facilitate identification of bladder and prostate cancer (Roy, Louis *et al.* 2008). Furthermore, this study demonstrated that MMP-2 and 9 activities in the urine of individuals with bladder tumours appeared to correlate with grade. Zymography showed gelatinolytic bands in the urine from individuals with bladder tumour, whereas only traces of MMP were detected in the urine of healthy subjects. However the majority of cancerous urine samples showed MMP-9 lytic activity but only a few contained MMP-2. The MMP-9 content was enhanced in the urine from patients with high-grade and advanced-stage bladder tumours (Roy, Louis *et al.* 2008), suggesting that elevated MMP-9 levels in bladder cancer patients correlate with a more advanced stage.

This study examined the effect of SF, IB and their conjugates on the secretion of MMP-2 and MMP-9, to elucidate their possible antimetastasis effect. Zymography revealed that the UM-UC-3 cell line constitutively secreted high levels of MMP-9 and lower levels of MMP-2, and that treatment of 12 μ M of SF, IB and SF-NAC reduced MMP-

2 and MMP-9 activities. Showing that 12 μ M of SF and IB can down regulate the production/secretion of MMP-2 and MMP-9 enzymes and also 12 μ M of SF-NAC can down regulate the production/secretion of the MMP-2 enzyme in the UM-UC-3 cell line. In a similar experiment to this using SK-Hep1 human hepatoma cells (Hwang and Lee 2006), MMP-2 and MMP-9 activity were demonstrated to be reduced in a dose dependent manner at concentrations of 0.1-5 μ M by the presence of the ITC AITC and its conjugate AITC-NAC. However in this experiment the AITC-NAC demonstrated a more potent inhibitory effect than the parent compound AITC at the same molar concentration. MMP-9 activity was suppressed by 20% and 50% by treatment with 5 μ M of AITC and 5 μ M AITC-NAC, respectively. MMP-2 activity was suppressed by 70% and 80% by 5 μ M treatments of AITC and 5 μ M AITC-NAC, respectively. Zymography also revealed that the SK-Hep1 cell line constitutively secreted high levels of MMP-9 and low levels of MMP-2 (Hwang and Lee 2006).

Human brain endothelial cells (HBMECs) are essential components of the blood brain barrier (BBB). While the secretion of MMP-9 by brain tumours has been well studied, secretion of MMP-9 by HBMECs themselves following treatment with a carcinogen (phorbol 12-myristate 13-acetate) has been little explored. In this study an increase of MMP-9 secretion upon treatment with phorbol 12-myristate 13-acetate was observed in HBMECs and SF was shown to inhibit both the excretion of MMP-9 enzyme and gene expression of MMP-9, although MMP-2 was not altered by the SF treatment (Annabi, Rojas-Sutterlin *et al.* 2008). Also broccoli and watercress extracts have been shown to be responsible for inhibition of MMP-9 activity and invasiveness of human MDA-MB-231 breast cancer cells (Rose, Huang *et al.* 2005). The effect of SF on the inhibition of lung metastasis in B16F-10 melanoma cells was studied. Gelatin zymographic

analysis showed that SF could inhibit the activation of matrix metalloproteinases in the B16F-10 melanoma cell line and proliferation assays showed that SF could inhibit the proliferation of B16F-10 melanoma cells *in vitro*. This suggests that SF reduced the invasion of B16F-10 melanoma cells by the inhibition of matrix metalloproteinases, thereby inhibiting lung metastasis. It was also shown in this study that *in vivo* metastatic control in C57BL/6 mice was found to be significantly lowered in the SF treated animals. There was 95.5% inhibition of lung tumour nodule formation and 94.06% increase in the life span of metastatic tumour bearing animals (Thejass and Kuttan 2006). Molecular signalling pathways leading to activation of endothelial cell proliferation and degradation of the basement membrane were analyzed by RT-PCR in the HMEC-1 cell line, an immortalized human microvascular endothelial cell line. It was suggested that SF could affect basement membrane integrity, as it suppressed transcription of endothelial metalloproteinase-2 and the tissue inhibitor of metalloproteinase-2 (Bertl, Bartsch *et al.* 2006). However when studying human umbilical vein endothelial cells SF induced a dose-dependent decrease in the proliferative activity of the endothelial cells, which was dependent on cell apoptosis and also inhibited tube formation on Matrigel™, but did not affect MMP production (Asakage, Tsuno *et al.* 2006). As all experiments were conducted in a single cells line, potential cell specific effects are not known.

In summary the UM-UC-3 cell line was employed in this study as it is known to be from a high grade tumour and therefore was thought to also have a high metastatic potential. However the study revealed that this cell line actually exhibited a low metastatic potential and therefore the capacity of ITCs to reduce invasiveness could not be assessed. Although, the inhibitory effects on MMP 2 and 9 and the reduction in cell motility observed in this current study suggests that these dietary factors may be responsible for inhibition of

metastasis by reducing MMP production and cell motility in this cell line and may contribute to the suppression of carcinogenesis by diets high in cruciferous vegetables. In the following chapter the *in vivo* effects of cruciferous vegetables are investigated following a human dietary intervention study which enabled the comparison of global gene expression before and after an acute intervention of a broccoli soup high in both SF and IB.

Chapter Seven

*A Human Dietary Intervention Trial With
Purple Sprouting Broccoli Naturally High
In Sulforaphane And Iberin.*

7.0 Human Dietary Intervention Trial.

7.1 Introduction.

The first aim of this study was to compare gene expression in bladder tissue, before and after a 4 day intervention of purple sprouting broccoli soup. To quantify changes in gene expression in bladder tissue from patients with recurring bladder cancer, following the consumption of purple sprouting broccoli containing naturally higher levels of iberin and sulforaphane. The use of whole genome array analysis may enable the identification of genes which are modulated by the short intervention and can be selected for further studies *in-vitro*.

Secondly in order to elucidate the mechanisms of chemoprevention of ITCs in bladder cancer, it is necessary to conduct a long term human intervention. To be able to do this, firstly a pilot study has to be carried out. Pilot studies are a crucial element for a good full study design. By conducting this pilot study it was hoped to fulfil a range of important functions, such as assessing the feasibility of a full-scale study, assessing whether the research protocol is realistic and workable, assessing the likely success of proposed recruitment approaches and identifying logistical problems which might occur using these proposed methods. This was a novel pilot study and as such had a number of limitations.

7.2 Specific materials and methods

7.2.1 Blood Samples

Following consent, samples of blood (maximum of 20 ml) were taken from each volunteer and put into either pre-labelled EDTA tubes or pre-labelled PAXgene™ tubes. The PAXgene™ blood RNA tube was intended for the collection, storage and transport of blood and stabilization of intracellular RNA. Sample codes were doubly encrypted to protect the volunteer's identity. Following collection, blood samples were transferred to the IFR laboratory to be stored for RNA and DNA extraction at a later date.

PAXgene™ Sample

PAXgene™ samples were stored and total RNA isolated and purified from the nucleated blood cells with PAXgene™ Blood RNA kit (Becton, Dickinson) at a later date. The quantity of RNA was assessed by absorbance at 260nm using a Beckman Coulter spectrophotometer and the quality of purified RNA assessed with the Agilent 2100 Bioanalyser. RNA was hybridized onto human Affymetrix U133Plus 2.0 probe arrays at the Nottingham Arabidopsis Stock Centre (NASc), University of Nottingham, and analysed for confirmation of expression via RT-PCR (section2).

Note- The major focus of this study was to quantify changes in gene expression in bladder tissue in patients with recurring bladder cancer. Therefore the RNA extracted from nucleated blood cells was stored anonymously in an IFR -80°C freezer until a later date for analysis when resources are available.

7.2.2 Biopsy Samples

Pre-intervention Biopsy

The Surrounding 'benign' bladder tissue biopsies taken during a flexible cystoscopy were obtained for research purposes only and were not part of routine procedure (refer to chapter one for detailed explanation of a flexible cystoscopy). Samples were immediately immersed in RNALater™ (Ambion) at the point of collection and transported to the tissue bank at the NNUH, where they were stored at 4°C. The samples were collected from the tissue bank and transported to IFR for analysis.

Post-intervention Biopsy

Two samples of surrounding 'benign' bladder tissue were taken during the TURBT/TURB for research purposes, in addition to routine biopsies taken for histological examination (refer to chapter one for a detailed explanation of the TURBT and TURB procedures). Samples were immersed in RNALater™ at the point of collection and transported to the tissue bank at the NNUH, where they were stored at 4 °C. Benign and cancerous tissue was routinely taken during the TURBT/TURB for histological examination. The samples were classified in accordance with the tumour node metastases (TNM) 1992 system and histologically graded according to the World Health Organisation criteria by NNUH histopathologist, Professor Richard Ball.

Biopsies placed in RNALater™ were incubated at 4 °C overnight before being analysed. RNALater™ stabilizes and protects RNA, and thus gene transcription, in fresh tissue specimens, so that the RNA can be extracted at a later stage. Changes in gene expression can occur during sampling, harvesting and handling, and during RNA isolation. These changes can occur very rapidly, within seconds after harvest. Stabilizing samples

minutes later is often insufficient to prevent artefacts and preserve the gene-expression pattern. There are two major types of artefacts that can occur. Cell death and enzymatic degradation of RNA result in artefactual non-specific and specific reduction of mRNA species. At the same time, specific genes can be induced or down-regulated during handling and processing of the sample. The combination of these two effects can result in a transcription profile that differs considerably from the true *in vivo* gene expression pattern. Therefore immediate protection with RNAlater™ technology ensures that downstream analyses truly reflect the expression profile of the intact tissues. Total RNA was extracted directly from the pre and post biopsies following the overnight incubation at 4°C in RNAlater. Total RNA isolation was performed using RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. RNeasy Micro Kit was the kit of choice due to the small amounts of tissue received, varying from 1 - 5 mg of bladder tissue. The RNeasy Micro Kit can purify RNA (maximum 45 µg) from very small amounts of tissue or cells (from a single cell to a maximum of 5mg). Preliminary investigation has demonstrated that although the procedure is difficult for the small biopsy and variable from sample to sample, sufficient RNA of high quality can be obtained from a biopsy sample of ≤5 mg in weight. The protocol for fibrosis tissue was used due to the nature of bladder tissue. RNA purification from fibrous bladder tissues can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. This protocol was a modification of the normal protocol of RNA extraction from tissue and included a proteinase K digestion to remove proteins which can interfere with RNA purification.

Although the post intervention samples tended to be much larger (10 mg-20 mg), a ≤5 mg sample was dissected from the biopsy and analysed using the same RNeasy Micro Kit to ensure consistent pre and post sample handling. RNA extractions were hybridised

onto Affymetrix Microarray and analysed for confirmation of expression via RT-PCR (refer to chapter two for detailed methods).

7.2.3 Supply of Vegetables

Seeds were supplied by Elsoms Seeds Ltd, Pinchbeck Road, Spalding, Lincolnshire, PE11 1QG, UK. The Red Head (Red H4) purple sprouting broccoli was grown and harvested by Olga Ltd, Boston, Lincolnshire, and processed by Christian Salvesen. It has attractive strong spears produced from a vigorous plant. Up until the last few years, the majority of purple sprouting broccoli varieties available were open pollinated. The line Red H4 is one of many 'parent' lines which are used to produce filial generation (F1) hybrids, its origin was an open pollinated variety called Red Head which has been commercially available for about 15 years and is quite variable in its characteristics. However, like most varieties at this time, it is only available during a short harvest period between March and early May. Breeding programmes are used to extend harvest time, increase uniformity, increase yield and improve ease of harvest.

The Red H4 hybrids offspring is a new uniform variety with specific and/or desirable characteristics from either or both parents. It has taken about 8 years to produce the uniform line Red H4 by the process of single plant selection over a number of generations. To produce consistent F1 hybrids, the original cross must be repeated each season. As in the original cross, this is usually done through controlled hand-pollination, and explains why F1-seeds are so expensive. Once a uniformed parent line is produced Elsoms Seeds test for glucosinolates levels and the Red H4 was shown to be a better line for its glucosinolate content.

The broccoli was made into a soup and packaged into 250 g portions and stored frozen at -20 °C until required. Commercial soup was bought from a supermarket to be used as the control group following ITC quantitative analysis (Waitrose broccoli and potato soup).

7.2.4 Broccoli soup preparation and storage

Soup kitchen

Following harvest of broccoli cultivars, individual soup portions were prepared at the Institute of Food Research (IFR) by the Human Nutrition Unit's diet cooks in accordance with Environmental Health guidelines for the storage and cooking of food provided to study participants.

The broccoli was washed in mineral water and left to drain in a colander. 500 g of broccoli florets were weighed out and placed into a plastic bowl and 750g of water was added to each portion of broccoli. A lid was placed on the bowl and microwaved on HIGH (700 W) for 2 minutes. The broccoli was homogenised with water using the 'mix' setting on the Kenwood New York Smoothie Maker for approximately 20 seconds, and finishing the mixing on the 'smooth' setting for approximately 10 seconds. The mixture was transferred into a large container, carefully blended together by hand with a spoon and portioned into 250 g bags. The 250 g bags were labelled and stored in the -20 °C freezer.

7.2.5 Analysis of Sulforaphane and Iberin levels in soup samples by UV/HPLC

A 250g bag of the homogenised soup portion was defrosted overnight in the fridge and one hour prior to analysis the soup was left out of the fridge to equilibrate to room temperature. For the control group soup, the commercial soup was heated according to the manufacturer's recommendations. For the broccoli group soup the 250 g portion of purple sprouting

broccoli was mixed with 150g of heated commercial soup and analysed. Both soup mixtures were mixed well and a 1 ml aliquot removed and placed into a 2 ml screw top microcentrifuge tube. The homogenate was centrifuged at 13,000 g for 20 minutes and 0.5 ml of the supernatant was removed with a 1ml syringe. This supernatant was filtered through a 0.2 µm PVDF aqueous filter into a glass HPLC vial. SF and IB standards were made by serial dilution of a 50 mM stock solution. For a 400µM standard 2.8µl of the 50 mM stock solution was added to 347.2µl of MilliQ-purified water. A double dilution of 150µl of standard plus 150µl of MilliQ-purified water was made to make the remaining standards. A calibration curve was run using 20µl injections of 400 µM, 200 µM, 100 µM, 50 µM and 25 µM of SF and IB. Test samples were diluted 1:10 and run directly after the calibration curve.

7.3 Study design

7.3.1 Study Protocol

A flow chart of the overall study design is given in figure 7.1. This pilot study consisted of a parallel dietary intervention trial studying male patients with superficial recurring transitional cell carcinoma of the bladder. Volunteers were allocated one of the two dietary groups: (i) consuming a soup consisting of 400g ITC-enriched purple sprouting broccoli /commercial soup mix per day for 4 days between diagnosis and treatment (n=3) or (ii) consuming 400g of a commercial soup only, for the 4 days between diagnosis and treatment (n=1). The commercial broccoli and potato soup was analysed and no SF or IB was found to be present in the soup. This soup was used for the control group and also to mix with the purple sprouting broccoli soup to enhance palatability.

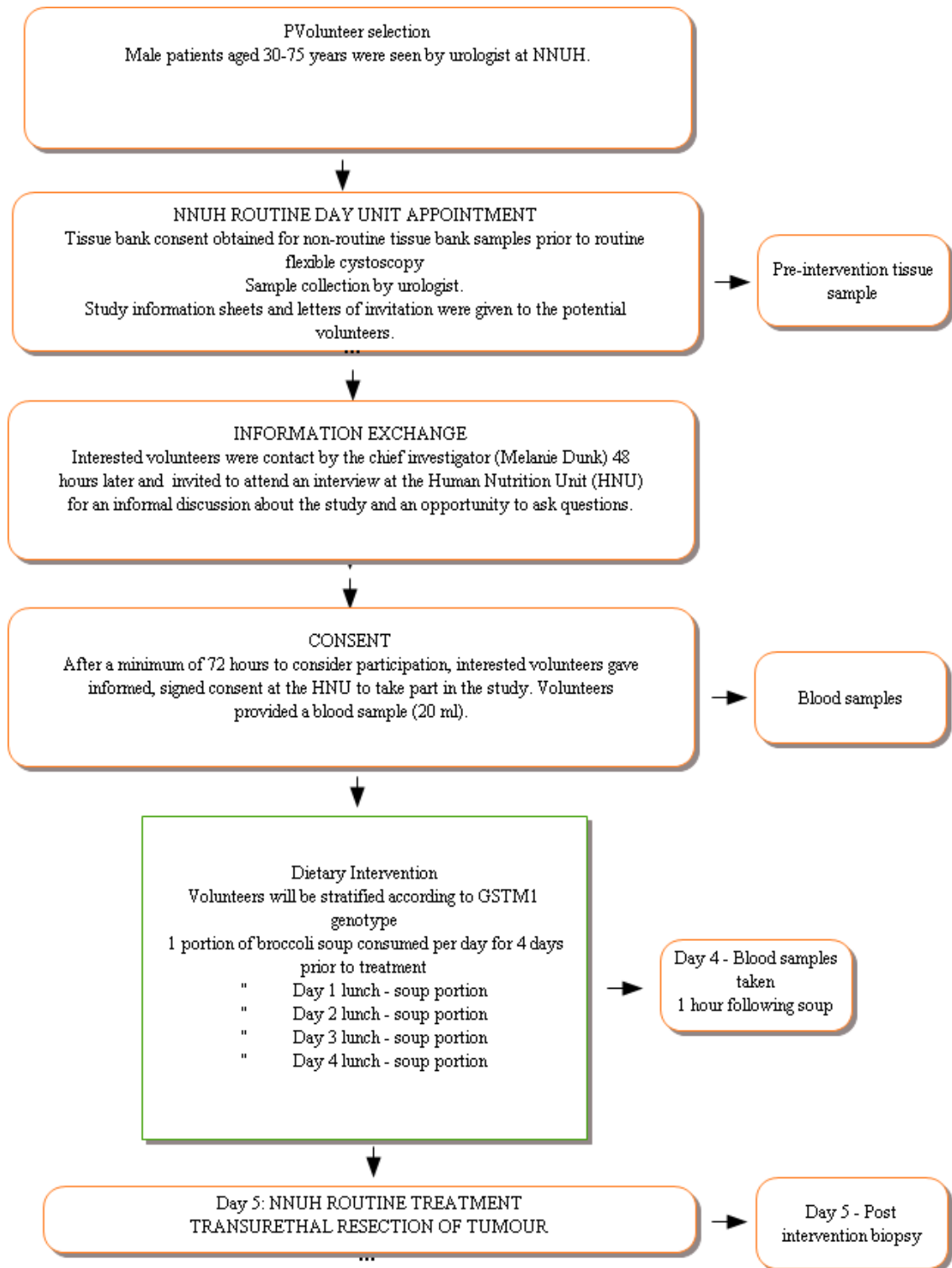


Figure 7.1 Study design

7.3.2 Patient Recruitment

The recurrence rate of treated bladder cancer is high in comparison to other malignancies and therefore patients attend regular surveillance cystoscopy, which was thought would enable relatively easy recruitment of volunteers. There is a short time period of approximately three weeks between diagnosis and the treatment of recurring bladder cancer, as opposed to the first presentation of bladder cancer, where there is only one week between diagnosis and treatment. Patients with recurring low grade bladder cancer were chosen for this study as the three week period gave an acceptable time to intervene, whereas to intervene in the one week period would have been impossible.

Men aged 30-75 years who were deemed to be 'at risk' from recurring bladder cancer attend the NNUH urology day unit for their routine surveillance cystoscopy every six months. They were seen by Robert Mills, consultant urologist or another member of his medical team. Volunteer recruitment was a two stage process:

7.3.3 Collection and storage of bladder tissue for research purposes by the NNUH tissue bank/histopathology department

Firstly patients presenting with recurring bladder cancer at the time of their surveillance cystoscopy were asked by the urology medical team to consent to the collection and storage of benign bladder tissue for research purposes, on behalf of the NNUH tissue bank/histopathology department. The tissue bank consent form was used at this stage, because it was known which patients would have a recurrence until the cystoscopy had been performed. Furthermore, it was crucial that tissue was removed at this time as this was the only opportunity to remove bladder tissue before the removal of the bladder tumour at a later date. Although the rate of bladder cancer recurrence is high, some

patients will not have a recurrence and to identify and gain patient consent onto the study before cystoscopy would have been impractical and insensitive.

Following a verbal explanation by the consultant urologist, patients were given a tissue bank information sheet and asked to sign a tissue bank consent form (annex 10.1) agreeing that if a recurrence was seen a biopsy of benign bladder tissue may be taken at the time of the flexible cystoscopy for the purpose of extracting RNA. The tissue bank form has been re-worded to allow the NNUH trust to use the bladder tissue non-anonymously, at its discretion, within this bladder study. The tissue bank consent form was signed by all complying volunteers at the time of the cystoscopy, in the presence of the consultant and copies accompanied all tissue transported to the tissue bank. It was made very clear that if the patient decided to donate the samples to the hospital tissue bank this did not mean they were committed to participating in the study in any way.

The amount of tissue removed at the time of the flexible cystoscopy was only very small, due to the narrow tube inserted into the urethra. Optimisation studies showed that the quality of the RNA was very variable from these small tissue samples. Therefore it was very important to extract RNA and check the quantity and quality, before contacting the patient and allowing them to consent to the intervention study. The tissue was released to the IFR for analysis to determine the quality and quantity of RNA.

Patients who met the following two criteria were given an invitation letter (annex 10.2) and volunteer information sheet (annex 10.3) about the pilot study during their routine surveillance cystoscopy appointment by Mr Robert Mills or one of his team. 1) Reoccurring bladder cancer was detected at the routine surveillance cystoscopy appointment and 2) if they had consented and donated a biopsy of benign bladder tissue to the tissue bank. If patients were interested in participating in the pilot study they were

informed that they would be contacted after 48 hours by the chief investigator (Melanie Dunk) by telephone. 48 hours was given to the patients to not only read the information sheet regarding the study and consider taking part, but also to come to terms with their diagnosis. The chief investigator's telephone number was supplied to all potential volunteers in case they had any questions or required further information at any stage.

7.3.4 Study Consent

Interested patients were invited to attend the HNU at IFR where the chief investigator explained what the study involved and the patients had the opportunity to ask questions. Following a 72 hour consideration period, interested volunteers contacted the chief investigator to confirm their intention to participate. It was hoped that recruitment would continue until a maximum of six volunteers in both treatment groups had completed the study. However due to difficulties in patient recruitment and time restraints, only four patients were recruited. Figure 7.2 is a flow diagram showing the route of patient selection.

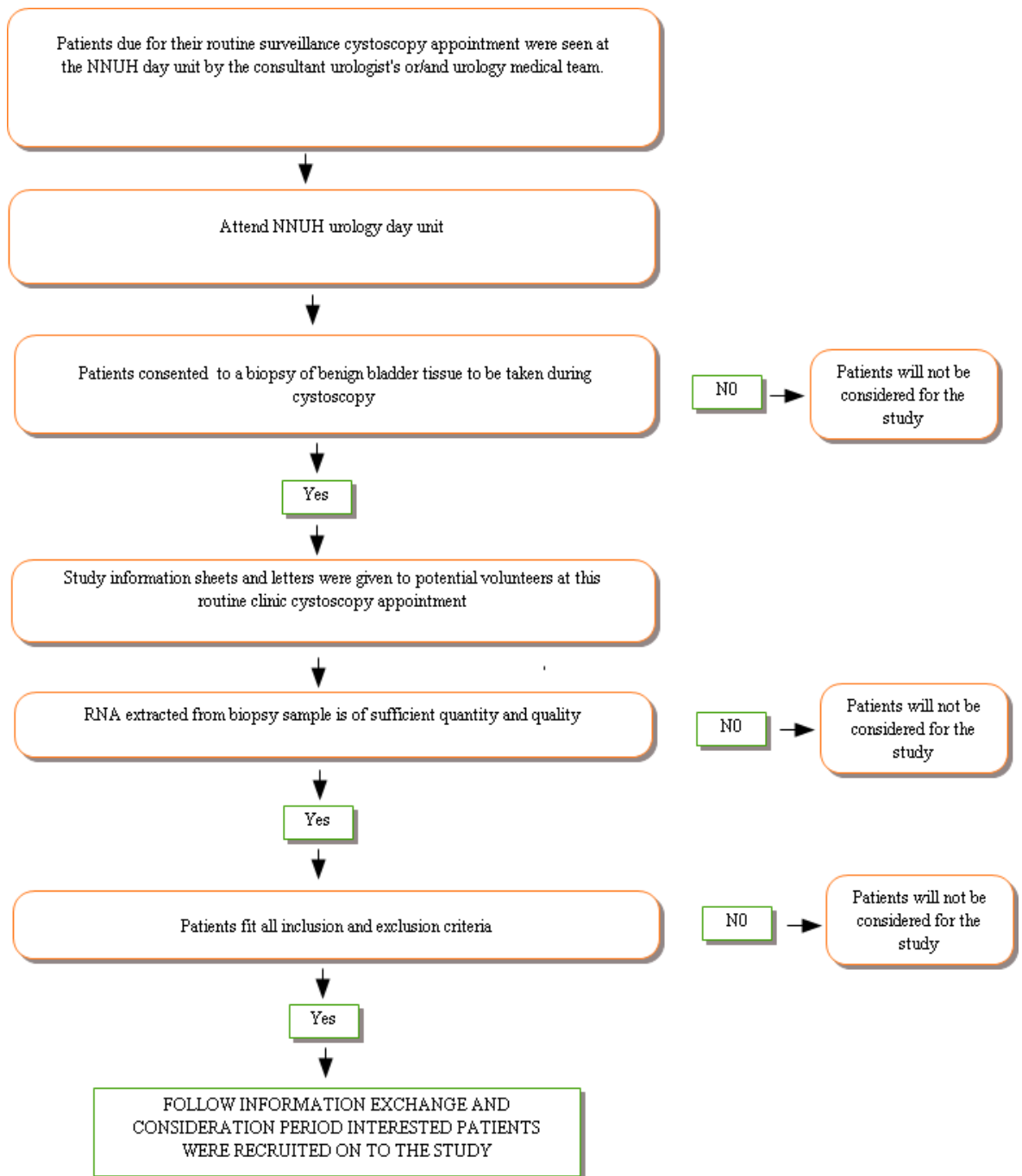


Figure 7.2 Patient selection

7.3.5 Inclusion and exclusion criteria

Inclusion criteria:

- Diagnosed with recurring transitional cell carcinoma of the bladder at time of cystoscopy
- A tissue bank sample had been donated at the time of their flexible cystoscopy
- Males aged between 30 and 75 years
- Smokers and non-smokers

Exclusion criteria:

- Undergone chemotherapy treatment within the previous 12 months
- Received any blood product transfusion in the last 4 months
- Type 1 diabetic and non-diet-controlled types 2 diabetics
- Acute infection (assessed by consultant urologist)
- The results of the health declaration and/or screening questionnaire form indicate that patient was not eligible to take part in this study (assessed by consultant urologist)
- Allergies to any ingredients in the soup
- If the tissue bank sample did not yield sufficient good quality RNA

7.3.6 Consent and study procedures

All volunteers responding positively following the period of consideration (minimum 72 hours) were invited to the HNU to sign a consent form and to give blood sample. On arrival at the HNU, the chief investigator went through the study consent form with the

volunteer (annex 10.4) and encouraged any questions they may have had at this stage; after which the volunteer signed the consent form agreeing to participate in the study.

A copy of the consent forms were kept in the study records; the patients' hospital notes and a copy was given to the volunteer to keep. Patients' general practitioners were informed by letter (annex 10.5) of their patient's participation in the study. The volunteers were required to sign a medical declaration form (Annex 10.6) and the study scientist completed a short screening questionnaire with the volunteer (Annex 10.7).

After signing the study consent form, blood samples totalling a maximum of 20 ml were taken by venepuncture by an appropriately trained HNU Research Nurse. Four blood samples of 2.5 ml were put into PAXgene™ blood RNA tubes. This sample was required for RNA extraction for Microarray gene expression profiling of white blood cells at a later date. The remaining blood was put into EDTA tubes for DNA extraction to determine genotypes for GSTT1, GSTM3 and GSTpi genes at a later date. Volunteers were allocated into one of the two dietary groups.

Once recruited onto the study, volunteers were assigned a code, only the chief investigator, approved by the Ethics Committee, being able to link codes to volunteers. Samples to be used for determining GST genotypes were doubly encrypted so that individuals could not be linked to the resulting genotype. All personal information was kept confidential and known only to the chief investigator.

7.3.7 Collection of bladder Biopsy Samples

Benign biopsies of the cancerous bladder were taken before and after the 4-day dietary intervention. The collection of all biopsy samples occurred at the NNUH. The first biopsies (a maximum of two) were taken during the flexible cystoscopy examination. The post intervention biopsies (a maximum of four) were taken at the time of TURB/TURBT. Small amounts of tissue (10-20 mg each) were removed pre intervention and small amounts of tissue (0.5-1 mg each) were removed post intervention. All patients received an explanation of the procedure by their consultant prior to examination and a standard information sheet. Figure 7.3 is a flow diagram representing sample collection.

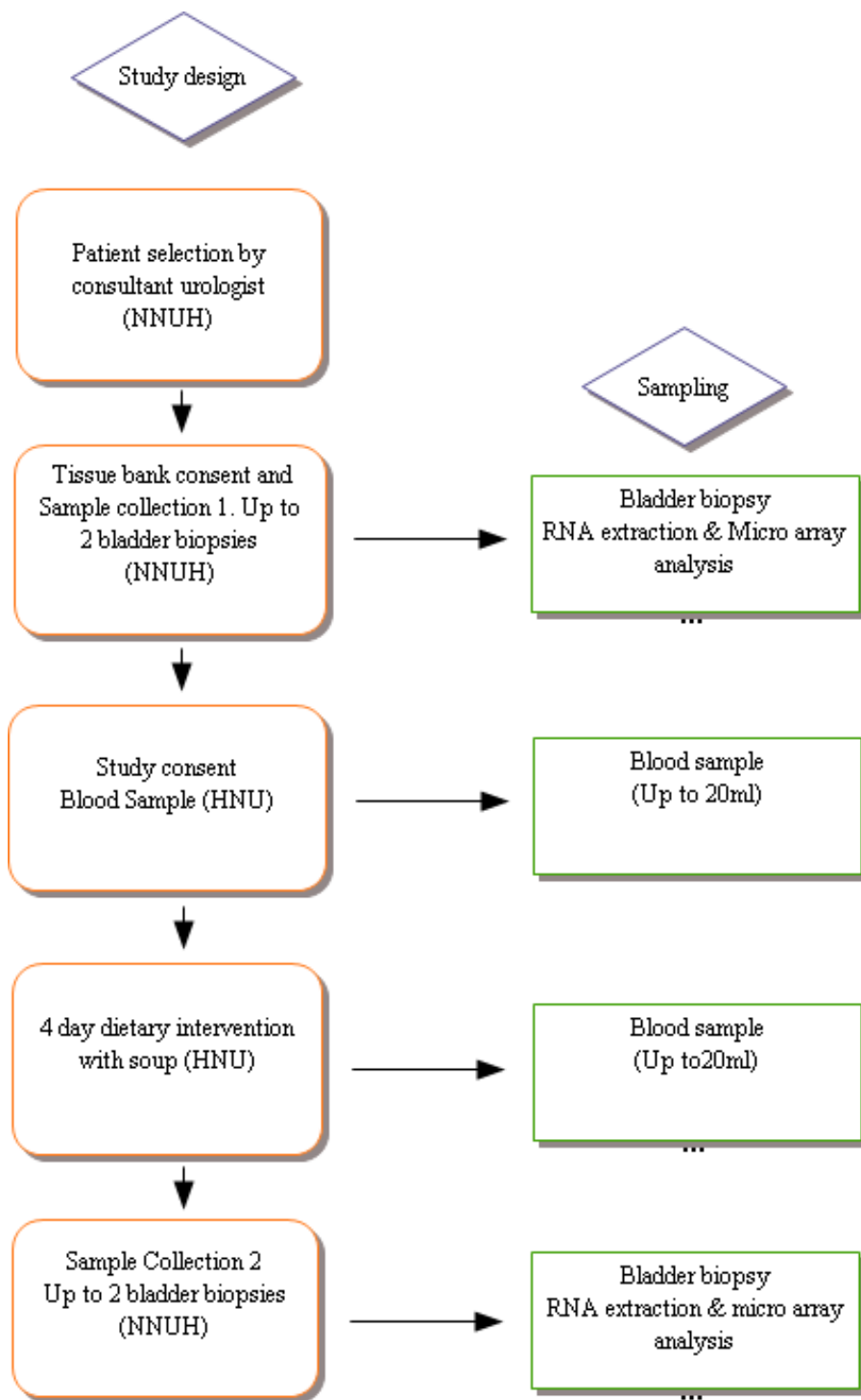


Figure 7.3: Plan of Sample Collection

* **Note-** The major focus of this study was to quantify changes in gene expression in bladder tissue in patients with reoccurring bladder cancer. Therefore the RNA extracted from nucleated blood cells was stored anonymously in an IFR -80°C freezer for analysis until a later date, when resources are available.

7.3.8 Dietary Intervention

After giving fully informed, signed consent, the volunteers were selected for either broccoli group or control group. Under supervision at the HNU, the volunteers allocated into the control group were asked to consume four portions (400 g each) of commercial soup over four consecutive days as part of their normal diet. Under supervision at the HNU, the broccoli group were asked to consume four portions (250 g of high ITC broccoli soup mixed with 150 g of commercial soup) of the special purple sprouting broccoli (PSB) soup, selectively grown for its enhanced ITC (SF and IB) content over four consecutive days as part of their normal diet. The volunteers managed to consume all their soups over the four day period.

The 250 g portions of purple sprouting broccoli soup were defrosted overnight in the fridge. One hour prior to consumption, the soup was left out of the fridge to equilibrate to room temperature. The commercial soup was heated according to the manufacturer's recommendations. For the broccoli group the 250 g portion of purple sprouting broccoli was mixed with 150g of heated commercial soup and served immediately. The control group had 400 g of heated commercial soup only. All volunteers were given a bread roll to eat with their soup. Volunteers were asked to refrain from eating any food for 1 hour before and after their soup, on the day of dietary intervention. A small aliquot of each volunteer's soup was collected on each day to analyse the level of ITCs.

7.3.9 Safety considerations

Risk factors: There can be a small amount of discomfort associated with taking blood and a small bruise may develop at the site of needle insertion, however the HNU nurses are trained and very experienced at taking blood and no adverse effects were seen or reported.

During the cystoscopy and the transurethral resection of the bladder tumour (TURBT), additional biopsies were taken for research purposes. Taking biopsy samples is painless but there is a small risk of damage to the bladder, infection and bleeding. Most would have some bleeding as a result of a biopsy that is not cauterised but perhaps < 1% would have either significant bleeding or infection (information obtained from Mr Robert Mills, consultant urologist). Should any bleeding or infection have occurred the patient would have been treated appropriately by the consultant urologist at the NNUH or their GP. However no adverse effects were seen or reported.

Food safety: The HNU at IFR complies with Environmental Health guidelines for the storage and cooking of food provided to study participants. These guidelines are an integral part of IFRs recognition as ISO 9001:2000 compliance and constitute part of our process working to the standards of Good Clinical Practice and Environmental Health Standards.

Toxicity: There is no evidence from animal or human studies that broccoli is harmful. However, toxic effects of indolyl glucosinolate have been identified in animals, causing goitre in animals with a deficiency in dietary iodine. The mechanism of this action is not well understood and there is no evidence of such acute effects in humans with intakes of glucosinolate below 50 mg/day. This level far exceeds the concentration consumed by volunteers in this study. As broccoli and broccoli sprouts are being used as vehicles for the delivery of glucosinolates and isothiocyanates in many human studies and clinical trials, a formal phase I study looking at the safety, tolerance, and pharmacokinetics of broccoli sprouts was carried out in 2006. A placebo-controlled, double-blind, randomized clinical

study of broccoli sprout extracts containing either 25 μmol of glucosinolate, 100 μmol of glucosinolate, or 25 μmol of isothiocyanate was conducted on healthy volunteers (Shapiro, Fahey *et al.* 2006). These doses are much higher than that ingested in this pilot study. Thirty-two haematology and biochemistry tests were done before, during and after the treatment period and no significant or consistent abnormal events (toxicities) associated with any of the sprout extract ingestions were observed (Shapiro, Fahey *et al.* 2006).

7.4 Statistical Power of the intervention study

It is not possible to calculate the power for differences in gene expression between the two intervention groups because to date there are no relevant publications that describe the effects of the ITC iberin on gene expression *in vivo*. This was a pilot study designed to provide data from which to calculate power for future studies in this field. The nature of a power calculation is to obtain the probability of detecting a difference that is true as opposed to a false negative result. It is arguable as to the appropriateness of such calculations when applied to Affymetrix gene expression data because the number of genes is so large (>47,000). Therefore the study is more concerned with controlling false positive results (type I error).

7.5 Ethical approval for research study

Research involving human subjects may not be carried out at the IFR without prior scientific peer approval from the IFR Human Research Governance Committee (HRGC) and a properly constituted human research ethics committee such as the Norfolk Research Ethics Committee. Also, as this study involved recruitment of Norfolk and Norwich University Hospital (NNUH) patients, the proposal was also submitted to the East Norfolk and Waveney Research Governance Committee for scientific peer review and NNUH research management approval. The Norfolk Research Ethics Committee is part of the National Research Ethics Service (NRES) formally called Central Office of Research Ethics Committees (COREC). They provide a standardised and robust ethical review to protect the safety, dignity and well being of research participants as well facilitating ethical research within the NHS. The NNUH East Norfolk and Waveney Research Governance Committee ensures the area of the hospital being utilised for the project is an appropriate environment for research into human physiology, disease or the development of new treatments. Figure 7.4 shows a flow chart developed by the senior research nurse at the HNU of the processes involved in submitting a human study for scientific and ethical approval. Annex 11.8 – 11.17 show the correspondence between the chief investigator and the above committees to gain ethical approval for the human intervention trial.

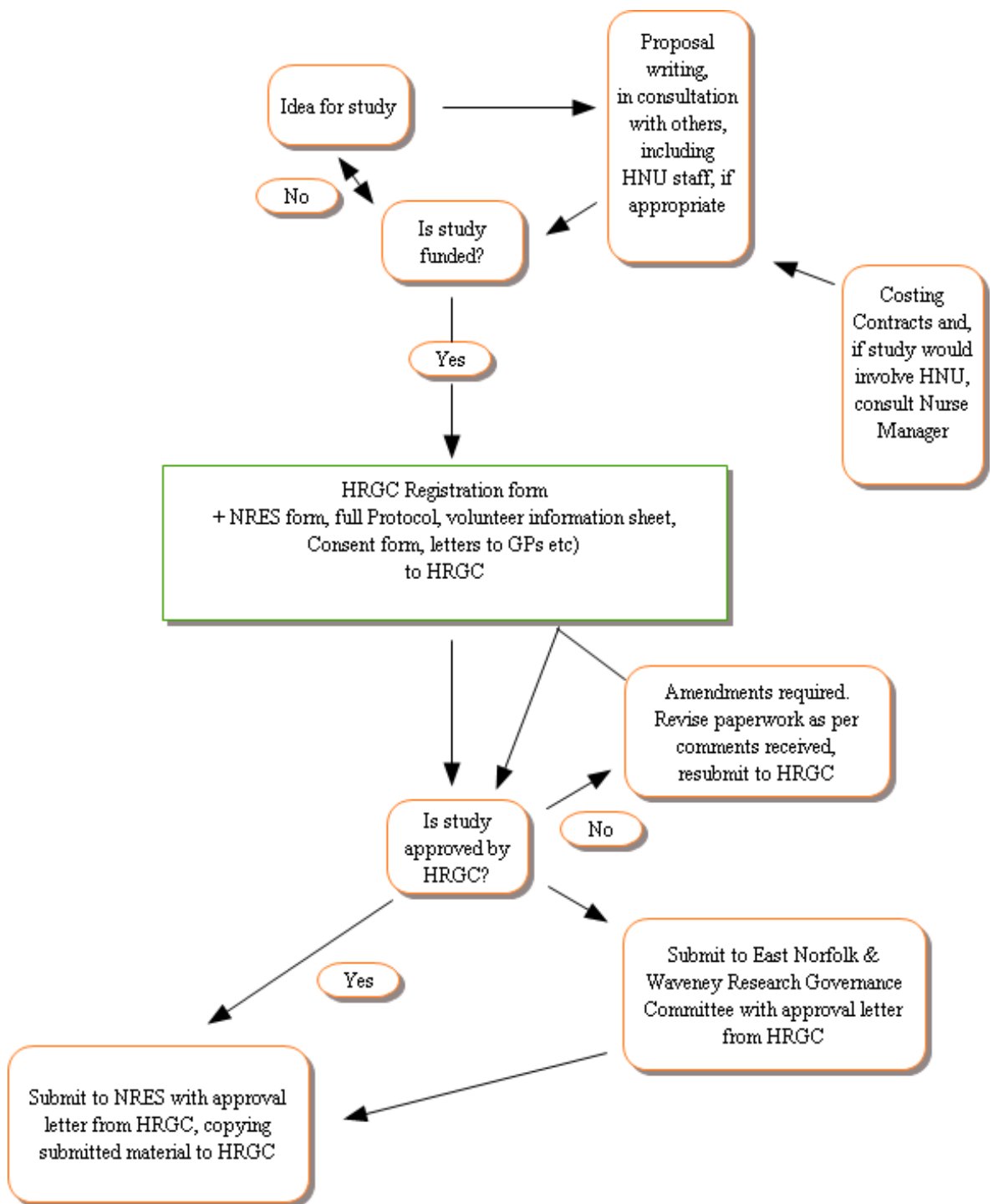


Figure 7.4 Flow chart for submitting a human study for ethical approval

7.6 Results

7.6.1 Intervention bladder tissue Affymetrix Array

Using whole genome array analysis enables the detection of changes in gene expression of novel genes due to the acute intervention of purple sprouting broccoli and these genes can be investigated in more depth in the characterised cell culture models. Although it must be highlighted that micro array analysis and the demonstration of particular gene expression cannot necessarily predict the production of a functional protein.

RNA was successfully extracted from seven pre and post dietary intervention bladder tumour biopsy samples for Affymetrix microarray analysis. Unfortunately one post sample was not received. Data analysis was performed in **R**. **R** is distributed by the Comprehensive R Archive Network (CRAN) and is available from the URI: <http://cran.r-project.org>. Normalisation of the signal intensities across all the arrays was performed in **R** to achieve signals that would be comparable between them. Following calculation of expression values for each probe set the data were submitted to higher-level analysis. Pair-wise comparisons between the pre and post samples were performed in **R** to identify genes that were differentially expressed more than two-fold at a statistical significant level ($p < 0.005$). Sample and gene clustering was performed, the former to identify any similarity between the two groups and the latter to identify genes with similar patterns of expression across all samples. The resulting dendrogram in figure 7.5, where red illustrates an increase in probe expression and green represents a decrease in probe expression. Larger gene lists were produced by specifying less stringent statistical criteria and lower fold changes. In contrast much smaller gene lists were produced with more stringent criteria. However a gene list of 18 was thought to be a manageable list to investigate further.

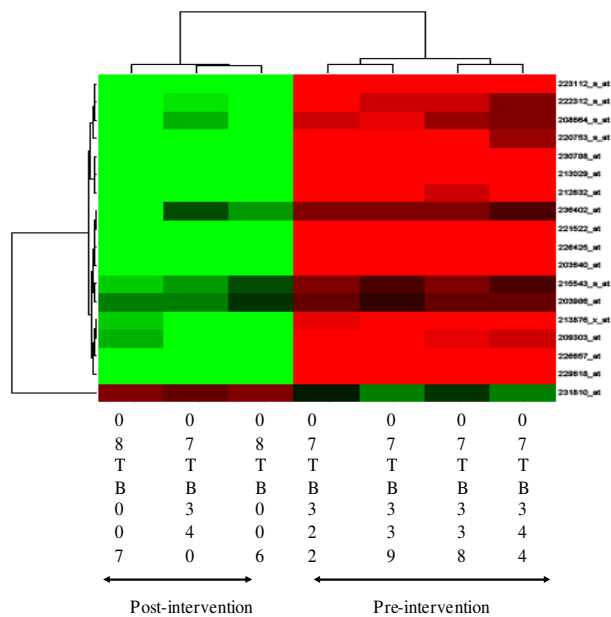


Figure 7.5 Hierarchical clustering dendrogram of 18 genes (P value of ≥ 0.005). Comparing pre dietary intervention samples with post dietary intervention samples.

The 18 genes are listed in table 7.1, and it should be noted that at this statistical criteria the majority of gene modulation, when comparing pre with post, is a down regulation of probe intensity in the post dietary intervention samples. It can also be seen that the COL6A1 gene previously investigated (chapter 5) is not significantly modulated at the statistical criteria, however collagen, type I, alpha 2 which is also a abundant ECM protein is down regulated.

<i>Accession No</i>	<i>Gene title</i> ¹	<i>Fold change</i> ²	<i>P-value</i> ³
NM_002160	Tenascin C (hexabrachion)	-2.18	0.0008
NM_020991	Chorionic somatomammotropin hormone 2	-2.11	0.001
NM_006347	Peptidylprolyl isomerase H (cyclophilin H)	-2.19	0.002
	Phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homolog, drosophila)	-2.08	0.004
NM_000923			
NM_003638	Integrin, alpha 8	-2.25	0.0008
NM_177966	2'-phosphodiesterase gb:X86400 H.sapiens mRNA for gamma X864000 subunit of sodium potassium ATPase.	-2.03	0.002
X864000		-2.95	0.005
NM_014095	gb:NM_014095.	-2.13	0.0042
NM_000024	adrenergic, beta, receptor kinase 2	-2.35	0.002
AA628535	Collagen, type I, alpha 2	-4.01	0.001
NM_020403	Protocadherin 9	-2.01	0.004
AA469917	Chromosome 8 open reading frame 72	-2.28	0.0002
NM_001099678	leucine rich repeat containing 58	-2.01	0.004
NM_001714	Bicaudal D homolog 1 (Drosophila)	-2.12	0.005
BF447682	gb:BF447682	-2.06	0.004
AK054653	hypothetical protein LOC153577	-2.35	0.003
NW_927317	cystatin pseudogene	-2.01	0.002
NW_923572	hypothetical protein LOC284661	-2.04	0.0004

Table 7.1 A list of probes altered in bladder tissue when comparing superficial pre and post intervention samples (18 probes)¹. The fold change shown represents the mean gene expression ratio calculated using R between the pre and post intervention cancer tissue. The negative values represent a down-regulation and as such correspond to green of the heat map in figure 7.1.² Genes that were differentially expressed more than two-fold at a statistical significant level ($p < 0.005$)³.

7.7 Discussion

7.7.1 Dietary Intervention Study

Some of the genes uncovered in this intervention study were very interesting, such as COL1A2 and Tenascin-C. However the recruitment was not as successful as one would have hoped i.e. although the rate of reoccurrence of TCC is high and as such, the amount of patients seen for routine surveillance is high, the uptake into the intervention study was low. There may be several contributing factors to this, such as the exclusion criterion was too strict. Although men are at more risk of bladder cancer, including women into the study could have increased recruitment numbers significantly. Secondly, the time of year may have had an impact on the patient's decision to participate in the study. Unfortunately recruitment coincided with Christmas and New Year and as such may have deterred patients from being involved. This was not planned and if time restraints were not an issue the study would have been delayed until January. Thirdly, the feedback from many patients suggested that they were discouraged about having to travel to the IFR/HNU on four consecutive days prior to their operation. Many of the patients who had shown an interest in being involved in the study lived some distance away from the NNUH/IFR and therefore felt they could not travel in every day. As a consequence all patients who participated in the study were from the nearby Norwich area. In hindsight, travelling to the participants' homes would have improved recruitment. Finally, considering the target patients/participants had very recently been diagnosed with reoccurring bladder cancer, it is not surprising that the recruitment was slow. This may have been corrected if the recruitment period was longer; however time restraints forced the study to come to an end.

7.7.2 Gene ontology

Chorionic somatomammotropin hormone 2

This gene encodes for the chorionic somatomammotropin hormone 2, is a member of the somatotropin/prolactin family of hormones and plays an important role in growth control. This particular family member is expressed mainly in the placenta and is up regulated during development and increases by term of pregnancy. Genetic variation in the somatomammotropin hormone 2 gene have been associated with low body weight at birth and in infancy, and metabolic syndrome later in life (Day, Chen *et al.* 2004), but to my knowledge has not been associated with cancer.

Peptidylprolyl isomerase H (cyclophilin H)

The protein encoded by this gene is a member of the peptidyl-prolyl cis-trans isomerase (PPIase) family. The protein has been shown to possess PPIase activity and may act as a protein chaperone that mediates the interactions between different proteins inside the spliceosome; however there are no associations with cancer.

Phosphodiesterase 4C, cAMP-specific (PDE4A) (phosphodiesterase E1 dunce homolog, *Drosophila*)

There is very little literature regarding this gene, however it has been suggested that the expression of a specific isoforms of PDE4A is reduced in patients with bipolar disorder (Fatemi, Reutiman *et al.* 2008). There are no associations with cancer.

Integrin, alpha 8 (ITGA8)

The protein encoded by this gene is an extracellular matrix receptor of the integrin family and is known to regulate cell adhesion, migration, and proliferation (Prols, Hartner *et al.* 1999) and appear to be the major receptors by which cells attach to extracellular matrices. It has been reported that alpha8 integrin could play an important role in maintaining tissue integrity in the glomerulus during glomerular injury. Alpha8 integrin is involved in the regulation of the mesangial cell phenotype and seems to promote adhesion, but inhibit migration and proliferation of mesangial cells (Bieritz, Spessotto *et al.* 2003). Also a polymorphism of the ITGA8 promoter modifies the progression of renal failure in autosomal-dominant polycystic kidney disease (Zeltner, Hilgers *et al.* 2008). There appears to be some work regarding renal abnormalities, however more specific to bladder cancer, the expression rate of integrin beta 4 subunit in renal cell carcinoma lines are lower than that in other urological tumour cell lines (Takiuchi, Kanokogi *et al.* 1994).

Collagen, type I, alpha 2 (COL1A2)

The COL1A2 protein is a component of the extracellular matrix and is involved in matrix integrity. It is found in most connective tissues and is abundant in bone, cornea, dermis and tendon. Type I collagen is the most abundant bone protein and considering the most frequent site of prostate cancer metastasis, along with many other cancers, is the bone, a study set out to determine whether prostate cancer bone metastasis is mediated by binding to type I collagen. They concluded that collagen I attachment mediated by alpha(2)beta(1), which is the type I collagen receptor, initiates motility programs through RhoC and suggest a mechanism for prostate cancer metastasis to the bone (Hall, Dai *et al.* 2006). In this bladder study the COL1A2 gene was seen to be down regulated 4 fold in the micro

array analysis (table 7.1). A study involving quantitative methylation-specific PCR and RT-PCR showed that COL1A2 gene inactivation/down regulation through CpG hypermethylation may contribute to proliferation and migration activity of bladder cancer. Comparing 67 bladder cancer specimens (BCs) and 10 normal bladder epithelia it was determined that the methylation index of COL1A2 was significantly higher in the 67 BCs than in the 10 normal bladder epithelia ($p=0.0011$) (Mori, Enokida *et al.* 2009). Like COL6A1, which was explored in chapter five, this collagen gene/protein also appears to be very relevant to bladder cancer invasiveness and metastasis.

Protocadherin 9 (PCDH9)

This gene belongs to a subfamily of the cadherin superfamily, but little appears to be known about its functions. One study suggests that PCDH20 hypermethylation may be a factor in the carcinogenesis of non-small-cell lung cancer (Imoto, Izumi *et al.* 2006). However there is little literature regarding PCDH9 and cancer.

Tenascin-C will be discussed in the following chapter.

There is little literature about the following genes functions and no association with cancer:

2'-phosphodiesterase

gb:X86400 H.sapiens mRNA for gamma subunit of sodium potassium ATPase.

gb:NM_014095.adrenergic, beta, receptor kinase 2

Chromosome 8 open reading frame 72

leucine rich repeat containing

Bicaudal D homolog 1 (Drosophila)

gb:BF447682

hypothetical protein LOC153577

cystatin pseudogene

hypothetical protein LOC284661

In summary, this novel short term intervention trial was successful as a pilot study. It highlighted some important issues relating to the study protocol which need to be changed before embarking on a full long term study, such as the inclusion/exclusion criteria and logistic issues creating problems with patients attending the HNU. However, this small pilot study did suggest that sequential sampling of bladder tissue before and after dietary intervention may provide insight to how certain diets may be able to reduce bladder cancer incidence and progression. Some very interesting genes were uncovered in relation to bladder cancer and this preliminary data in combination with the first array data, suggest that effects on the extracellular matrix may be of importance. In particular tenascin-C was a gene which was found to be increased with the severity of cancer grade, as seen by the first array analysis however the expression of this gene is decreased following the intervention with the broccoli soup. Tenascin-C is an extracellular matrix glycoprotein and promotes cell migration and proliferation. This gene has been shown to be expressed in bladder tumour stroma and positively correlated with tumour grade, stage and proliferative activity. Furthermore, tissue remodelling during carcinogenesis is associated with expression of specific tenascin-C splice variants and this will be explored in the following chapter.

Chapter Eight

*The Relationship Of Tenascin-C Expression
With The Grade Of Bladder Cancer.*

8.0 Relationship Of Tenascin-C (Tn-C) Gene Expression With The Grade Of Bladder Cancer

8.1 Introduction

While the broccoli intervention did not induce genes involved in phase II detoxification of xenobiotics, as may have been expected from studies with cell models, the intervention did highlight the decreased expression of an important extracellular matrix gene encoding for a protein called Tenascin-C. The expression is in an opposing manner to that observed with tumour progression. That is, results from the first array analysis, comparing superficial to invasive TCC in chapter three, showed a 4.5 increase in this gene expression, while the results from the second array analysis comparing expression pre and post broccoli intervention, showed a 2.18 decrease in Tn-C expression. In neoplastic tissues the alternative splicing of the Tn-C pre-mRNA is deregulated leading to the generation of a number of repeats which are absent from all normal adult human tissues, but are present in cases of tissue re-modelling, such as in foetal tissues and many types of solid tumours, ultimately leading to varied forms of the Tn-C protein. Among these repeats, domain A1, D and C are said to be particularly suitable markers for anti-angiogenesis targeting. The aim of this final chapter is to identify five splice variants of the Tn-C gene in 8 UM-UC-3 cell culture samples and 29 tissue samples and to assess whether there is a higher degree of these splice variants being expressed in the more invasive tissue/cell type.

8.2 Specific materials and methods

8.2.1 RNA extraction from UM-UC-3 cell culture and human bladder tissue

RNA was extracted from cell cultures and human bladder tissue using RNeasy Blood and Tissue Kit (Qiagen) according to the protocol described in the handbook for the purification of RNA from animal or cells spin-column protocol method, (section 2). RNA was extracted from eight treated and untreated cell cultures and 29 bladder tissue biopsies of varying grades. The diagnosis and histological staging of the tissue samples were established according to the WHO classification (Section 1) (Oosterlinck, Lobel *et al.* 2002).

8.2.2 Cycling conditions for Tn-C primers

Primers were used as designed by Mighell *et al.* (Mighell, Thompson *et al.* 1997) and Bell *et al.* (Bell, Pringle *et al.* 1999) and ordered from Sigma Genosys (Table 8.1). The reactions were carried out in triplicate. The samples were run on a Whatman Biometra® T gradient thermocycler. Cycling conditions for Tn-C primers, as described by Berndt *et al.* (Berndt, Anger *et al.* 2006), were 50 °C reverse transcription for 30 minutes, incubation at 95 °C for 15 minutes and PCR at 94 °C for 1 minute, 57 °C for 1 minute and 72 °C for 1 minute, for a total of 40 cycles. Ten µl of RT-PCR were mixed with the appropriate amount of 6X loading dye (Promega) and the product were electrophoresed through a 2% agarose gel in Tris-acetate (TAE) buffer (0.04 M Tris acetate 0.001 M EDTA) against 100 bp ladder (Promega). The length of the potential RT-PCR products were calculated according to published data of human Tn-C studies (Sriramarao and Bourdon 1993). The RNA was visualied using Ethidium bromide chelation to the RNA and observed under UV light.

Primers for RT-PCR analysis of splicing domains of Tenascin-C

Primer	Sequence	Reference*
T1	AGGCAGACACAAGAGCAAGC	Mighell <i>et al.</i> (1997)
T6	GCTACCCCCTAGTACTGATTTTATTGTCTA	Mighell <i>et al.</i> (1997)
T8	CATTGGCTATGAGGTTATGG	Mighell <i>et al.</i> (1997)
T9	CTGGTCTGAGTCTTGGTTCCGTCC	Mighell <i>et al.</i> (1997)
T12	TGTAATGACAAAGGCAGTGA	Mighell <i>et al.</i> (1997)
T15	TGAGATTGTGAGCCTCTTCC	Mighell <i>et al.</i> (1997)
T16	GCTGCCTCTACTGTGTCAGC	Mighell <i>et al.</i> (1997)
T25F	TGAACAAAATCACAGCCCAG	Bell <i>et al.</i> (1999)
T27R	CAGTGGAACCAGTTAACGCC	Bell <i>et al.</i> (1999)
β -Actin 1	CCTTCCTTCCTGGGCATG	QIAGEN GmbH
β -Actin 2	GAGCAATGATCTTGATCTTC	QIAGEN GmbH

*Mighell (Mighell, Thompson *et al.* 1997)

Bell (Bell, Pringle *et al.* 1999)

Table 8.1 RNA was amplified using primers for Tn-C and for Tn-C₁ splicing domains as described by Bell *et al.* and Mighell *et al.* To evaluate the RT-PCR system and RNA integrity, amplification of β -actin mRNA was performed as described by Katenkamp *et al.* To assess the whole amount of Tn-C mRNA a primer pair mapping the C-terminal domains F3-F5 was amplified (Berndt, Anger *et al.* 2006).

Primers		Target splicing domains	bp
5'	3'		
1 T1	T16	5-A1-A3	454
2 T1	T15	5-A1-A2-A3	737
3 T6	T12	B-ADI	346
4 T6	T9	B-B-D-6	531
5 F8	T9	C-D-6	519
6 25F	27R	Tn-C _(s)	322
7 β -actin 1	β -actin 2		

Table 8.2 Summary of primers for RT-PCR analysis and their target splicing domains.

	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Probe sequence (5'-3')
Tenascin C 216005_at	TCACAGGAGCTTTGGATGCA	AATTGTCCAAGGTAACACAGTCTGTAA	CAGGCTGGCGCTCTGATCCTGAC
Tenascin C 201645_at	GGGCCAACGGCAACAG	CAGAAACATGTTGGAGACTGATGTC	ATGGGCCTCACCTCCTCTGTGATTCTT

Table 8.3 Primer and probe designed and used in the real time RT-PCR analysis of the two Affymetric probe areas areas 216005_at and 201645_at

8.3 Results

8.3.1 Comparison of Tenascin-C expression between the two Affymetrix microarray studies (Chapter 3 and chapter 7)

From table 8.4 it may be seen that, not only does the expression of Tn-C increase with the severity of cancer grade, as seen by the first array analysis (chapter 3), but the expression of this gene is decreased following the intervention with the broccoli soup (chapter 7).

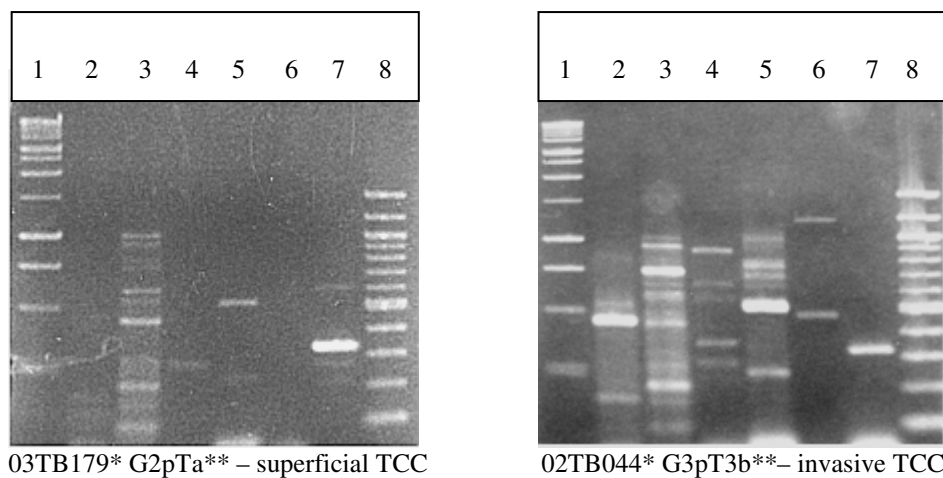
<i>Broccoli Dietary intervention</i>	<i>Superficial versa invasive bladder cancer</i>
Tenascin-C gene expression fold change	
2.18 decrease following dietary Intervention	4.51 increase in invasive tissue when compared to superficial tissue

Table 8.4 Comparison of Tenascin-C expression (fold change) between the two micro array studies.

The microarray dietary intervention data showed the expression of Tn-C is opposite to that observed with microarray data generated when comparing superficial bladder cancer samples with invasive bladder cancer samples.

8.3.2 Differential expression of Tenascin-C splicing domains in treated cell cultures and human bladder tissue

The Tn-C transcript, exhibiting a length of 332 bp (constant region, lane 7, in all gels, see annex 11.18) was very distinctive in all of specimens under investigation and independent from their diagnostic status. Figure 8.1 gives two examples of RT-PCR gels showing the different splicing domain patterns of RNA extracted from a superficial and an invasive bladder tissue biopsy. All gels can be seen in the appendix (a total of 48 gels), annex 11.18.



* Sample number; ** Histological grade of tissue biopsy

Figure 8.1 Example RT-PCR gels showing the different splicing domain patterns of RNA extracted from a superficial and an invasive bladder tissue biopsy. The biolab 100bp DNA ladder is in lanes 1. Lanes 2 – 6 represent splicing domains 5-A1-A2, 5A1-A2-A-3, B-ADI, B-D6 and C-D-6 respectively. Lane 7 represents a region which is always constant in the Tn-C gene and therefore serves as a positive control for the presences of the Tn-C gene. Lane 8 represents the positive control β -actin.

Generally all 39 samples showed Tn-C splice variant synthesis on different levels. Table 8.5 shows qualitative analysis of the five splice variants and in general the low grade (superficial) TCC samples expressed less Tn-C slice variants than the invasive TCC. All the samples expressed the constant region of the Tn-C gene (332bp, lane 7) and therefore

demonstrated the presences of the gene. The high grade UM-UC-3 cell culture samples expressed all five splice variants and the prior treatment with ITC did not modulate the expression of Tn-C variants. Samples 04TB125 and 02TB125, which are high grade TCC, both samples being graded as G3pT3b, also expressed all five splice variants. In accordance with published data, only high grade TCC tends to express the C-D-6 splice variant, lane 6 (Berndt, Anger *et al.* 2006), 04TB125, 02TB044, 04TB093 and cell culture samples were the only samples to express C-D-6. Interestingly, while the broccoli intervention (samples before (b) and after (a) in red, Figure 8.2) did not modulate the expression of Tn-C splice variants, in general the samples expressed more splice variants than the other TCC graded as superficial and as such gave a phenotype more comparable to that of the invasive TCCs. Samples 03TB146, 03TB165, 07TB020 and 04TB269, all graded as superficial TCC only expressed one splice variant, 5-A1-A2, lane 2, B-ADI, lane 4, B-ADI and B-D-6, lane 6 respectively. Apart from the intervention samples and sample 02TB023, which expressed three of the five splice variants, the maximum splice variants expressed by the superficial samples was two and predominately B-D-6, lane 5. The work of Berndt *et al.* reported that the B-ADI domain was rarely expressed; however examination of the work indicates that the expression of the B-ADI domain is actually only rarely expressed in the superficial TCC, which is consistent with my work. Only two high grade TCC samples did not express this splicing domain and nine out of eleven of the superficial TCC also did not express this splicing domain. The C-D-6 splice variant of the B-D region is rarely expressed in any of the samples at any grade except the cell culture samples. Only samples 02TB044, 04TB125 and 02TB093 expressed the C-D-6 domain. It has been reported that this splicing domain appears only to be expressed in tumours with

compact invasion patterns (Berndt, Anger *et al.* 2006). In accordance with this observation, samples 02TB044 and 04TB125 are graded as G3pT3b and as such are undifferentiated cells which have invaded the bladder wall giving rise to an extravesical mass which could be seen macroscopically by the urologist. Sample O2TB093 is also undifferentiated cells and the tumour would have begun to invade the bladder wall also.

	Grade	Splicing domains						
		1	2	3	4	5	6	7
		A1-A3 region			B-D region		constant	Ctl
		5-A1-A2	5-A1-A2-A3	B-ADI	B-D-6	C-D-6	Tn-C	β-actin
Bladder cells following treatment with:		453 bp	737 bp	346 bp	531 bp	519 bp	332 bp	204 bp
sf ctl (DMSO)	High	√	√	√	√	√	√	√
Sf	High	√	√	√	√	√	√	√
ib ctl (DMSO)	High	√	√	√	√	√	√	√
Ib	High	√	√	√	√	√	√	√
sf-nac ctl (DMSO)	High	√	√	√	√	√	√	√
sf-nac	High	√	√	√	√	√	√	√
sf/ib ctl (DMSO)	High	√	√	√	√	√	√	√
sf/ib	High	√	√	√	√	√	√	√
Human bladder tissue:								
03TB135	High	√	x	√	√	x	√	√
03TB163	Low	√	x	x	√	x	√	√
03TB180	High	√	√	√	√	x	√	√
03TB189	High	√	√	√	√	x	√	√
03TB192	Low	x	x	√	√	x	√	√
03TB195	High	√	x	√	x	x	√	√
04TB018	High	√	x	x	√	x	√	√
04TB023	Low	√	x	√	√	x	√	√
04TB035	High	√	√	√	√	x	√	√
04TB099	High	√	√	√	√	x	√	√
04TB269	Low	x	x	x	√	x	√	√
04TB280	High	√	x	√	√	x	√	√
04TB192	Low	x	x	√	√	x	√	√
03TB165	Low	x	x	√	x	x	√	√
02TB067	low	√	x	x	√	x	√	√
02TB044	High	√	√	√	√	√	√	√
02TB093	High	√	x	√	x	√	√	√
03TB179	Low	x	√	x	√	x	√	√
04TB119	High	√	√	√	√	x	√	√
04TB125	High	√	√	√	√	√	√	√
04TB021	Low	√	x	x	√	x	√	√
03TB132	Low	√	x	x	√	x	√	√
03TB146	Low	√	x	x	x	x	√	√
07TB0322	Low	√	√	√	√	x	√	√
07TB0338	Low	√	√	√	√	x	√	√
07TB0339	Low	x	√	x	√	x	√	√
07TB0344	Low	√	√	√	√	x	√	√
07TB0340	Low	√	√	√	√	x	√	√
08TB0007	Low	x	√	x	√	x	√	√

Table 8.5 Identification of five splice variants of the Tn-C gene in 8 UM-UC-3 cell culture samples and 31 tissue samples. To assess whether there is a higher degree of these splice variants being expressed in the more invasive tissue/cell type.

Sample	Grade	A1-A3 region		B-D region			constant	Ctl
		5-A1-A2	5-A1-A2-A3	B-ADI	B-D-6	C-D-6	Tn-C	β -actin
		453 bp	737 bp	346 bp	531 bp	519 bp	332 bp	204 bp
sfn ctl	High							
Sfn	High							
ib ctl	High							
Ib	High							
sfn-nac ctl	High							
sfn-nac	High							
sf/ib ctl	High							
sf/ib	High							
03TB135	High		Not expressed			Not expressed		
03TB180	High							
03TB189	High							
03TB195	High		Not expressed		Not expressed			
04TB018	High		Not expressed	Not expressed				
04TB035	High							
04TB099	High							
04TB280	High		Not expressed					
02TB044	High							
02TB093	High		Not expressed		Not expressed			
04TB119	High							
04TB125	High							
03TB163	Low	Not expressed	Not expressed	Not expressed		Not expressed		
03TB192	Low	Not expressed	Not expressed			Not expressed		
04TB023	Low	Not expressed	Not expressed			Not expressed		
04TB269	Low	Not expressed	Not expressed	Not expressed		Not expressed		
04TB192	Low	Not expressed	Not expressed			Not expressed		
03TB165	Low	Not expressed	Not expressed			Not expressed		
02TB067	Low	Not expressed	Not expressed			Not expressed		
03TB179	Low	Not expressed	Not expressed			Not expressed		
04TB021	Low	Not expressed	Not expressed			Not expressed		
03TB132	Low	Not expressed	Not expressed			Not expressed		
03TB146	Low	Not expressed	Not expressed			Not expressed		
07TB0322 b	Low							
07TB0338 b	Low							
07TB0339 b	Low	Not expressed		Not expressed				
07TB0344 b	Low							
07TB0340 a	Low							
08TB0007 a	Low	Not expressed		Not expressed				
08TB0018 a	Low	Not expressed						
06TB0208	Low	Not expressed	Not expressed			Not expressed		
07TB020	Low	Not expressed	Not expressed			Not expressed		

Indicates the splice variant is being expressed
 Indicates the splice variant is not being expressed
 Indicates intervention samples

Figure 8.2 Graphical representation of five splice variants of the Tn-C gene in 8 treated UM-UC-3 cell culture samples and 31 tissue samples.

8.3.3 Mapping of the Tenascin-C gene to identify the location of splicing domains and the Affymetrix probe 201645_at and 216005_at locations for RT-PCR analysis.

The Tn-c gene was mapped to identify the locations of all splicing domains and the Affymetrix probes 201645_at and 216005 to enable the design of primer and probes for RT-PCR analysis. To identify all relevant sequences, genomic, mRNA and protein the NCBI Sequence Viewer (www.ncbi.nlm.nih.gov/projects/sviewer) and the Human Genome Browser was used (www.genome.ucsc.edu/cgi-bin/hgGateway). BLAST was used to identify where the array sequences for the Affymetrix probe locations are on the gene and also exons AD1 and AD2.

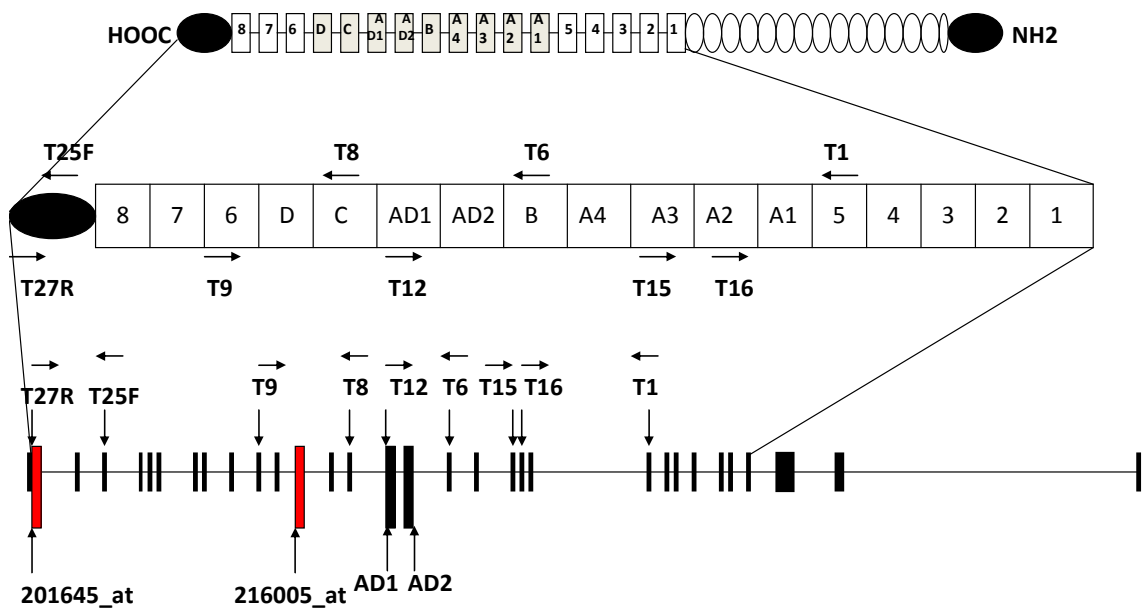


Figure 8.3 Mapped Tenascin-C gene to locate primers, splicing domains and Affymetrix probes 201645_at and 216005_at locations (Figure created by Dr Caroline Furniss of the Institute of Food Research).

8.3.4 Confirmation of dietary intervention array analysis (chapter 7)

When comparing superficial TCC with invasive TCC there was a significant 3.24 fold increase in Tn-C expression in the microarray analysis in the invasive group at the Affymetrix probe location 201645_at (figure 8.4) and a 2.18 fold decrease in Tn-C expression at this probe location post dietary intervention (figure 8.4 and chapter 7). The microarray analysis at the Affymetrix probe 21005_at did not show any significant difference between the invasive and superficial sample groups or the pre and post dietary intervention. To confirm these findings the Tn-C gene was mapped for the Affymetrix probes 216005_at and 20645_at target locations (Figure 8.3), primer and probes were designed and optimised (Table 8.3). Tn-C gene expression was determined via RT-PCR in RNA extracts from six pre and post dietary intervention tissue samples (figures 8.4 and 8.5) and 20 remaining tissue samples of varying grade (figures 8.6 and 8.7). Unfortunately the 20 remaining tissue samples were not analysed in triplicate due to insufficient RNA sample/tissue sample and as such do not have error bars.

The Tn-C gene at the Affymetrix 201645_at probe location showed a significant (* $p < 0.05$) decrease in expression in patient one and two following the dietary intervention (post biopsy sample). Patient three showed no change in Tn-C gene expression (figure 8.4). There was no significant change in Tn-C gene expression at the 216005_at Affymetrix probe location (figure 8.5).

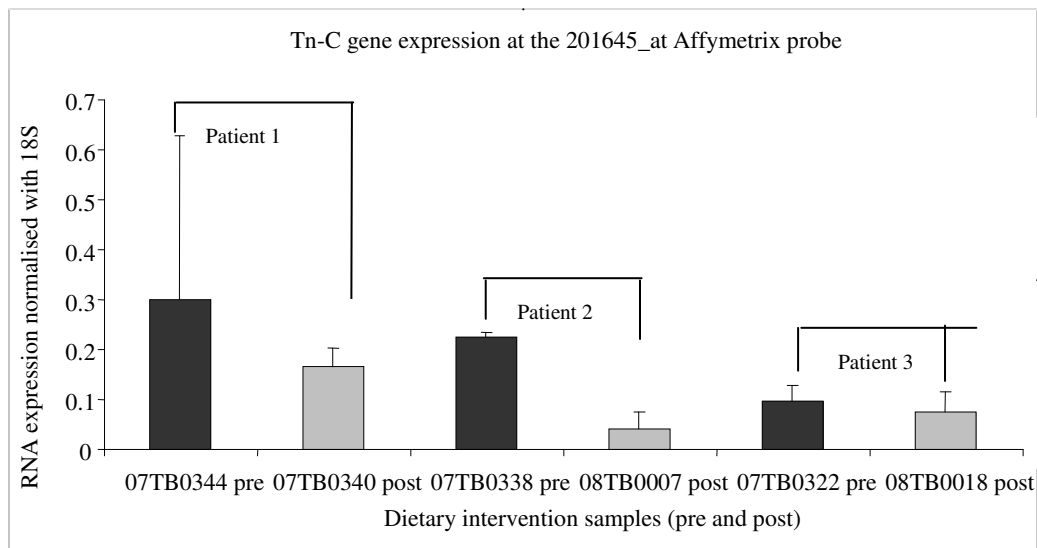


Figure 8.4 Tn-C gene expression in six pre and post dietary intervention tissue biopsies (three individuals) at the 201645_at probe target. Data is shown from three biological replicates. The error bars represent the SD of these biological replicates, * $p < 0.05$, $n = 3$.

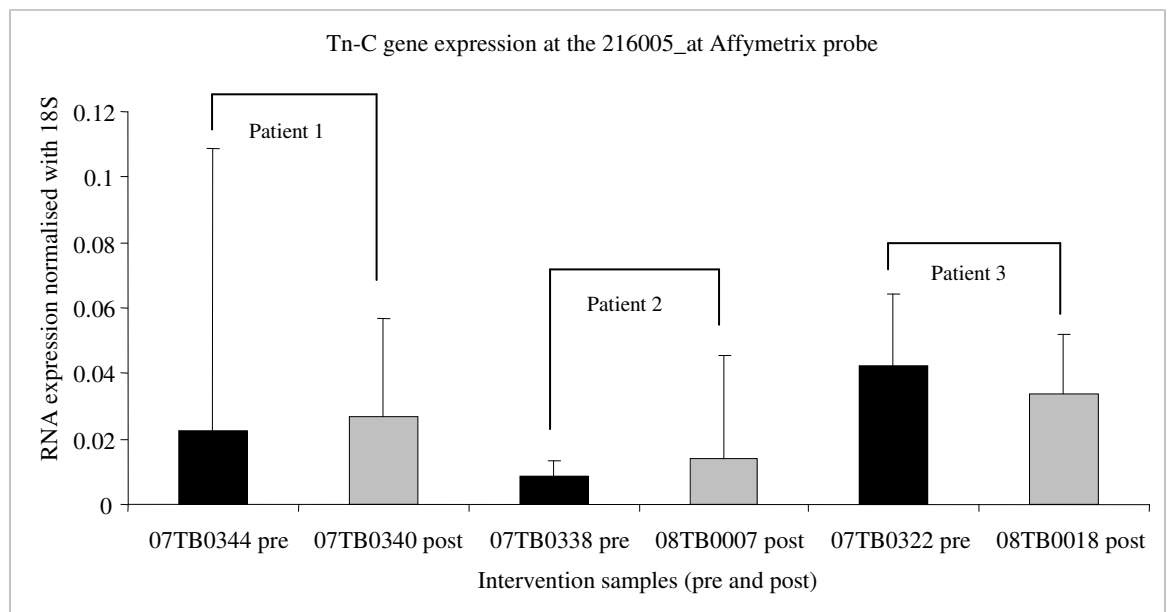


Figure 8.5 Tn-C gene expression in six pre and post dietary intervention tissue biopsies (three individuals) at the 216005_at probe target. Data is shown from three biological replicates. The error bars represent the SD of these biological replicates $n = 3$.

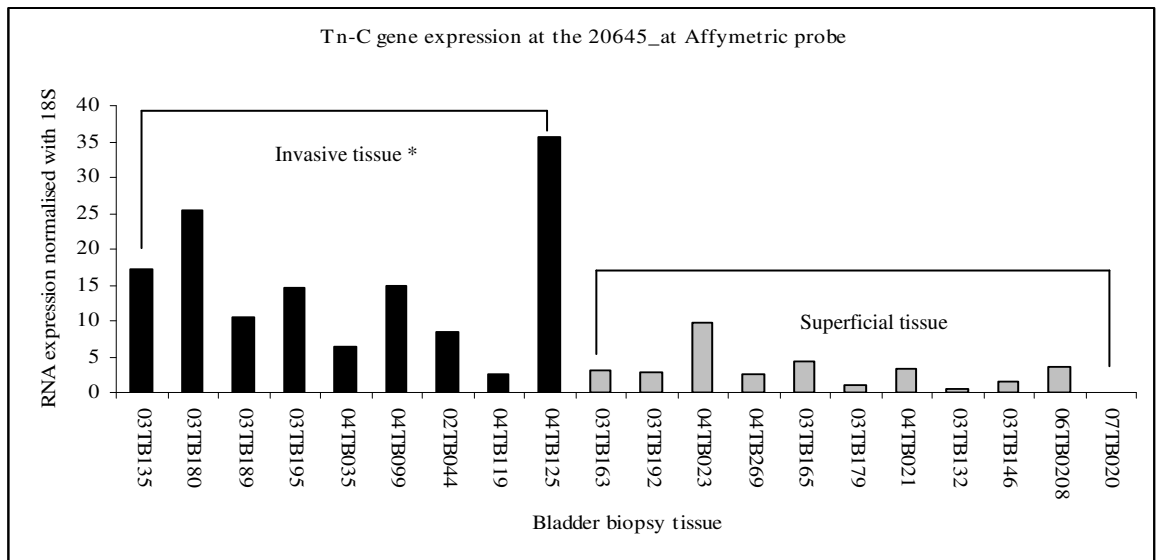


Figure 8.6 RT-PCR of the 20645_at Affymetric probe target region of the Tn-C gene in 20 bladder tissue biopsy samples.

Tn-C mean gene expression is significantly increased ($*p<0.05$) in the invasive samples compared to the mean Tn-C gene expression in the superficial samples at the Affymetric probe location 20645_at. Tn-C mean gene expression was also significantly increased ($*p<0.05$) in the invasive samples compared to the mean Tn-C gene expression in the superficial samples at the Affymetric probe location 216005_at.

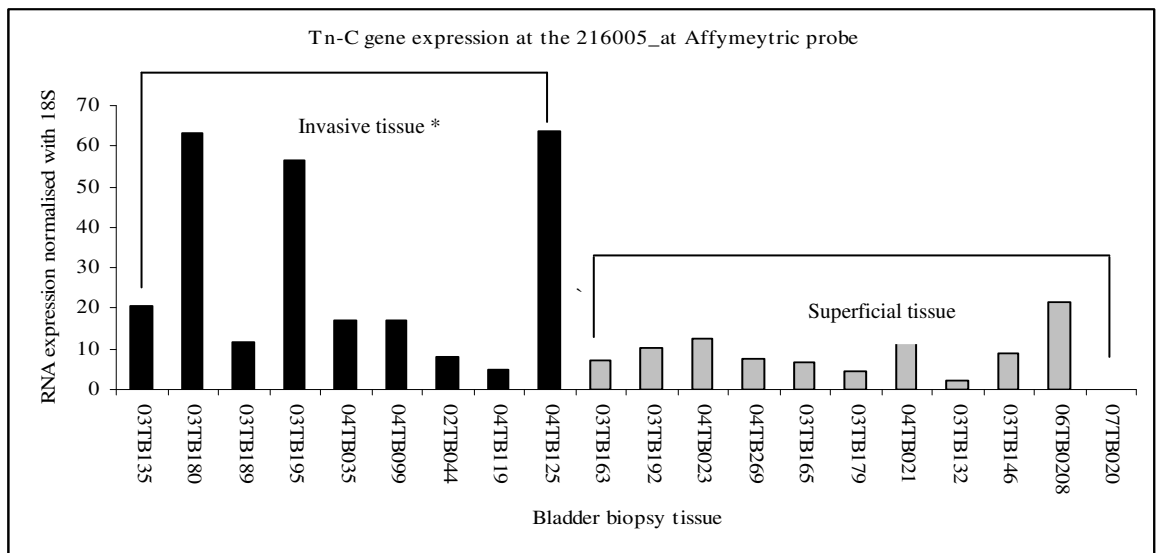


Figure 8.7 RT-PCR of the 216005_at Affymetric probe target region of the Tn-C gene in 20 bladder tissue biopsy samples.

8.4 Discussion

Tenascin-cytotactin (Tn-C), also known as hexabrachion, is from a family of large multimeric extracellular matrix glycoproteins; Tenascin -C, -R, -X and -W are all present in mammalian connective tissue (Chiquet-Ehrismann 2004). Six monomers are disulphide linked at their *N*-terminal from a central core as seen in the rotary shadowing image, figure 8.8 below.

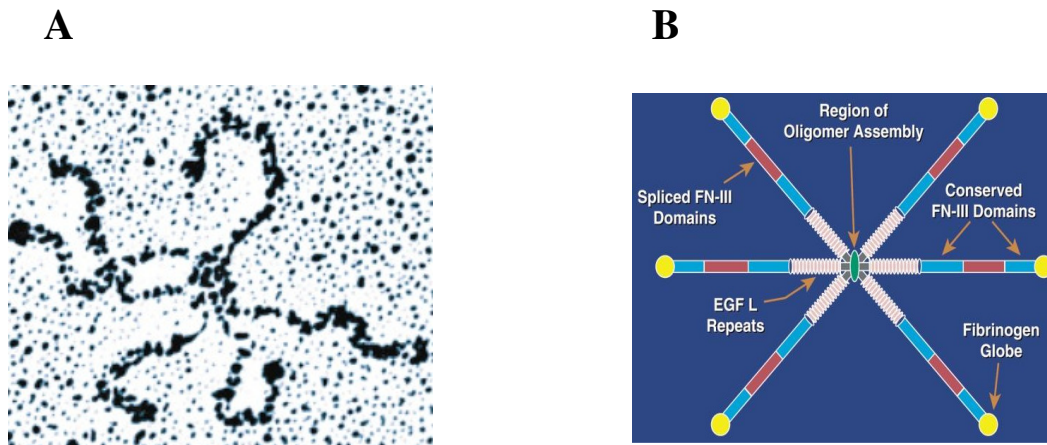


Figure 8.8 A Rotary shadowing image showing the highly symmetrical structure of the hexabrachion (Jones and Jones 2000).

B A key feature of Tn-C is the assembly into hexamers creating a six-armed structure of the hexabrachion (Jones and Jones 2000)

The subunits are composed of structural domains that include the globular amino terminal domain, 14.5 epidermal growth factor like repeats, a carboxy-terminal sequence with homology to the globular domain of β - and γ -chains of fibrinogen (Bell, Pringle *et al.* 1999) (Erickson 1993), as seen in figure 8.3. There also eight constant fibronectin type III-like domains (FN III); nine additional fibronectin type III-like domains can be included or omitted in a combinatorial manner by alternative splicing repeats, generating structurally and functionally different Tn-C isoforms (Siri, Carnemolla *et al.* 1991; Borsi, Balza *et al.* 1995; Berndt, Anger *et al.* 2006). These low splicing large Tn-C (Tn-C_L) are preferentially expressed during tissue remodelling processes including embryogenesis,

organogenesis, wound healing and carcinoma development and are known to modulate cancer adhesion to ECM (Bell, Pringle *et al.* 1999; Hauptmann, Budianto *et al.* 2003; Tse and Kalluri 2007).

It is this alternative splicing within the stretch of FN III domains which results in the great number and diversity in Tn-C isoforms. Therefore, depending on the variant generated by this alternative splicing, each subunit may have a molecular mass in the range of 190-300 kDa (Kammerer, Schulthess *et al.* 1998).

8.4.1 Confirmation of Tn-C array results by RT-PCR analysis of Affymetric probe locations 201645_at and 216005_at

Comparing superficial TCC with invasive TCC there was a 3.24 fold increase in Tn-C expression at the Affymetrix probe location 201645_at in the invasive sample group when compared to the superficial group. This confirms the array results; indicating that the expression of Tn-C increases with the grade and severity of bladder cancer. The Affymetrix probe 21005_at also showed a mean significant increase in the invasive sample group compared with superficial sample groups. This was not shown by the array results, as no significant difference at the probe location was seen in the array analysis.

8.4.2 Differential expression of Tn-C splicing domains in TCC biopsy tissue and UM-UC-3 cell line in relation to grade.

In order to reveal information about possible differentional expression of the Tn-C splice variants in TCC and bladder cell culture, an examination of the mRNA expression of five splice variants were investigated via RT-PCR. Figure 8.1 represents just two RT-PCR gels from superficial and invasive TCC and all gels can be seen in the appendix (a total of 48

gels). Tn-C is an adhesion-modulating extracellular matrix molecule that is highly expressed in tumour stroma and stimulates tumour cell proliferation and is said to promote cell migration and proliferation demonstrated *in vitro* (Yoshida, Yoshimura *et al.* 1999; Orend and Chiquet-Ehrismann 2000). Tsunoda *et al.* found that Tn-C variants were found at only low levels in normal breast tissues, but were highly expressed at invading sites of intraductal cancers and in the stroma of invasive ductal cancers and in addition the splice variants of these proteins, which are generally absent in normal adult tissues, become predominant in cancerous tissue (Adams, Jones *et al.* 2002; Tsunoda, Inada *et al.* 2003).

Tn-C has been shown to stimulate invasion line via up-regulation of MMP-1 expression through activation of mitogen-activated protein kinase (MAPK) cascade activation in a chondrosarcoma cell line. The analysis of gene expression in JJ012 cultured cells grown under different conditions indicated a significant 100% increase of MMP-1 mRNA levels in the cells treated with Tn-C bp320, compared cells treated with Tn-C bp220. Tn-C bp320 was not a splicing domain analysis in this experiment and Tn-C bp220 is a constant region of the Tn-C gene (such as 332bp in my experiments) and as such served as a control (Galoian, Garamszegi *et al.* 2007). Furthermore invasion activity assays demonstrated a 3-fold difference in invasion potential compared in cells treated with Tn-C bp 320 in comparison with the control.

Functional analysis of the Tn-C gene in mice has revealed its role in regulating the migration of tumour cells and the formation distant new tumour tissue. The research also found that reduced expression of Tn-C resulted in a decrease in tumour growth, tumour relapse following surgical removal and reduced metastasis in the lung (Albini, Mirisola *et al.* 2008). As in my research, Tn-C has been shown to be expressed in bladder tumours and was positively correlated with tumour grade and also appears to

correlate with proliferative activity, as seen in the invasive grades of TCC. Patients treated with TURBT alone and who had low expression of Tn-C, had a longer tumour-free interval than those with high expression of Tn-C. This observation may be poignant when considering the intervention biopsies/patients and some of the other superficial bladder biopsies with high Tn-C splicing domain expression studied in this RT-PCR investigation. This may be of significance, as all patients taking part in the intervention study had a reoccurrence of TCC; for two patients this was the third reoccurrence of TCC. All patients underwent TURBT post intervention and it is evident from the results that they have high levels of Tn-C splicing domains. It may be postulated that the presence of these splicing domains may contribute towards this observed short tumour-free intervals in these patients. It would be of interest to follow up these patients to discover whether they continue to have shorter tumour-free interval in comparison to patients with low levels of splicing domains.

In summary, these findings suggest that differential expression of Tn-C splicing variants in these TCC samples increase with grade of TCC tumour. Treatment of cell cultures with 12 μ M SF or 12 μ M IB and the consumption of broccoli high in SF and IB did not modulate Tn-C splicing domain expression in this experiment. However, these findings along with other study findings suggest that the detection of Tn-C splicing domains or perhaps particular Tn-C splicing domain expression patterns could be useful for the assessment of and diagnosis of muscle invasion TCC, tumour surveillance, as well as target structures for antibody based tumour detection and therapy. Moreover, the over expression of Tn-C may identify groups of patients with poor tendency to disease recurrence and longer relapse-free time.

Chapter Nine

General Discussion

Chapter 9: General Discussion

Bladder cancer is the fourth most common cancer among males and the eleventh most common cancer among women in the UK (Cancer Research UK). Additionally, bladder cancer has the highest recurrence rate of any malignancy (Grossman, Soloway *et al.* 2006) and most patients will experience either recurrence or progression. Therefore the need for accurate and diligent surveillance is paramount and consequently biological markers in the diagnosis and monitoring of reoccurring bladder cancer is of the utmost importance (Mohammed, Khan *et al.* 2008).

Many epidemiological studies have shown that the consumption of cruciferous vegetables can reduce the risk of developing bladder cancer and it is thought that isothiocyanates may play an important role in mediating the chemo preventative activity of cruciferous vegetables (Larsson, Andersson *et al.* 2008; Kim and Park 2009). SF is one of the prominent ITCs in calabrese and IB is one of the prominent ITC in purple sprouting broccoli. While SF is the most extensively studied ITC, there is very little literature regarding the biological effects of IB and cancer in general, and none regarding IB and bladder cancer. IB may have comparable biological effects to SF due to its similar structure (Chambers, Bacon *et al.* 2009).

There were five main aims of this thesis;

- 1) To characterise superficial and invasive bladder tissue. To compare global gene expression profiles between superficial and invasive transitional cell carcinoma and define gene expression profiles or selective genes that are characteristic of aggressive clinical behaviour in invasive bladder tumours for further studies *in-vitro*.
- 2) To characterise human bladder cell cultures and assess the effects of sulforaphane and iberin and their *N*-acetylcysteine conjugate on phase II gene expression.

- 3) To use whole genome array analysis to enable the identification of genes which are modulated by a short term dietary intervention in bladder tissue from patients with recurring bladder cancer, and to select genes for further studies *in-vitro*.
- 4) To investigate the effects of sulforaphane, iberin and their conjugates on the cellular motility of the characterised UM-UC-3 cell line and their potential capability to reduce invasion into extracellular matrix.
- 5) To evaluate the differential expression of Tn-C splicing domains in human transitional cell carcinoma of the bladder with respect to the invasive behaviour.

In this thesis there are two main objectives, firstly what are the molecular differences between superficial and invasive bladder tissue, and secondly what molecular changes are induced in bladder tissue by ITCs/Broccoli; specifically, can these compounds induce a more 'superficial profile' in bladder tissue and bladder cell cultures. Through the use of Affymetrix arrays, to quantify differences in global gene expression profiles between superficial and invasive transitional cell carcinoma, clear molecular differences between the two forms of cancer were observed. Non hierarchical analysis clustered gene expression into two clinically distinct groups; the superficial transitional cell carcinoma of the bladder (Ta- T1) into one group and the muscle invasive transitional cell carcinoma of the bladder (\geq T2) into another (Figure 3.1). This analysis identified, for the first time, two clinically distant phenotypes in accordance to their grade. In most invasive bladder cancer, muscle invasion is present at the time of first diagnosis and as such is classified as the primary tumours, in the remainder of patients muscle invasion is caused by progression of superficial tumours to invasive tumours (also known as progressive tumours). Prognosis of patients with progressive tumours is worse than prognosis of patients with primary invasive tumours and therefore it is of great importance to detect superficial tumours in their pre-

invasive stage, when they can be treated successfully and microarray analysis may be a good diagnostic tool. A list of probes which altered in bladder tissue when comparing superficial bladder cancer with invasive bladder cancer was generated (Table 3.3); identifying some putative markers that may be useful in diagnosis of progressive tumours and as such improve prognosis. The array analysis also supported the field effect theory of urothelial cell carcinogenesis by highlighting molecular changes in tissue which appeared histologically benign; sample 6, in the dendrogram (Figure 3.1), exhibited an invasive-like pattern in the bladder tissue taken from a histologically 'normal' area of the cancerous bladder of patient 02TB040. It has been shown that multiple coexisting tumours often arise before clinical symptoms become apparent and that separate tumours may or may not share a similar histology. Recurrences occur in more than 60% of patients and over 50% of these reoccur at a higher grade and are found at sites remote from the primary tumour. Therefore the field effect theory may not only account for the frequent development of genetically unrelated tumours within the bladder, but also the high rate of reoccurrence of bladder TCC. Obviously it is of clinically important to develop genetic detection for recurrent or residual tumour cells, and these results provide evidence to suggest that diagnosis and treatment of bladder cancer should not only be focused on the tumour but also on the field from which it developed. Furthermore the array analysis highlighted a significant fold change in COL6A1 gene expression that exhibited an increase in expression in the invasive bladder tissue compared to the superficial bladder tissue (Figure 3.1). This finding was investigated further using RT-PCR analysis of independent TCC biopsy samples and the results indicated a definite trend towards an increase of COL6A1 gene expression with the grade of TCC (Figures 3.2 and 5.2). The expression of this gene has significantly different means when superficial TCC tissue is compared with invasive

TCC tissue, being much greater in higher grade samples, thus rendering it a possible powerful tumour marker in TCC. To the best of my knowledge, COL6A1 has never been described in bladder cancer and these results warrants further investigation. Finally in chapter three I sought to characterise GST genotype in the invasive and superficial TCC, although in hindsight perhaps too much emphasis was made in this area considering the limitations due to sample size. However due to the few tissue samples analysed in this study, any firm conclusions regarding grade of TCC and GST genotype/ gene expression was not established.

Multiple mechanisms have been suggested to be activated in the response to ITCs such as, Nrf2-mediated induction of phase II detoxification enzymes, suppression of cytochrome P450 enzymes, induction of apoptotic pathways, suppression of cell cycle progression, inhibition of angiogenesis and anti-inflammatory activity (Juge, Mithen *et al.* 2007; Hayes, Kelleher *et al.* 2008). Most studies regarding ITCs and bladder cancer have mainly centred on the ability of ITCs to induce apoptotic pathways and cell cycle suppression (Algaba, Trias *et al.* 2003; Tang and Zhang 2004; Tang and Zhang 2004; Tang and Zhang 2005; Tang, Li *et al.* 2006). The human bladder cell lines UM-UC-3 and RT4 were characterised and the effects of sulforaphane and iberin and their *N*-acetylcysteine conjugate on phase II gene expression was assessed. The UM-UC-3 cell line showed a significant up regulation in GSTP1 gene expression, whilst the RT4 cell line showed no significant change (Tables 4.4 and 4.5). Both cell lines showed up regulation of GSTM3 expression and this expression was also up regulated by treatment with SF-NAC in the RT4 cell line. NQO1 expression was up regulated in both cell lines by treatment with SF, IB and their conjugates (Tables 4.4 and 4.5). This illustrates that different cell lines responded differently to detoxifying gene inducers, such as ITCs. Another cell based study

also concluded this, with seven well-established mammalian cell lines, which had different origins (Jiang, Chen *et al.* 2003). This study suggested that as different cell lines responded differently to individual detoxifying gene inducers, including ITC, that the selection of the appropriate cell line is important for screening potential chemo preventive agents. However whilst it is important to characterise cell cultures so that it is known that it has the capacity to respond to the particular external stimuli of interest, caution must be excised in only selecting hyper-sensitive cell lines, giving rise to miss-leading results.

COL6A1 is an extracellular matrix protein and like other collagen proteins plays an important role in maintaining the integrity of the ECM (see section 1). The array analysis in Chapter three highlighted a significant fold change in COL6A1 gene expression showing for the first time a significant increase in COL6A1 gene expression in the invasive bladder tissue compared to the superficial bladder tissue (Figure 3.1). This finding was investigated further using RT-PCR analysis of independent TCC biopsy samples and the results indicated a definite trend towards an increase of COL6A1 gene expression with the grade of TCC (Figure 3.2, 5.1 and 5.2). To the best of my knowledge this bladder study is the only studies to identify differential expression of COL6A1 with tumour grade.

Furthermore the modulation of COL6A1 expression in the previously characterised human bladder cell was investigated by exposing the cell lines to nutritionally relevant doses of SF, IB and their conjugates were investigated. It was demonstrated that COL6A1 gene expression is significantly down regulated following 48hr treatments of 12 μ M of SF, IB and SF-NAC in both UM-UC-3 and RT4 human bladder cell line (figure 5.3 and 5.4), although the UM-UC-3 cell line showed a greater degree of down regulation of COL6A1 gene expression. To conclude; COL6A1 gene expression was shown to be up-regulated in the invasive bladder TCC tissue in comparison to the superficial TCC and treatment with

ITCs was able to down regulate COL6A1 gene expression in the human bladder cell lines. For the first time it has been shown that COL6A1 gene expression increases with the severity of bladder cancer *in vivo* and the gene can be down regulated by physiologically relevant doses of SF and IB *in vitro*. These results suggest that both IB and SF may have important effects on the extracellular matrix (ECM), as collagen is a dominant component.

Very few studies have investigated the effects of ITCs on the transition from superficial bladder cancer to invasive bladder cancer and its subsequent modulation of the ECM. The effect of SF and IB on markers of progression and invasion of cultured human bladder cells was investigated in Chapter six. Results suggest that SF, IB and SF-NAC have the capability to reduce the mobility of the UM-UC-3 cell line following a 24 hour period of incubation (Figure 6.2). Invasion analysis demonstrated that treatment with these ITCs do not modulate UM-UC-3 cell invasion and furthermore the resulting low invasion index suggests that UM-UC-3 cells have a low-invasive phenotype suggesting that the UM-UC-3 cell line has little metastatic potential (Table 6.1). Further studies would involve using a more invasive cell line to investigate the possible inhibitory effects of these ITCs on cell invasion. Finally the effects of SF, IB and their conjugates on the secretion of MMP-2 and MMP-9 were investigated to elucidate their possible antimetastasis effect. The results indicated that the UM-UC-3 cell line secretes high levels of MMP-9 and lower levels of MMP-2, and that treatment with SF, IB and SF-NAC reduced MMP-2 and MMP-9 activities in this cell line, figure 6.8. In this study, it was shown for the first time the antimetastatic effects of SF and IB in human cancerous bladder cells. SF and IB had an inhibitory effect on cell migration. Protein levels of MMP-2 and MMP-9 were reduced by SF and IB. Enhanced level of MMPs is known to facilitate metastasis and cell invasion of tumour cells by removal of physical barriers and the observed down regulation of these

enzymes by ITCs suggests that these dietary factors may be responsible for inhibition of metastasis by reducing MMP production and cell motility in this cell line and may contribute to the suppression of carcinogenesis by diets high in cruciferous vegetables. These findings suggest that SF and IB have potential as an antimetastatic agent.

The biological activities of cruciferous vegetables and ITCs have been almost exclusively investigated in cell and animal models. In chapter seven I sought to extend this study to an *in vivo* pilot dietary intervention study with bladder tissue I obtained before and after a short diet of interest. With the use of whole genome array analysis to enable the identification of genes which were modulated by the short term dietary intervention. In contrast to the results of the previous *in vitro* study, phase II gene expression was not modulated in this short term intervention study. Moreover, to my knowledge this is the first dietary intervention undertaken with bladder cancer patients using whole genome arrays. The recruitment of patients into the dietary intervention study was more difficult than expected and due to the sensitive nature of the patients, was a challenging research area. The pilot study was very successful in identifying important issues relating to the study protocol. With protocol adjustment, this will hopefully lead to a successful, novel long term study of the effects of broccoli consumption on bladder cancer. It was notable that certain genes involved in regulation of the ECM such as collagen genes and Tn-C which were seen to be modulated in the first array from superficial to invasive tissue (Chapter three), were also modulated by the dietary intervention study. Further to this, Tn-C splicing domains were investigated in bladder tissue and findings suggest that differential expression of particular Tn-C splicing variants in TCC samples increase with grade of TCC tumour.

10.0 annex

10.1 Tissue bank information and consent form

Norfolk and Norwich University Hospital



NHS Trust

Affix an addressograph label here or complete the following details:

Patient's name.....

.....

Date of birth.....

Hospital number.....

Consent form for the collection and storage of human tissue and/or fluids for research

I agree that the following tissue or other material may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

Benign and cancerous bladder tissue
EDTA blood sample 20ml (two teaspoons)

I also agree that (Please initial small boxes, as appropriate):

This tissue or other material becomes the property of the Norfolk & Norwich University Hospital NHS Trust ("the Trust")

The Trust may store this tissue or other material in a Tissue Bank

The Trust may use this tissue or other material at its discretion within the research programme of Ms Melanie Dunk and Professor Richard Mithen of the Institute of Food Research.

The Trust may disburse this tissue or other material at its discretion to other approved tissue banks and/or companies in properly approved research programmes

Information about my case may be kept on the Tissue Bank database

Such information may be passed to Ms Melanie Dunk and Professor Richard Mithen of the Institute of Food Research in connection with research, and may be published anonymously with any research findings

I agree that appropriately qualified staff employed by the Trust may review my hospital case notes, as appropriate, for the purposes of research using my donated tissue

I confirm that:

I have read and understand the Information Sheet for Patients, Version 8, dated 11 November 2006

The issues have been explained to me, and that I have had the opportunity to ask questions.

Signed _____

Date _____

Relationship to patient (if relevant) _____

I have explained the request for tissue for research purposes and answered such questions as the patient (parent/guardian) has asked.

Name _____

Medical / Nursing Practitioner

Signed _____

Date _____

Affix an addressograph label here or complete the following details:

Patient's name.....

.....

Date of birth.....

Hospital number.....

Consent form for the collection and storage of human tissue and/or fluids for research

CONSENT FOR TAKING EXTRA SAMPLES FOR RESEARCH

Please initial the appropriate box for each item:	YES	NO
<p>In addition to the removal of tissue, blood or other fluid samples as a necessary part of my procedure, I also consent to the removal of additional tissue, blood or other fluid samples from the operation site during my procedure PROVIDED THAT SUCH REMOVAL CAUSES ME NO HARM now or in the future, is limited to what I and the doctor treating me (or a research nurse or nurse practitioner delegated by him/her) have discussed and agreed, and which is specified below. *</p> <p><i>Please initial appropriate box</i></p>		

*Additional samples to be taken: a maximum of two benign bladder tissue biopsies and one 20ml (two teaspoons) blood sample. _____

Patient name _____

Signed _____

Date _____

Relationship to patient (if relevant) _____

I have explained the request for tissue for research purposes and answered such questions as the patient (parent/guardian) has asked.

Name _____

Medical / Nursing Practitioner

Signed _____

Date _____

Medical / Nursing Practitioner

(i)

(ii)

(iii) **WHAT WILL HAPPEN**

During your procedure it will be necessary to remove tissue, blood or other samples for diagnosis and treatment of your condition. The samples will be sent to the Pathology Laboratories where they will be tested to decide exactly what they are and whether any further treatment will be necessary. This is a standard part of treatment. **Only as much tissue or fluid as is needed will be removed.**

1) MEDICAL RESEARCH AND
WHY THIS PROGRAMME IS
IMPORTANT

When all the routine tests have been done, if any samples are left over, with your consent,

they could be donated for use in medical research. As part of a research program set up by the Norfolk and Norwich University Hospital NHS Trust, the James Paget Healthcare NHS Trust, the School of Biological Sciences at UEA and the Institute of Food Research, some of the sample or material extracted from it will be stored in a Tissue Bank for use by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease, and to

develop new methods of prevention or new treatments for the benefit of future patient care. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care, in which case there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your case notes as part of this research. It is important to see how you progress after the donation of tissue or other samples, and to see how your condition relates to what we learn in the research project(s).

The research may also involve training doctors and scientists in scientific medicine, and may lead to higher qualifications for them (e.g. PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS

If you agree, we may send stored material or products derived from it to other approved tissue banks or companies to support their research programmes or the research programmes of those companies' clients. Such outside organisations will provide financial support for our Tissue Bank, to help it recover its operating costs.

Continued..... We are not, however, allowed to sell tissue or other samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL

The Tissue Bank acts as a custodian of the samples it holds and releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been properly

approved by the Trust's Research Governance Committee and by an NHS Research Ethics Committee before it starts. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participating patients, and issues of consent and confidentiality. We will release tissue or other samples to commercial companies only if they work to appropriate ethical and scientific standards.

i) **DONATING EXTRA
SAMPLES FOR RESEARCH**

In certain circumstances you may be asked by the doctor treating you (or by a doctor, research nurse or nurse practitioner working with him/her) to consider donating tissue or other samples in addition to those to be removed as part of your diagnostic investigation or treatment. **Such extra samples will be taken only if you give your consent and if their removal does not**

cause you any harmful effects now or in the future.

2) **YOUR RIGHTS**

If your samples are stored, information about your case will be kept on a computer in the Tissue Bank. This will help us understand what your illness was like and relate what we find in the laboratory to what happens to patients. Under the Data Protection Act you are entitled to ask to see what is recorded about you by applying to the Chairman of the Human Tissue Bank Committee, Norfolk & Norwich University Hospital, c/o Dept. of Histopathology, Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records and any information needed for research purposes will be made anonymous before it is given to the researcher.

You will have the opportunity to discuss with a doctor issues relating to the possible use of your tissue or fluids for research purposes. He or she will answer any questions you may have.

If you decide that you want your tissue,

etc., to be stored in the Tissue Bank and used for research purposes, you will be asked to sign a special Consent Form confirming your decision and stating that you have read and understood this sheet.

When you sign the form you will give the ownership of the tissue or other samples to the Norfolk & Norwich University Hospital NHS Trust. The tissue or other samples will then belong to the Trust, which will store it for an indefinite period of time and will be able to decide how it should be used for research. It will also have the right to dispose of unused stored material in an appropriate and ethical manner following normal procedures.

If you do not want your tissue to be stored in the Tissue Bank please tell us and do NOT sign the special Consent Form.

If you do not sign this form, the tissue or other samples will still be sent to the laboratory to undergo those tests that are necessary for your care but they will not be used for research

purposes. All unused tissue from your procedure will be disposed of using normal hospital methods. We will respect your decision and it will not affect in any way the treatment you receive.

10.2 Patient letter



and

Norfolk and Norwich University Hospital NHS Trust

Melanie Dunk, BSc PDipBiomedSc
Phytochemicals and Health programme
Institute of Food Research
Norwich Research Park
Colney
Norwich
NR4 7UA

Tel: 01603-255048 (direct)

Fax: 01603-507723

Email: melanie.dunk@bbsrc.ac.uk

Web: www.ifr.ac.uk

Date

Dear

[The 'broccoli and bladder' study at the Institute of Food Research.](#)

I enclose the information about my current study, as your details may fit the necessary criteria. This will depend on the findings of this routine day unit appointment with your urologist and analysis of any biopsy taken at that time. Perhaps you would kindly let me know as soon as conveniently possible whether you are interested in participating in this study. Please don't hesitate to call me on the number above if you have any queries regarding the study.

Thank you.

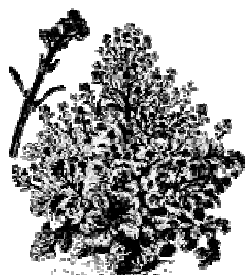
Yours sincerely,



Melanie Dunk BSc PDipBiomedSc (study scientist)

10.3 Volunteer information sheet

Norfolk and Norwich University Hospital 
NHS Trust



Volunteer information sheet

(b)

(c) GENE EXPRESSION IN

(d) BLADDER CANCER FOLLOWING CONSUMPTION OF HIGH
GLUCOSINOLATE BROCCOLI: a pilot study

Short title: **Broccoli and the bladder**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

- **Part 1** tells you the purpose of this study and what will happen if you take part
- **Part 2** gives you more detailed information about the conduct of the study

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

This information sheet is yours to keep.

Thank you for reading this.



Contact Details:

Research Scientist

Melanie Dunk

Phytochemicals & Health Programme

Institute of Food Research

melanie.dunk@bbsrc.ac.uk

Tel: 01603 255048

Research Nurses

Human Nutrition Unit

Institute of Food Research

Tel: 01603 255305



PART 1 of the information sheet

What is the purpose of the study?

Cruciferous vegetables, such as purple sprouting broccoli contain

Phytochemicals (plant chemicals) called glucosinolates. During chopping, chewing and digestion, these phytochemicals change into substances called isothiocyanates. Many scientists believed that these isothiocyanates play a role in preventing cancer.

Isothiocyanates have been shown to hinder the growth of bladder cancer cells. However, there is no evidence that consuming broccoli for the course of this study will have any direct medical benefits with regards to your treatment or prognosis. In this study we will be using a variety of purple sprouting broccoli which is naturally high in these isothiocyanates. This broccoli will be made into a soup for participants to eat, once a day for 4 consecutive days. This study will also form part of the scientist's PhD.

Why should I take part in the study?

It is unknown to what extent broccoli can really fight bladder cancer, and how much a person needs to eat to prevent or slow down the progression and re-occurrence of bladder cancer.

Finding answers to these questions could help us create functional foods that benefit health beyond providing just basic nutrition.



Why have I been invited?

You have been invited to participate because you may fit the criteria for this study.

We are aiming to recruit a total of

- 10 patients

- *aged 30-75 years*
- *male*

Please note: You will **not** be able to volunteer if:

- The screening questionnaire indicates that you are not suitable to take part in this study.
- Results from your cystoscopy suggest you are not eligible for the study.
- You have undergone chemopreventative therapy within the previous 12 months
- You are unable to give written, informed consent to take part
- You have been diagnosed with diabetes which can not be controlled by your diet
- You have any known allergies to any of the ingredients in the soup
- You have an acute infection requiring treatment
- The donated tissue bank sample is of insufficient quality

- You have received a blood product transfusion within the previous four months
- You are related to someone in the study team i.e. spouse, partner or immediate family member

Do I have to take part?

It is up to you to decide.

If you are interested in participating in the study you should contact the researchers named on this information sheet. Please feel free to say no by not responding to the letter we have sent you. Do not worry, no one will contact you and try to persuade you to join the study.

After you have replied to tell us you are interested in participating, a member of the study team will contact you.

You are free to withdraw from the study at any time, without giving a reason.

An expression of interest does not mean you are committed to participating in the

study and a decision to withdraw or not to take part will not affect the standard of care you receive.

What will happen to me if I take part?

If you decide to take part in the study you will be required to attend the Human Nutrition Unit (HNU) at the Institute of Food Research (IFR) which is near to the hospital on six occasions, four of these will be consecutive days to eat broccoli soup. During the study the study team will keep in contact with you to check that you are ok and to discuss any questions you may have about the study.

For the purpose of this study we will be collecting four extra biopsies from your bladder at the same time as your routine biopsies are taken (at the NNUH) and we will also be taking blood samples of 20ml (4 teaspoons), one at the second visit and one on day four of the intervention period. The blood samples will be taken at the Human Nutrition Unit by the Human Nutrition Unit

research nurses. Where possible appointments at the Human Nutrition Unit will be made to suit you. More information about each visit is listed below:

Visit 1 - Informal meeting to discuss the study.

The meeting will last for about one hour, during which time the researchers will go through this information sheet with you and answer any questions you may have.

After this meeting you will be given at least **72 hours** to decide whether or not you wish to take part in the study.

During this time we will not contact you.

After the 72 hours if you decide to take part contact Melanie Dunk and an appointment will be arranged for you to attend for visit 2.

Visit 2 - Screening and Informed Consent.

This visit will take place at the Human Nutrition Unit and will be approximately

60-90 minutes. Before we complete the screening questionnaire and take your blood sample you will be asked to sign a consent form agreeing to participate in the study and a medication declaration form. These forms will also be signed by the researcher and you will be given a copy of them to keep.

Once you have signed the consent form you are still free to withdraw from the study at any time and without giving a reason. The study scientist will complete the screening questionnaire with you and the Human Nutrition Unit nurse will then take a 20ml (4 teaspoons) blood sample from a vein in your arm. The nurses in Human Nutrition Unit are trained and experienced in taking blood.

Visit 3 - Study days

Once a date has been set for your operation, you will be contacted by Melanie Dunk who will give you any instructions in preparation for the study days. You will be asked to visit the Human Nutrition Unit at lunchtime on

four consecutive days to eat the broccoli soup. You will be allocated into either the control group or the broccoli group partly by chance (randomly) and partly depending on your genetic makeup. (See genetic testing in section 2). You will be required to eat one portion of soup each day for four days and on the fourth day you will also have the second 20ml (4 teaspoons) blood sample taken by the Human Nutrition Unit nurses. Ideally the dietary intervention will commence the week prior to your surgery. Therefore dates will be arranged once an operation date has been set by the NNUH.

Access to your personal information

Once recruited onto the study you will be given a code number which will be used to make your information and samples anonymous. Your personal information will only be accessed by the research team, Human Nutrition Unit nurses and your GP. Although all research is subject to Inspection and Audit and your records may be accessed

for this purpose. All your personal information will remain confidential.

Blood and bladder tissue sample

The sample you donated to the NNUH tissue bank at the time of your routine cystoscopy will be brought over to the IFR for testing. Also, in addition to the routine biopsies taken for hospital laboratory diagnostic examination during the transurethral resection of the bladder tumour (TURBT), two tumour samples and two surrounding benign bladder tissue samples will be taken for research purposes.

The tissue and blood samples donated by you will be examined to find out if the compounds present in the purple sprouting broccoli have had a protective effect on the bladder.



Expenses and payments.

Participating in these studies is done on a voluntary basis. However we do recognise that being involved in the study can cause you some inconvenience and that there are travel costs associated with you visiting the HNU. Travelling expenses to and from the HNU will be reimbursed on production of a receipt for buses or trains or at 35p/mile for private cars. If you require transport to and from the HNU please let us know. We will order a taxi and pay for it. Travelling expenses to and from the NNUH will not be reimbursed.

Will my GP be informed?

Yes, it is routine practice for us to inform your GP that you are participating in a study at IFR. This is one of the things you are agreeing to when you sign the consent form. Although analysis of your samples in

this study will not have any prognostic or predictive results with regards to your illness or treatment and therefore your GP will not receive any of your results.

What are the risks or side effects from participating in this study?

There can be a small amount of discomfort associated with taking blood. Obviously this affects some people more than others but the discomfort occurs generally only on insertion of the needle. You should not experience pain during the procedure or afterwards. You may develop a small bruise at the site of the blood sample but this will fade like any bruise. The HNU nurses will be happy to answer any questions you may have about this procedure.

During the cystoscopy and the transurethral resection of the bladder tumour (TURBT), additional biopsies will be taken for research purposes. Taking biopsy samples is usually painless but there is a small risk of

damage to the bladder, infection and bleeding. This may be slightly increased by taking additional biopsies. Most would have some bleeding as a result of a biopsy but perhaps less than 1% would have either significant bleeding or infection. Should either of these occur you may need to be seen by a doctor and may require treatment. Your consultant will be happy to answer any questions you may have about this procedure.

What are the potential benefits of taking part?

There are no direct benefits for you taking part in this study. However, the information we find out from this study will help us to find out the beneficial effects of eating broccoli. This research will also contribute to a higher qualification (PhD) and expand scientific knowledge, which is very important for future research.

What if there is a problem whilst I am on the study?

Any complaint about the way you have been dealt with during the study will be addressed. Detailed information on this is given in Part 2 of this information sheet.



Will my taking part be kept

confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. More details about this are in **Part 2**.

This completes Part 1 of the information sheet. If the information in Part 1 has interested you and you are considering taking part, please read the additional information in Part 2 before making any decision.

PART 2 of the information sheet

What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples, but we may need to use the data collected up to your withdrawal. Withdrawal from the study will not affect the standard of care you receive.

Complaints

If you have a concern about any aspect of the study, you should ask to speak to Melanie Dunk who will do her best to answer your questions. Alternatively you may wish to speak to Ms Dunk's academic supervisor, Professor Richard Mithen (255259). If you remain unhappy

and wish to complain formally about any aspects of the study at IFR, you can do this through the IFR Human Research Governance Committee chairperson – Dr David Hughes (255345). If your concern is about any aspect of your care within the NNUH you may contact the patient advice and liaison service (PALS) staff (289036) or the complaints manager (287475) who will do their best to help you.

Harm

Up to the point of tissue transfer release to IFR researchers from the NNUH, NHS indemnity will apply. All procedures carried out in the Human Nutrition Unit at IFR will be covered by IFR indemnity.

IFR accepts responsibility for carrying out trials and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the trial, with the exception of those claims arising out of negligence by the participant. Like all

publicly funded bodies, the institute is unable to insure and thus cannot offer advance indemnity cover for participants. The institute will not fund any legal costs arising from any action unless awarded by a court.

Will my taking part in this study be kept confidential?

Any information which is collected about you during the study will be kept strictly confidential and stored in locked filing cabinets at the Institute of Food Research (IFR).

When you are recruited onto the study you will be issued with a volunteer code number. This number will be used on your samples and information so that no one will be able to work out that they are yours.

Access to your personal records is restricted to the study team, the HNU research nurses and your GP. The data will be kept for 15 years in a secure

archive at IFR and will be destroyed by incineration.

What will happen to the samples I give?

Your samples will be assigned a code number which will be used to make your samples anonymous. These samples will be securely stored at IFR. All of the blood and tissue samples you provide will be used to obtain study specific information, measurements and analysis. Any samples remaining after all the information has been collected will be stored under their code numbers for possible further anonymous research at a later date.

Genetic tests

This study will predominately involve genetic analysis of both blood and bladder tissue. We will be investigating if the protective compounds present in broccoli can switch off or switch on genes in the bladder tissue, after a short intervention study. This is called gene

expression analysis. Also we will be looking for a particular group of genes which form part of the body's defence system against environmental substances and carcinogenic compounds. These genes can alter toxic compounds and help their discharge from the body. This kind of analysis is called genotyping. However, this genetic analysis of your blood and bladder tissue would not have any significance for you, your relatives or the treatment you receive.

What will happen to the results of the research study?

As a volunteer you are valuable to us but we are unable to tell you any of your individual results. Some results will be published anonymously. At the end of the study we will try to provide feedback about what we have found as a result of your help and what it may mean for the future research.

Who is organising and funding this study?

This study is being organised by Melanie Dunk and Professor Richard Mithen. The study is sponsored and funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

Who has reviewed this study?

This study has been reviewed, and given favourable opinion by The Institute of Food Research (IFR) Human Research Governance Committee (HRGC), Norfolk Research Ethics Committee (NREC) and East Norfolk & Waveney Research Governance Committee (ENWRGC).

These committees are made up of independent groups of people to protect your safety, rights, wellbeing and dignity.

Taking part in the research is entirely voluntary! You are free to withdraw from the study at any time.

11.4 Study consent form



Institute of Food Research



Norfolk and Norwich University Hospital



NHS Trust

and

INFORMED CONSENT FORM FOR RESEARCH STUDY

Please affix an addressograph label here or complete the following details:
Patient's name.....
.....
Date of birth.....
Hospital number.....

Full Study Title: GENE EXPRESSION IN BLADDER CANCER FOLLOWING CONSUMPTION OF HIGH GLUCOSINOLATE BROCCOLI

Vol. Initial

Have you read the Volunteer Information Sheet; Version No:Date:YES/NO

Do you agree that you do not fall within the basic exclusion criteria listed for this research study?.....YES/NO*

*If you have answered NO to this question we are unable to accept you on this study.

Have you had an opportunity to discuss this study and ask questions; including the exclusion criteria

and your responsibilities as a volunteer?.....YES/NO

With whom have you discussed the information for this research study?
.....

Have you received sufficient information about the study?
.....YES/NO

Have you received satisfactory answers to all your questions?
.....YES/NO

Do you understand that you are free to withdraw from the study:

- at any time
- without having to give a reason for withdrawing
- without withdrawal affecting future participation in other research studies.....YES/NO

Are you aware that your personal information will be held confidentially?.....YES/NO

Do you agree to us informing your General Practitioner of your participation in this study and

of all clinical results?.....

YES/NO*

* If you have said NO to this question then we are unable to accept you on this study.

Name and address of your General Practitioner?

.....
.....
.....

Do you understand that all research is subject to Inspection and Audit*.....YES/NO

*Although your records may be accessed for this purpose your personal information remains confidential

Do you agree to take part in this study?.....YES/NO

Signed: (Name in BLOCK Letters:.).....Date:.....

Date of Birth:.....Scientist (I confirm that the volunteer above has been given a full verbal and written explanation of the study)

Signed:..... Name in BLOCK Letters:
Date:.....

10.5 General Practitioners letter



Institute of Food Research



and

Norfolk and Norwich University Hospital 
NHS Trust

Melanie Dunk, BSc PDipBiomedSc
Phytochemicals and Health programme
Institute of Food Research
Norwich Research Park
Colney
Norwich
NR4 7UA

Tel: 01603-255048 (direct)

Fax: 01603-507723

Email: melanie.dunk@bbsrc.ac.uk

Web: www.ifr.ac.uk

Date!!

Dear Doctor

This is to inform you that your patientdate of birth.....has consented to participate in a human nutrition study at the Institute of Food Research.

The pilot study; a human intervention trial studying gene expression in bladder cancer following consumption of high glucosinolate broccoli has been approved by the **Norfolk Research Ethics Committee** and the East Norfolk & Waveney Research Governance committee. The study co-ordinator Melanie Dunk can be contacted on the above number if you require further information.

We anticipate your patient will complete this study by.....

Yours sincerely,

Melanie Dunk BSc PDipBiomedSc (study scientist)

10.6 Medical declaration form



Institute of Food Research

Norfolk and Norwich University Hospital 

NHS Trust

and

MEDICATION/MEDICAL CONDITIONS DECLARATION

AGREEMENT

**Full Study Title: GENE EXPRESSION IN BLADDER CANCER FOLLOWING
CONSUMPTION OF HIGH GLUCOSINOLATE BROCCOLI**

Certain illnesses and medication may affect the outcome of research studies.

Therefore, we would like you to inform the study organisers if you

- start taking medication
- suffer from any illness

Please sign below to confirm that you have agreed to this request.

I.....consent to inform the study organiser of the

commencement of any medication/medical changes whilst participating in the

study

Signature of volunteer.....Volunteer number.....

Date.....

Signature of scientist.....

Contact Details

Research Scientist

Melanie Dunk

Phytochemicals & Health Programme

Institute of Food Research

Tel: 01603 255048

10.7 Volunteer Screening questionnaire



Institute of Food Research

and

Norfolk and Norwich University Hospital



NHS Trust

BROCCOLI AND THE BLADDER

CONFIDENTIAL

Volunteer Screening Questionnaire

Volunteer code number.....

Sex:.....

Date of birth:.....

Age:.....

Are you currently on any the following:

If yes, give details below each relevant section of brand, dosage, frequency, when started etc.

Prescribed medication: Y N

.....
.....
.....

Dietary Supplements: Y N

Herbal remedies: Y N

.....
.....
.....
.....
.....
.....

Do you OR have you ever smoked? Y N If yes how much do you smoke.....

Have you had a major operation in the last year: Y N

If yes give details below:

.....

.....

.....

.....

.....

Have you received a blood or blood product transfusion in the last 4 months Y N

Volunteer code number:.....

1 of 2

Are you currently suffering from any illness/injury: Y N

(Other than bladder cancer)

If yes give details below:

.....

.....

.....

.....

Have you any known allergies: Y N

Food:.....

Drugs:.....

Other:.....

....

.....

.....

Special dietary requirements: Y N

If yes

state:.....

.....

.....

Do you agree to us informing your General Practitioner of your participation in the study or of any results found: Y N

If you have answered NO to this question then we are unable to accept you on this study.

Name and Address of your General Practitioner:

.....

.....

.....

.....

.....

.....

Telephone number:.....

Form completed by (print):.....

Signature:.....

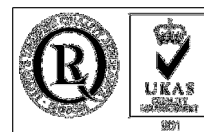
Date:.....

Volunteer code number:.....

10.8 Coorspondance from the IFR Research Governance Commmitte (HRGC)



Institute of Food Research



Article II. Direct Dial Tel: +44 (0) 1603 255345 Direct Dial Fax: +44 (0) 1603 255167 E-mail: davidA.hughes@bbsrc.ac.uk

2 March 2007

Ms M Dunk
H1 Programme
IFR

Dear Melanie,

HRGC ref: IFR01-2007 Short title: Broccoli and the bladder

As you know, your revised protocol was discussed at today's meeting of the IFR Human Research Governance Committee (HRGC). Following discussion, the committee's decision was that the proposal could be submitted to the East Norfolk & Waveney Research Governance Committee and the Norfolk Research Ethics Committee (Norfolk REC) once further minor changes have been made.

The Committee wish to congratulate you on the significant improvements you have made to the paperwork.

I list the main changes we request/comments to consider. Once again, please use the electronic version of this letter to detail your response to each of the bulleted points (eg. "Done", or any argument for not agreeing with any point) and please highlight the changes in your revised documents.

Protocol version 2, dated 22.02.07

- p2. As Amy Gasper may be taking consent from volunteers, please add her name to the "Researchers" at the top of this page.
- p8. Append to first sentence of "Hypothesis": "...in men" (and make "phase II enzyme" plural). Change "hypnosis's" to "hypotheses"!
- p8. bottom line, replace "as part of" with "in addition to the".
- p10. Typo bottom box. "Conserdation".
- p12. para 2. There will only be 2 criteria, since criteria 3, as it's written, will not be known at this time point. Therefore, please delete this 3rd criteria.

- p12 para 3. Change “disbursed” to “sent”.
- p12. The number of volunteers you wish to recruit to each group needs to be stated, in a form that allows you to keep recruiting until you reach your required number, eg. “Recruitment will continue until 5 volunteers in both treatment groups have completed the study.”
- p12. Inclusion criteria: remove “Smokers and ex-smokers” and “Diet-controlled diabetes”, as you are effectively stating this in the exclusion criteria.
- p13. We suggest you state in brackets why you are excluding “never smokers” (see previous HRGC letter).
- p13. bottom para. Remove “using a BD vacutainer safety lok blood collection set”, as this would limit the choice for the nurses if a situation arose where this could not be used.
- p14. top para. typo, should be “doubly”.

NNUH Tissue consent form:

- In sentence, “Such information may be passed in an anonymous form to Ms Melanie Dunk..”, delete “in an anonymous form”.

NNUH Tissue information sheet:

- Where you have typed “(Delete)”, use format font to strike through the text in bold type.

Volunteer information sheet:

- p1. Do you have an answerphone linked to your contact number? The Committee recommends this and that you state “(linked to answerphone)” after the phone number.
- p2. top para. Remove “crucial”, as this is still uncertain and conflicts with text in next section.
- p2. section 2. We suggest you remove the first sentence “It is recommended....” and begin with “It is unknown to what extent...”
- p2. last para. We suggest you insert “(near to the hospital)” after IFR.
- p3. top para. For clarity, we suggest you revise the beginning of the first sentence to “For the purpose of this study we will be collecting four extra biopsies from your bladder at the same time as any routine biopsies...” (and make teaspoon plural).
- p3. Visit 2. typo with ½ (or state “60-90 minutes”).
- p3. top of column 2. Clarification is needed that, as well as by genotype, the treatment allocation they will receive is random, eg. insert “..or the broccoli group partly by chance (randomly) and partly depending on your genetic makeup..”
- p3. Blood and bladder tissue sample. Correct spelling of “tumor” in 2 places.
- p4. typo in GP section. “you’re GP”.
- p5. Harm. We have been advised to remove the word “sympathetic” from the indemnity statement. Please also delete it in the COREC form sections A35-1to3.
- p6. Typos, Add “by the” before BBSRC, and capitals for “Governance Committee”.

COREC form:

- A3. typo, start date 2008!
- A7. Suggest you remove “crucial”.
- A8. Please convert “key polymorphic genes” into lay language.

- A10-1. Suggest you finish hypothesis sentence after "...enzyme gene expression." and then "The resulting changes may contribute..."
- A22. Remove last 3 inclusion criteria.
- A23. Insert "never smokers" and justify.
- A26. Insert "minimum" prior to "...72 hour consideration..."
- A39. Tick which of the devices the data will be stored on.
- A68. Suggest move the last 2 para to the top, as these are probably the most important ethical considerations.
- p46. The Director has delegated signing off the final box to Dr Mary Anderson, Head of Contracts, so please insert Mary's name and position at "Print Name:".

Please send me your response to this letter with the revised paperwork for Chairman's action to approve submission to the relevant committees. We will provide a letter to this effect, which you should submit with your paperwork, together with this letter.

Best wishes.

Section 2.01 David A Hughes **PhD, RNutr, Cert CRGCP**
Chair, IFR Human Research Governance Committee

cc. Prof R Mithen; HRGC members

10.9 Correspondance from the IFR Human Research Governace Committee post amendments



Institute of Food Research



Article III. Direct Dial Tel: +44 (0) 1603 255345 Direct Dial Fax: +44 (0) 1603

255167 E-mail: davidA.hughes@bbsrc.ac.uk

13 March 2007

Ms M Dunk
H1 Programme
IFR

Dear Melanie,

HRGC ref: IFR01/2007
Short title: Broccoli and the bladder

The revised documentation you have provided has been reviewed by Liz Lund and myself and we are happy to take Chairman's Action on behalf of the Human Research Governance Committee to give approval for this proposal to be submitted to the East Norfolk & Waveney Research Governance Committee and the Norfolk Research Ethics Committee (Norfolk REC). This approval implies that the Human Research Governance Committee is satisfied both with the originality and with the quality of the science proposed and considers that adequate expertise and manpower are available to undertake the work.

Please remember to mention in your covering letter that you have made an alteration to the information sheet that accompanies the tissue bank Consent Form.

Please enclose this letter, along with the previous correspondence from the Human Research Governance Committee (05.02.07 & 02.03.07), with your ethical submission.

Please provide me with a copy of the letters you receive from the Research Governance and Research Ethics Committees, giving their responses to your proposal.

Best wishes.

Yours sincerely,

David A Hughes, PhD, RNutr, CertCRGCP
Chair, IFR Human Research Governance Committee

10.10 Correspondance with Norwich Research Ethics Committee for ethical approval



Institute of Food Research



Melanie Dunk, BSc PDipBiomedSc
Phytochemicals and Health programme
Institute of food research
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Web: www.ifr.ac.uk

19TH March, 2007

Dear Katherine Norton and committee members,

Re: GENE EXPRESSION IN BLADDER CANCER FOLLOWING CONSUMPTION OF HIGH GLUCOSINOLATE BROCCOLI: a pilot study

I would like to submit a research protocol for the above pilot study for consideration by the Norwich Research Ethics Committee at their next meeting on the 2nd of April. This study has been reviewed and approved by the IFR Human Governance Committee and will be submitted to East Norfolk and Waveney Research Governance Committee for consideration at their next meeting on 4th of April.

The submission comprises the following documents:

Study Protocol version 3, dated 2nd March 2007

Annex 1 Tissue bank form* version 8, dated 2nd March 2007

Annex 2 Volunteers letter version 2, dated February 2007

Annex 3 Volunteers information sheet version 4, dated 2nd March 2007

Annex 4 Consent Form version 1, dated January 2007

Annex 5 GP letter version 1, dated January 2007

Annex 6 Medical declaration form version 1, dated January 2007

Annex 7 Health questionnaire version 1, January 2007
Annex 8 NNUH Cystoscopy information sheet version 1, January 2007
COREC form parts A & B and SSI form

* Please note that the NNUH tissue bank information and consent form has been modified to allow the trust to use tissue or other material in a non anonymous form at its discretion within this research study only.

Please note that the principle investigator and clinical director (Mr. Robert Mills, NNUH) signature are absent from the SSI. Mr. Rob Mills will be sending an email to the East Norfolk and Waveney Research Governance Committee to approve/authorize this study and signatures will be obtained prior to the meeting. (As advised by Julie Dawson)

I look forward to receiving feedback from the committee in due course

Yours sincerely,

Melanie Dunk BSc PDipBiomedSc (study scientist)

10.11 East Norfolk and Waveney Research Governance Committee



Institute of Food Research



Melanie Dunk, BSc PDipBiomedSc
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Email: melanie.dunk@bbsrc.ac.uk

Web: www.ifr.ac.uk

16TH March, 2007

Dear Julie Dawson and committee members,

Re: GENE EXPRESSION IN BLADDER CANCER FOLLOWING CONSUMPTION OF HIGH GLUCOSINOLATE BROCCOLI: a pilot study

I would like to submit a research protocol for the above pilot study for consideration by the East Norfolk and Waveney Research Governance Committee at the next meeting on the 4th April. This study has been reviewed and approved by the IFR Human Governance Committee and will be submitted to the Norwich Research Ethics Committee for consideration at their next meeting on the 2nd of April.

The submission comprises the following documents:

Study Protocol version 3, dated 2nd March 2007

Annex 1 NNUH tissue bank information and consent form * version 8, dated 2nd March 2007

Annex 2 Volunteers letter version 2, dated February 2007

Annex 3 Volunteers information sheet version 4, dated 2nd March 2007

Annex 4 Consent Form version 1, dated January 2007

Annex 5 GP letter version 1, dated January 2007

Annex 6 Medical declaration form version 1, dated January 2007

Annex 7 Health questionnaire version 1, January 2007

Annex 8 NNUH Cystoscopy information sheet version 1, January 2007

COREC form parts A & B and SSI form

* Please note that the NNUH tissue bank information and consent form has been modified to allow the trust to use tissue or other material in a non anonymous form at its discretion within this research study only.

Please note that the principle investigator and clinical director (Mr. Robert Mills) signature are absent from the SSI. Both will be sending emails to approve/authorize this study and signatures will be obtained prior to the meeting on the 4th April. (As advised)

I look forward to receiving feedback from the committee in due course

Yours sincerely,

Melanie Dunk BSc PDipBiomedSc (study scientist)

10.12 Correspondance with Norfolk Research Ethics Committee response letter

Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor, Aldwych House
57 Bethel Street
NORWICH
NR2 1NR

Telephone: 01603 286323

Facsimile: 01603 286573

11 April 2007

Ms Melanie Dunk
PhD student
Institute of Food Research
Norwich Research Park
Colney, Norwich
NR4 7UA

Dear Ms Dunk

Full title of study: **Gene expression in bladder cancer following consumption of high glucosinolate broccoli: a pilot study**
REC reference number: **07/Q0101/32**

The Research Ethics Committee would like to thank you and Dr David Hughes for attending the review of your application at the meeting held on 02 April 2007.

Ethical opinion

On behalf of the Committee, I am pleased to confirm a **conditional** favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

The favourable ethical opinion is subject to the following amendments being made:

- 1) In the Human Tissue Bank (HTB) consent form, two references are made (in the third text box on page 1 and in the sentence with the asterisk on page two) to the 4ml sample being equivalent to 2 teaspoons; this is incorrect. The correct amount is expressed in the participant information sheet as 20ml being equivalent to 2 teaspoons, therefore please amend the HTB consent form accordingly.
- 2) Please correct clause 6 of the HTB consent form so that it reads "...and may be published anonymously with any research findings."
- 3) Please ensure the presentation of the last sentence of the final paragraph of the HTB information sheet is corrected so that it makes sense.

- 4) In the letter to the volunteer, members suggest that the tense is changed as currently the letter is confusing.
- 5) Members suggest that the first paragraph of the participant information sheet (PIS) is amended to understate the benefits of eating broccoli as the consumption of broccoli will not change the course of study participants' disease. Members appreciate that this has been mentioned later on in the PIS but felt it should be clearly stated earlier on.
- 6) It is suggested that as this research is being undertaken by a PhD Student the Supervisor's contact details ought to be included in the PIS as well. It is therefore recommended you include Professor Mithen's details under 'Complaints' in Part 2 of the PIS.
- 7) During the discussion at the meeting Members expressed a concern that apart from the participant's name and the IFR volunteer number, there is no other identifier on the standard HRGC consent form. The concern being that once the consent form has been placed in the participants' hospital notes this could be lost/fall out and if there is more than one person with the same name it could be misfiled. In order to prevent this it was agreed at the meeting that the consultant responsible for placing the copy of the consent form in the hospital notes could be asked to place a 'short form patient sticker' on the form.
- 8) As the study PIS has both IFR and NNUH headers please also incorporate the NNUH header on the study consent form.
- 9) In the GP letter it was noted that the name of the Committee was incorrect and should read the 'Norfolk Research Ethics Committee'.
- 10) Please include the IFR and NNUH header on the Medication/Medical Conditions Declaration Agreement, the study title, and a section for the volunteer number and contact details of the researcher.

Please ensure revised documentation is presented to the Norfolk REC Office. A Site Approval Form (SF1) listing approved sites for this study will then be issued.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in paragraph Ethical Opinion above and the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Application	5.3 Checklist	19 March 2007
Application	5.3; Parts A&B	20 February 2007
Application	5.3; SSI - NNUH	
Investigator CV	1; Ms Melanie Dunk - CI	01 February 2007
Investigator CV	Professor Richard Mithen -	

	Supervisor	
Investigator CV	Mr Robert Mills - PI	01 October 2004
Investigator CV	Professor Richard Ball	
Protocol	3	02 March 2007
Covering Letter	From Melanie Dunk	19 March 2007
Summary/Synopsis	3; Protocol Pg 9 - Study Design	02 March 2007
Summary/Synopsis	3; Protocol Pg 10 - Patient Selection	02 March 2007
Letter from Sponsor	From David Hughes	13 March 2007
Peer Review	From D. Hughes, HRGC	13 March 2007
Compensation Arrangements	From David Hughes	13 March 2007
Questionnaire: Volunteer Screening	1; Annex 7	01 January 2007
Letter of invitation to participant	2; Annex 2	01 February 2007
GP/Consultant Information Sheets	1	01 January 2007
Participant Information Sheet: NNUH Standard Cystoscopy Info Sheet	1; Annex 9	01 January 2007
Participant Information Sheet: Broccoli Study	4; Annex 3	01 February 2007
Participant Information Sheet: NNUH collection, storage & use of Human tissue/fluids for research	8 Annex 1	11 November 2006
Participant Consent Form: Broccoli Study	1	01 January 2007
Participant Consent Form: NNUH Collection & Storage of Human Tissue/fluids for research	8; Annex 1	11 November 2006
Medication/Medical Conditions Declaration Agreement	1; Annex 6	01 January 2007
Correspondence	From D. Hughes HRGC	05 February 2007
Correspondence	From D. Hughes, HRGC	02 March 2007

R&D approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final approval from the R&D office for the relevant NHS care organisation.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

07/Q0101/32

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

**The Reverend Walter Currie
Chairman**

Email: janette.guymer@nnuh.nhs.uk

*Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments
Standard approval conditions*

Copy to: *HRGC, Institute of Food Research*
East Norfolk & Waveney Research Governance Committee
Norfolk Research Ethics Committee

Attendance at Committee meeting on 02 April 2007

Committee Members:

<i>Name</i>	<i>Profession</i>	<i>Present?</i>	<i>Notes</i>
The Reverend Walter Currie	Retired	Yes	Chairman
Dr Elizabeth Lund	Senior Research Scientist	Yes	Alternate Vice-Chair
Dr Robert Stone	General Practitioner	Yes	Vice-Chair
Mr William Allen	Retired	No	
The Reverend Bill Bazely	Senior Chaplain	No	
Mr Michael Flowerdew	Acupuncture Practitioner and Writer	Yes	
Miss Sheila Ginty	Senior Sister	Yes	
Mr Colin Green	Drugs & Therapeutics Pharmaceutical Advisor	Yes	
Mrs Belinda Hoste	Case Worker	No	
Miss Rosemary Jackson	Midwife	Yes	
Mrs Pamela Keeley	East Anglian Eye Bank Nurse Manager	Yes	
Mr Azad Mathur	Consultant Paediatric Surgeon	Yes	
Dr Michael Sheldon	Retired - Clinical Psychologist	Yes	

Also in attendance:

<i>Name</i>			<i>Position (or reason for attending)</i>
Mrs Janette Guymer	REC Manager	Yes	
Miss Katherine Norton	Assistant Administrator	Yes	Minuting Secretary
Mrs Samantha Podmore	Assistant Administrator	Yes	Minuting Secretary

10.13 Correspondance with Norfolk Research Ethics Committee reply with amendments



Institute of Food Research



Melanie Dunk, BSc PDipBiomedSc
Phytochemicals and Health programme
Institute of food research
Norwich Research Park
Colney
Norwich
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Tel: 01603-255048 (direct)

Fax: 01603-507723

Email: melanie.dunk@bbsrc.ac.uk

Web: www.ifr.ac.uk

17 April, 2007

Dear Reverend Walter Currie and committee members,

Re: GENE EXPRESSION IN BLADDER CANCER FOLLOWING CONSUMPTION OF HIGH GLUCOSINOLATE BROCCOLI: a pilot study

REC REF: 07/Q0101/32

I would like to re-submit this research protocol for the committee's favourable ethical opinion. This study was reviewed and given conditional favourable ethical opinion by the Norwich Research Ethics Committee on the 2nd of April 2007. The following documents have been amended in accordance with your recommendations:

Annex 1 Tissue bank form version 9, dated April 2007

Annex 2 Volunteers letter version 3, dated April 2007

Annex 3 Volunteers information sheet version 5, April 2007

Annex 4 Consent Form version 2, April 2007

Annex 5 GP letter version 2, dated April 2007

Annex 6 Medical declaration form version 2, dated April 2007

The following amendments were made and are highlighted in orange in the corresponding documents:

- 1) In the Human Tissue Bank (HTB) consent form- 4ml sample being equivalent to 2 teaspoons has been changed to 20ml being equivalent to 2 teaspoons.

2) HTB consent form- reads “ ...and may be published anonymously with any research findings.”

3) The presentation of the last sentence of the final paragraph of the HTB information sheet has been corrected so that it makes sense.

4) Letter to the volunteer - The tense has been changed.....‘I enclose information’

5) PIS - The first paragraph of section one has been amended to understate the benefits of eating broccoli.

6) PIS - Professor Richard Mithen’s details have been added under ‘Complaints’ in Part 2.

7) Three forms of patient identification – the consultant or a member of the urology team will be asked by the chief investigator to place a 'short form patient sticker' on the copy of the consent form before placing it into the patients hospital notes. An area has been made on the form as a reminder.

8) Study consent form - NNUH header has been incorporate.

9) GP letter - name of the Committee is corrected to read the 'Norfolk Research Ethics Committee'.

10) Medication/Medical Conditions Declaration Agreement - The IFR and NNUH header, the study title, and a section for the volunteer number and contact details of the researcher have been incorporated.

I do hope these amendments are agreeable and I look forward to receiving feedback from the committee in due course

Yours sincerely,

Melanie Dunk BSc PDipBiomedSc (study scientist)

10.14 Norfolk Research Ethics Committee approval letter

Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor, Aldwych House
57 Bethel Street
NORWICH
NR2 1NR

Telephone: 01603 286 397

Telephone: 01603 286 323

14 May 2010

Ms Melanie Dunk, PhD student
Institute of Food Research
Norwich Research Park
Colney, Norwich
NR4 7UA

Dear Ms Dunk

Full title of study: **Gene expression in bladder cancer following consumption of high glucosinolate broccoli: a pilot study**

REC reference number: **07/Q0101/32**

Thank you for your e-mail correspondence of 17/18 April 2007 and your letter dated 17 April 2007, responding to the Committee's conditions of approval and submitting revised documentation.

I can confirm that you have responded appropriately.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Application	5.3 Checklist	19 March 2007
Application	5.3; Parts A&B	20 February 2007
Application	5.3; SSI - NNUH	
Investigator CV	1; Ms Melanie Dunk - CI	01 February 2007
Investigator CV	Professor Richard Mithen - Supervisor	
Investigator CV	Mr Robert Mills - PI	01 October 2004
Investigator CV	Professor Richard Ball	
Protocol	3	02 March 2007
Covering Letter	From Melanie Dunk	19 March 2007
Summary/Synopsis	3; Protocol Pg 9 - Study Design	02 March 2007
Summary/Synopsis	3; Protocol Pg 10 - Patient Selection	02 March 2007

Letter from Sponsor	From David Hughes	13 March 2007
Peer Review	From D. Hughes, HRGC	13 March 2007
Compensation Arrangements	From David Hughes	13 March 2007
Questionnaire: Volunteer Screening	1; Annex 7	01 January 2007
Participant Information Sheet: NNUH Standard Cystoscopy Info Sheet	1; Annex 9	01 January 2007
Correspondence	From D. Hughes HRGC	05 February 2007
Correspondence	From D. Hughes, HRGC	02 March 2007
<i>*Letter of invitation to participant</i>	<i>3; Annex 2</i>	<i>01 April 2007</i>
<i>*GP/Consultant Information Sheets</i>	<i>2; Annex 5</i>	<i>01 April 2007</i>
<i>*Participant Information Sheet: Broccoli Study</i>	<i>5; Annex 3</i>	<i>01 April 2007</i>
<i>*Participant Information Sheet: NNUH collection, storage & use of Human tissue/fluids for research</i>	<i>9 Annex 1¹</i>	<i>01 April 2007</i>
<i>*Participant Consent Form: Broccoli Study</i>	<i>2</i>	<i>01 April 2007</i>
<i>*Participant Consent Form: NNUH Collection & Storage of Human Tissue/fluids for research</i>	<i>9; Annex 1</i>	<i>01 April 2007</i>
<i>*Medication/Medical Conditions Declaration Agreement</i>	<i>2; Annex 6</i>	<i>01 April 2007</i>
<i>*E-mail Correspondence of response to conditional favourable opinion letter</i>	<i>From M. Dunk</i>	<i>17/18 April 2007</i>
<i>*Hard copy of response to conditional favourable opinion letter</i>	<i>Original signature from M. Dunk</i>	
<i>*E-mail including correspondence to Ms Dunk</i>	<i>From ENWRGC</i>	<i>23 April 2007</i>
<i>* E-mail including correspondence to ENWRGC to REC</i>	<i>From M. Dunk</i>	<i>23 April 2007</i>
<i>* E-mail including correspondence confirming approval for the study</i>	<i>From ENWRGC Ref: 2007IFR01S</i>	<i>01 May 2007</i>

**Documents which have been forwarded and updated to meet the favourable conditional opinion and to be used on the study and as confirmation of support for the study.*

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation. You should ensure that R&D have received any amended study documentation as a result of the REC review.

¹ Please amend the version number in the title to correspond with the version number in the footer on this document

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

07/Q0101/32

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Janette Guymer
REC Manager

Email: janette.guymer@nnuh.nhs.uk

Enclosed: SF1 Form

*Copy to: HRGC, Institute of Food Research
East Norfolk & Waveney Research Governance Committee*

Norfolk Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	07/Q0101/32	Issue number:	1	Date of issue:	01 May 2007
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Chief Investigator:	Ms Melanie Dunk
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Full title of study:	Gene expression in bladder cancer following consumption of high glucosinolate broccoli: a pilot study
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This study was given a favourable ethical opinion by Norfolk Research Ethics Committee on 02 April 2007. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

<i>Principal Investigator</i>	<i>Post</i>	<i>Research site</i>	<i>Site assessor</i>	<i>Date of favourable opinion for this site</i>	<i>Notes ⁽¹⁾</i>
Mr Robert Mills	Consultant Urologist	Norfolk and Norwich University NHS Trust	Norfolk Research Ethics Committee	03/04/2007	
Ms Melanie Dunk	PhD Student	Institute of Food Research, Human Nutrition Unit	Human Research Governance Committee, Institute of Food Research	03/04/2007	07/Q0101/47 [IFR01/2007]

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/Co-ordinator)
(delete as applicable)

..... (Name)

10.15 East Norfolk and Waveney Research Governance committee response

East Norfolk and Waveney Research Governance Committee



Ms Melanie Dunk
Institute of Food Research
Norwich Research Park
Colney,
Norwich
NR4 7UA

Please reply to: Research Governance Committee Office
Research and Development Department
Level 3, East Block, Room 032
Norfolk and Norwich University Hospital NHS Trust
Colney Lane
Norwich
NR4 7UY
Direct Dial: 01603 287408
Internal: 3408
Direct Fax: 01603 289800

10 April 2007

e-mail: rdoffice@nnuh.nhs.uk
Website: www.norfolkhealthresearch.nhs.uk

Dear Ms Dunk

Re: 2007IFR01S (49-04-07) Gene expression in bladder cancer following consumption of high glucosinolate broccoli: a pilot study.

Thank you for submitting the above project to the East Norfolk and Waveney Research Governance Committee for scientific peer review and Research Management approval. The following comments were made as part of the scientific peer review and you are required to address these points before approval can be given.

- Members felt that the exclusion criterion stating that the result of the health declaration might indicate ineligibility to participate needed to be expanded.
- It was felt that participants taking food supplements containing broccoli extracts should also be excluded.
- Concern was expressed that the numbers in each arm are too small for sub-group analysis. However it was acknowledged that this is principally a pilot study to determine feasibility.

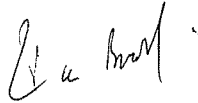
When you have had the opportunity to address these points and make the necessary changes please send your response to the Research Governance Committee office at the above address.

When submitting a response, please send revised documentation where appropriate highlighting the changes you have made either by underlining them or using an italic font and giving revised version numbers and dates. You should also note that the original text should not be deleted from the revised document but should be 'struck out'.

The Committee has delegated authority to the Chair to approve these amendments once they have been received. Subject to the Chair's agreement a formal approval letter will then be issued.

If you have any queries regarding this or any other project please contact Julie Dawson, Research Governance Administrator, at the above address. Please note, the reference number for this study is **2007IFR01S (49-04-07)** and this should be quoted on all correspondence.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Iain Brooksby'.

Dr Iain Brooksby
Director of Research & Development

10.16 East Norfolk and Waveney Research Governace committee reply letter

Melanie Dunk, BSc PDipBiomedSc
Phytochemicals and Health programme
Institute of food research
Norwich Research Park
Colney
Norwich
NR4 7UA

Tel: 01603-255048 (direct)
Fax: 01603-507723
Email: melanie.dunk@bbsrc.ac.uk
Web: www.ifr.ac.uk

19th April, 2007

Dear Dr Lain Brooksby and committee members,

Re: 2007IFR01S (49-04-07) **GENE EXPRESSION IN BLADDER CANCER FOLLOWING CONSUMPTION OF HIGH GLUCOSINOLATE BROCCOLI: a pilot study**

This study was submitted to the East and Waveney Research Governance Committee for scientific peer review and research management approval on 4th April. Three points were raised to be addressed prior to your approval. My supervisor, Professor Richard Mithen has discussed these with Julie Dawson and explained that due to time constraints the protocol has been submitted and approved by the Norfolk Research Ethics committee. She suggested that we wrote to you without amending the protocol.

Firstly, it was considered that the exclusion criterion stating that the result of the health declaration needed to be expanded. It was considered to be impractical to list all the potential medical conditions that may result in exclusion. An important function of the health declaration is that it requires the volunteer to disclose all medical conditions and medications both before and during the study. This enables the principle investigator to decide upon inclusion or exclusion based upon clinical judgement.

Secondly, it was advised that participants taking food supplements containing broccoli extracts should be excluded. We agree with this. Potential participants are required to disclose all dietary supplements in the screening questionnaire (annex 7), and appropriate decisions will be taken. The exclusion criteria (page 13) states that the results of the screening questionnaire may result in someone being ineligible for the study. Thus, we do not think any amendments are required.

Thirdly, we agree with the comments that the numbers in each arm are low. However, this is intended as a pilot study to assess feasibility. If successful, we seek to expand the intervention to larger numbers.

We hope that you will be satisfied with these responses, without the need to amend the protocol itself.

Yours sincerely,
Melanie Dunk BSc PDipBiomedSc (study scientist)

10.17 East Norfolk and Waveney Research Governance committee approval letter

East Norfolk and Waveney Research Governance Committee



Ms Melanie Dunk
Institute of Food Research
Norwich Research Park
Colney,
Norwich
NR4 7UA

Please reply to: Research Governance Committee Office
Research and Development Department
Level 3, East Block, Room 032
Norfolk and Norwich University Hospital NHS Trust
Colney Lane
Norwich
NR4 7UY

Direct Dial: 01603 287408
Internal: 3408
Direct Fax: 01603 289800

25 April 2007

e-mail: rdooffice@nnuh.nhs.uk
Website: www.norfolkhealthresearch.nhs.uk

Dear Ms Dunk

Re: 2007IFR01S (49-04-07) Gene expression in bladder cancer following consumption of high glucosinolate broccoli: a pilot study.

Thank you for your correspondence dated 19 April 2007 in response to the points raised by the East Norfolk and Waveney Research Governance Committee. I am satisfied that all the points have been fully resolved and I am pleased to inform you that the Research Governance Committee is now happy to support this aspect of the project.

Please note that this approval applies to the following sites.

- Norfolk & Norwich University Hospital NHS Trust

You are advised to contact the Research Ethics Committee Office where you wish to have your project ethically reviewed and arrange to make your submission; you will also need to supply them with a copy of this letter. Your nearest Research Ethics Committee is Norfolk. If you wish to use this Committee you should contact Katherine Norton on katheriner.norton@nnuh.nhs.uk or telephone 01603 286397, alternatively if you wish to use a different Ethics Committee please visit the Central Office for Research Ethics Committees website, www.nres.npsa.nhs.uk where further details of other committees can be found.

When you have received a 'favourable opinion' from the Research Ethics Committee office you will need to provide the R&D office with a copy of:

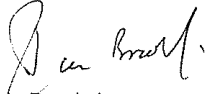
- All the final documents, with version numbers and dates, as listed in the 'favourable opinion' letter.
- A copy of the 'favourable opinion' letter from the Research Ethics Committee.

If you make any changes to the study as a result of the Ethical review please ensure that these changes are tracked by highlighting the changes you make either by underlining them or using an italic font and giving revised version numbers and dates. You should also note that the original or approved text should not be deleted from the revised document but should be 'struck out'.

When the Research Governance Committee office is in receipt of this information your project will be reviewed to verify that there are no further Research Management issues arising from any amended information following the Ethical review. You will then be issued with a formal approval letter and you will be able to commence your study.

If you have any queries regarding this or any other project please contact Julie Dawson, Research Governance Administrator, at the above address. Please note, the reference number for this study is **2007IFR01S (49-04-07)** and this should be quoted on all correspondence.

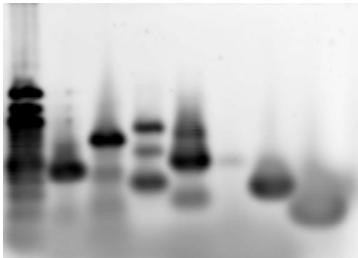
Yours sincerely



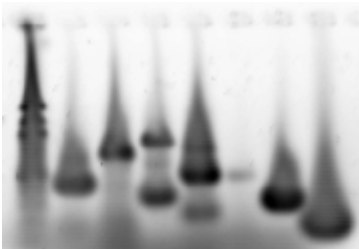
Dr Iain Brooksby
Director of Research & Development

10.18 Tenascin-C RT-PCR gels

1 2 3 4 5 6 7 8

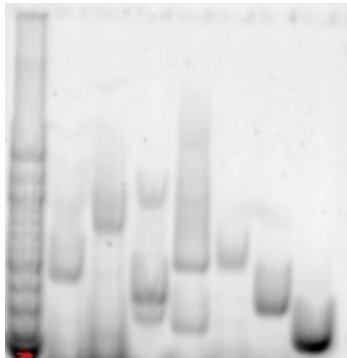
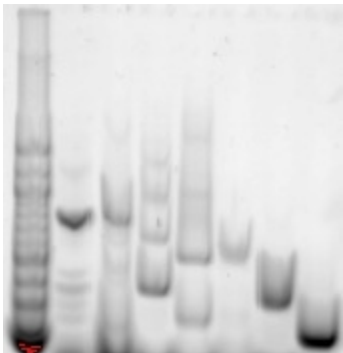


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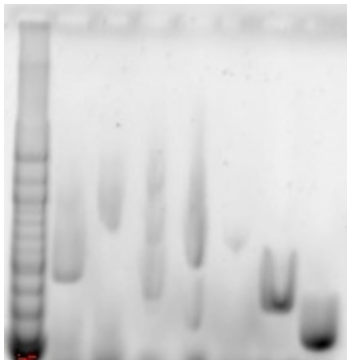
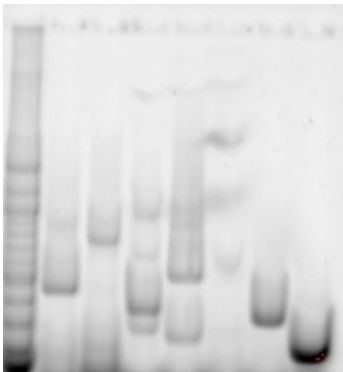
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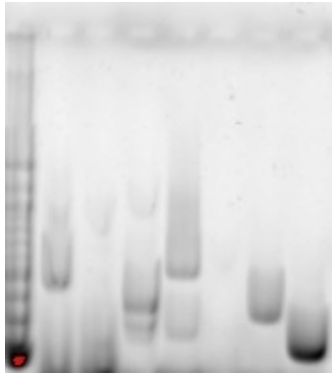
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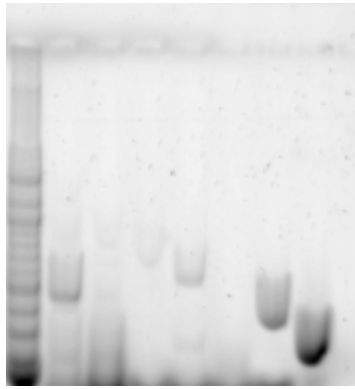


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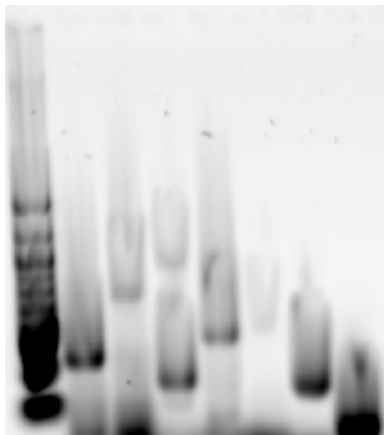
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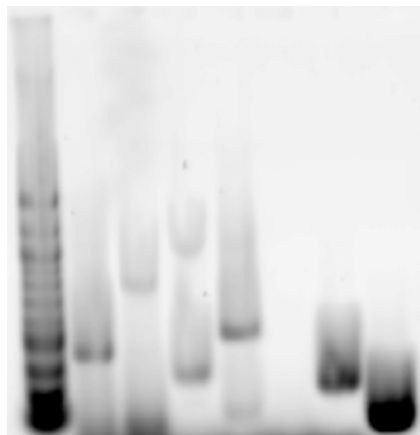
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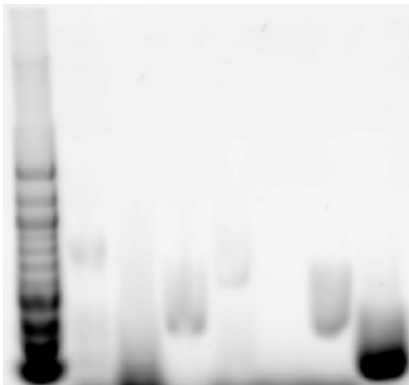
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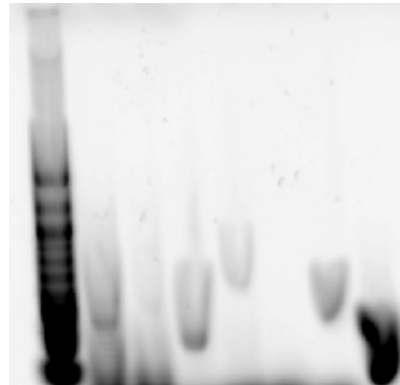
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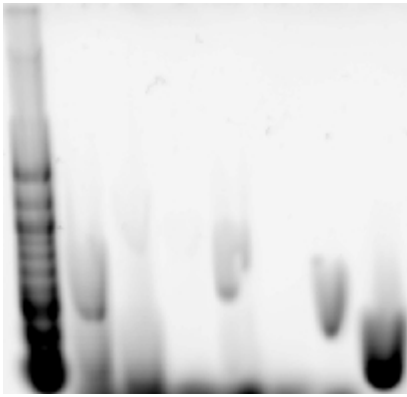
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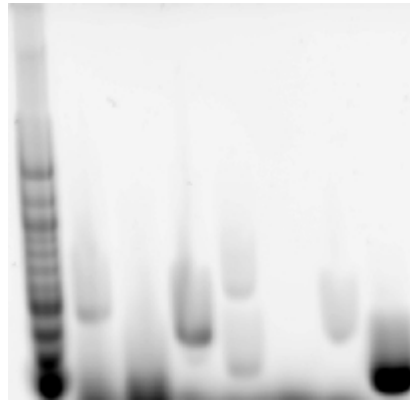
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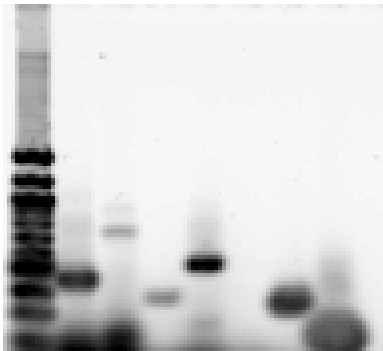
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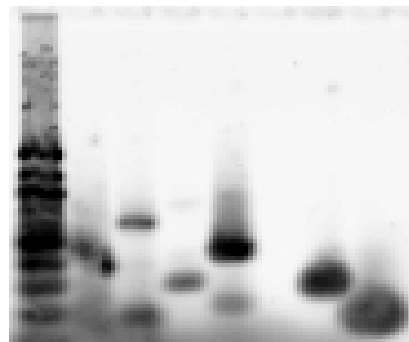
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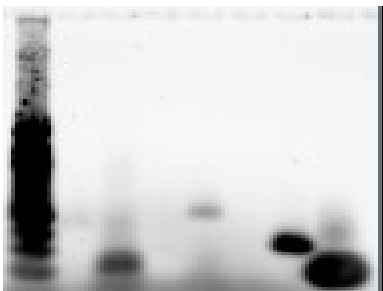
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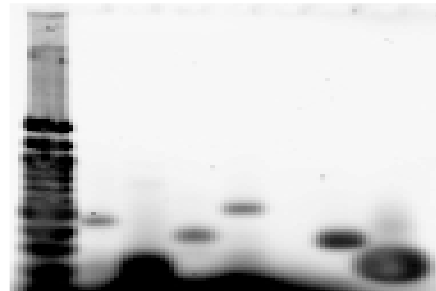
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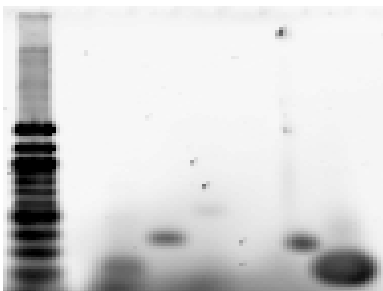
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19 - 04TB269



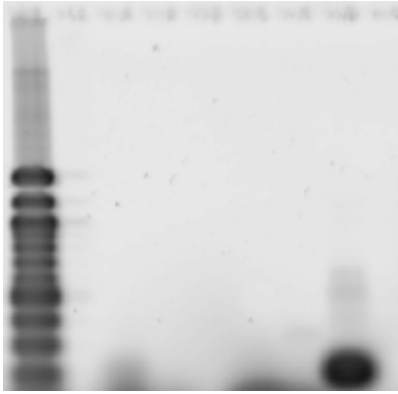
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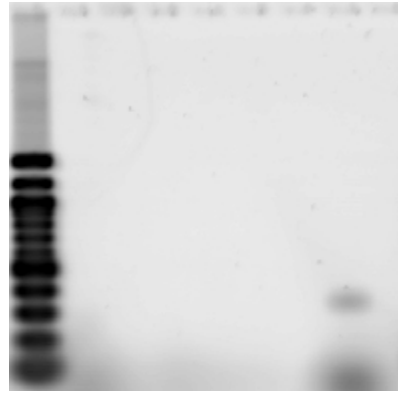
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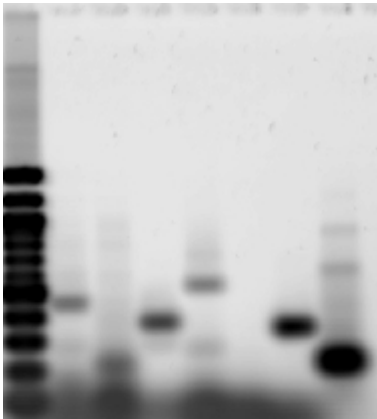
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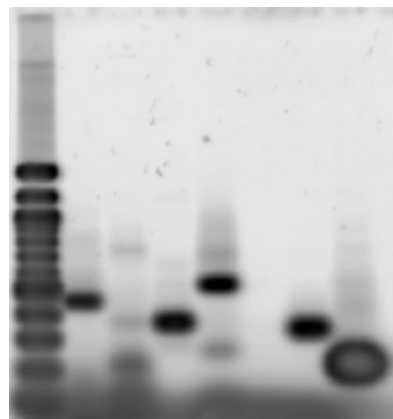
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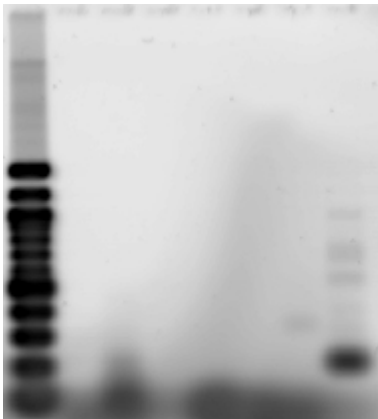
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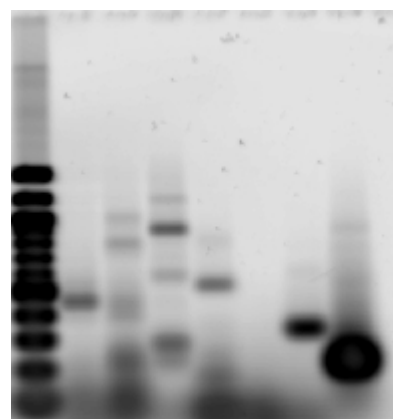
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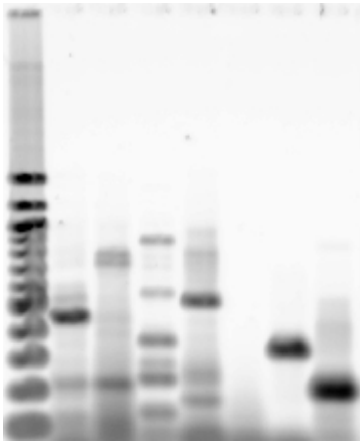
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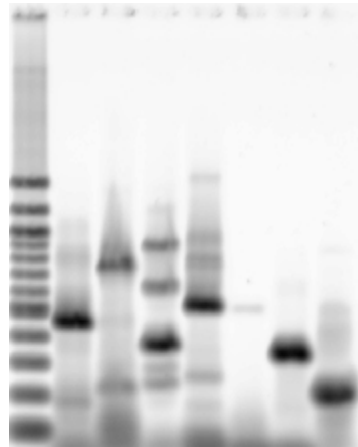
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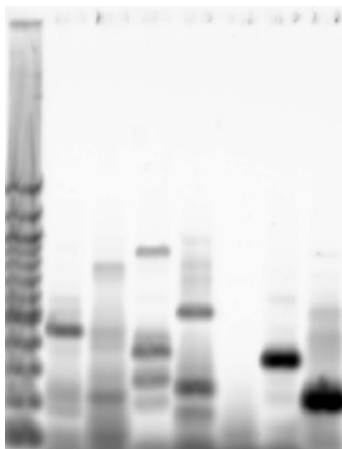
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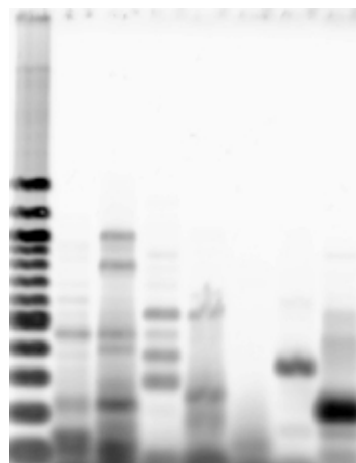
30- 04TB119



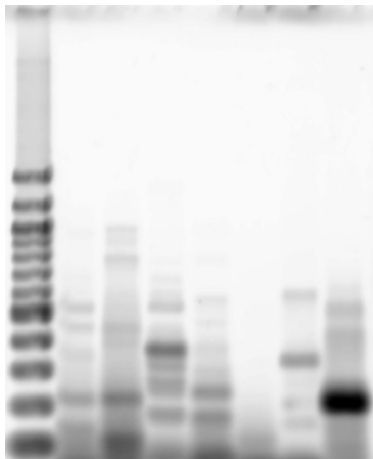
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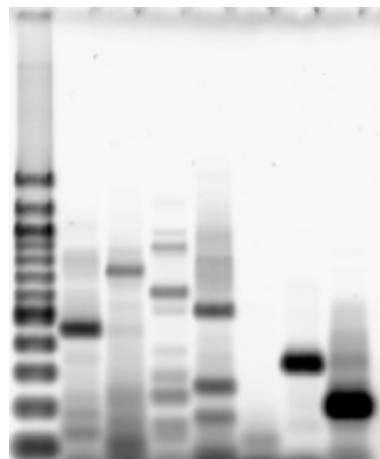
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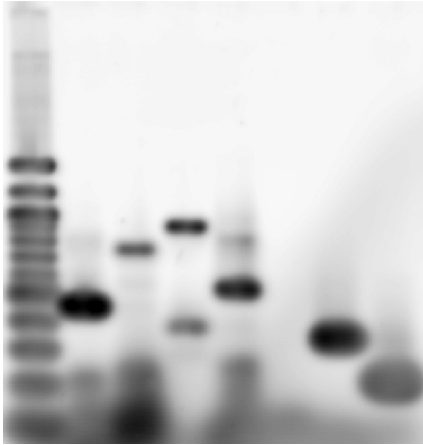
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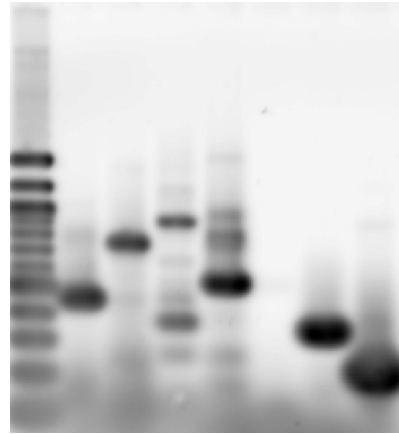
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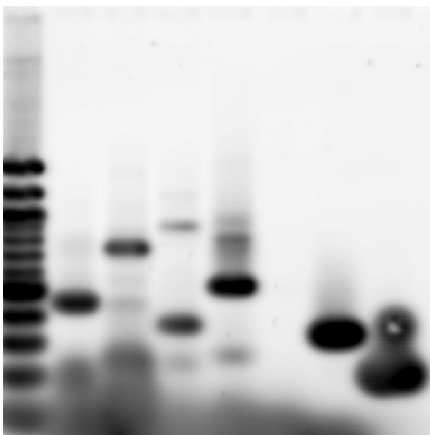
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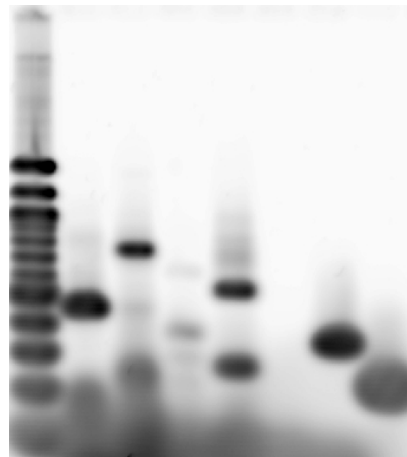
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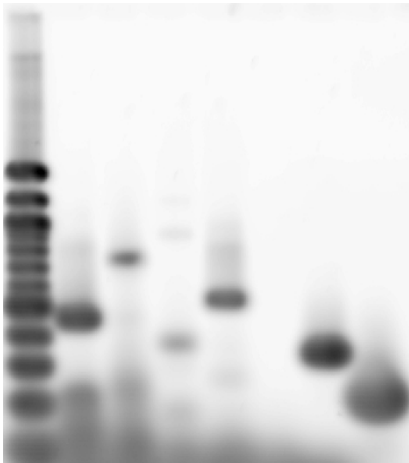
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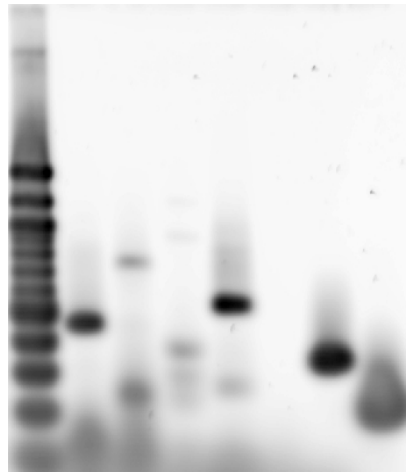
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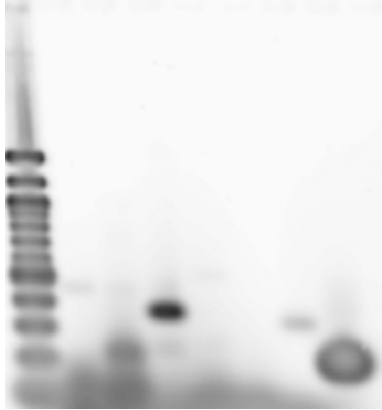
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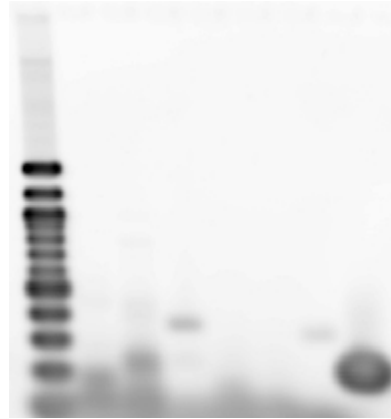
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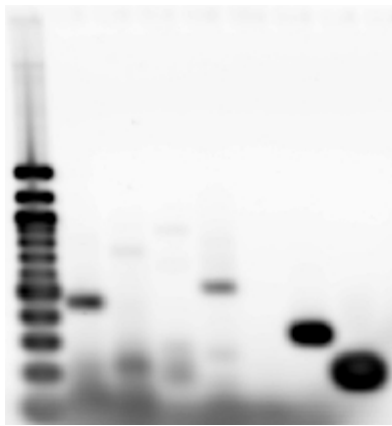
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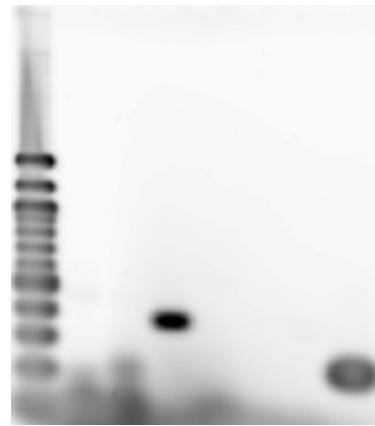
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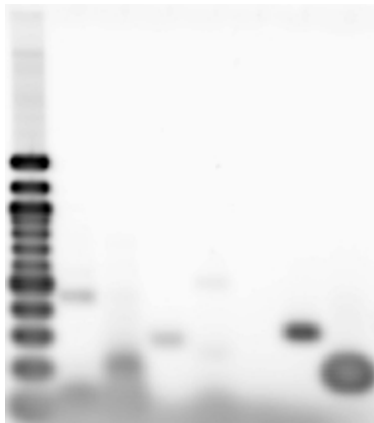
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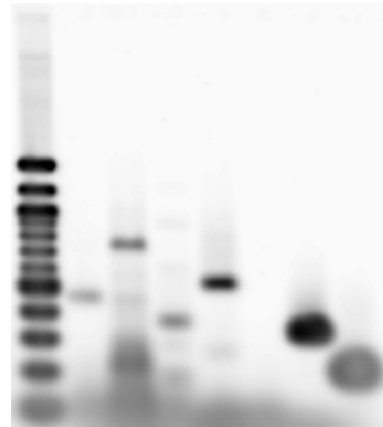
45 – 06TB0010



46 – 07TB0299



47 – 06TB0991



48 – 05TB0237

11.0 References

- Adams, M., J. L. Jones, et al. (2002). "Changes in tenascin-C isoform expression in invasive and preinvasive breast disease." Cancer Res 62(11): 3289-97.
- Albini, A., V. Mirisola, et al. (2008). "Metastasis signatures: genes regulating tumor-microenvironment interactions predict metastatic behavior." Cancer Metastasis Rev 27(1): 75-83.
- Algaba, F., I. Trias, et al. (2003). "TP53 in urologic tumors." Anal Quant Cytol Histol 25(3): 123-30.
- Ali-Osman, F., J. M. Brunner, et al. (1997). "Prognostic significance of glutathione S-transferase pi expression and subcellular localization in human gliomas." Clin Cancer Res 3(12 Pt 1): 2253-61.
- Amar, A. P., S. J. DeArmond, et al. (1994). "Development of an in vitro extracellular matrix assay for studies of brain tumor cell invasion." J Neurooncol 20(1): 1-15.
- Androulidaki, A., E. Dermitzaki, et al. (2009). "Corticotropin Releasing Factor promotes breast cancer cell motility and invasiveness." Mol Cancer 8: 30.
- Annabi, B., S. Rojas-Sutterlin, et al. (2008). "The diet-derived sulforaphane inhibits matrix metalloproteinase-9-activated human brain microvascular endothelial cell migration and tubulogenesis." Mol Nutr Food Res 52(6): 692-700.
- Arai, T., Y. Miyoshi, et al. (2006). "Association of GSTP1 CpG islands hypermethylation with poor prognosis in human breast cancers." Breast Cancer Res Treat 100(2): 169-76.
- Aref, S., E. Osman, et al. (2007). "Prognostic relevance of circulating matrix metalloproteinase-2 in acute myeloid leukaemia patients." Hematol Oncol 25(3): 121-6.
- Arima, Y., Y. Inoue, et al. (2008). "Rb depletion results in deregulation of E-cadherin and induction of cellular phenotypic changes that are characteristic of the epithelial-to-mesenchymal transition." Cancer Res 68(13): 5104-12.
- Asakage, M., N. H. Tsuno, et al. (2006). "Sulforaphane induces inhibition of human umbilical vein endothelial cells proliferation by apoptosis." Angiogenesis 9(2): 83-91.
- Bacon, J. R., G. Williamson, et al. (2003). "Sulforaphane and quercetin modulate PhIP-DNA adduct formation in human HepG2 cells and hepatocytes." Carcinogenesis 24(12): 1903-11.
- Baker, N. L., M. Morgelin, et al. (2005). "Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy." Hum Mol Genet 14(2): 279-93.
- Baldock, C., C. J. Gilpin, et al. (2002). "Three-dimensional reconstructions of extracellular matrix polymers using automated electron tomography." J Struct Biol 138(1-2): 130-6.
- Baldock, C., M. J. Sherratt, et al. (2003). "The supramolecular organization of collagen VI microfibrils." J Mol Biol 330(2): 297-307.
- Ball, S., J. Bella, et al. (2003). "Structural basis of type VI collagen dimer formation." J Biol Chem 278(17): 15326-32.
- Banyard, J., L. Bao, et al. (2007). "Collagen XXIII expression is associated with prostate cancer recurrence and distant metastases." Clin Cancer Res 13(9): 2634-42.
- Beaudeux, J. L., P. Giral, et al. (2004). "Matrix metalloproteinases, inflammation and atherosclerosis: therapeutic perspectives." Clin Chem Lab Med 42(2): 121-31.
- Beer, T. M., A. J. Evans, et al. (2002). "Polymorphisms of GSTP1 and related genes and prostate cancer risk." Prostate Cancer Prostatic Dis 5(1): 22-7.

- Bell, S. C., J. H. Pringle, et al. (1999). "Alternatively spliced tenascin-C mRNA isoforms in human fetal membranes." Mol Hum Reprod 5(11): 1066-76.
- Berendsen, C. L., W. H. Peters, et al. (1997). "Glutathione S-transferase activity and subunit composition in transitional cell cancer and mucosa of the human bladder." Urology 49(4): 644-51.
- Berking, C., R. Takemoto, et al. (2001). "Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling." Cancer Res 61(22): 8306-16.
- Berndt, A., K. Anger, et al. (2006). "Differential expression of tenascin-C splicing domains in urothelial carcinomas of the urinary bladder." J Cancer Res Clin Oncol 132(8): 537-46.
- Bertini, E. and G. Pepe (2002). "Collagen type VI and related disorders: Bethlem myopathy and Ullrich scleroatonic muscular dystrophy." Eur J Paediatr Neurol 6(4): 193-8.
- Bertl, E., H. Bartsch, et al. (2006). "Inhibition of angiogenesis and endothelial cell functions are novel sulforaphane-mediated mechanisms in chemoprevention." Mol Cancer Ther 5(3): 575-85.
- Bieritz, B., P. Spessotto, et al. (2003). "Role of alpha8 integrin in mesangial cell adhesion, migration, and proliferation." Kidney Int 64(1): 119-27.
- Borsi, L., E. Balza, et al. (1995). "The alternative splicing pattern of the tenascin-C pre-mRNA is controlled by the extracellular pH." J Biol Chem 270(11): 6243-5.
- Botteman, M. F., C. L. Pashos, et al. (2003). "The health economics of bladder cancer: a comprehensive review of the published literature." Pharmacoeconomics 21(18): 1315-30.
- Brinkman, M., F. Buntinx, et al. (2006). "Use of selenium in chemoprevention of bladder cancer." Lancet Oncol 7(9): 766-74.
- Buchard, A., J. J. Sanchez, et al. (2007). "Multiplex PCR detection of GSTM1, GSTT1, and GSTP1 gene variants: simultaneously detecting GSTM1 and GSTT1 gene copy number and the allelic status of the GSTP1 Ile105Val genetic variant." J Mol Diagn 9(5): 612-7.
- Bustin, S. A. (2002). "Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems." J Mol Endocrinol 29(1): 23-39.
- Cantor, K. P., C. F. Lynch, et al. (1992). "Bladder cancer, parity, and age at first birth." Cancer Causes Control 3(1): 57-62.
- Cengiz, M., A. Ozaydin, et al. (2007). "The investigation of GSTT1, GSTM1 and SOD polymorphism in bladder cancer patients." Int Urol Nephrol.
- Chaffer, C. L., J. P. Brennan, et al. (2006). "Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2." Cancer Res 66(23): 11271-8.
- Chaffer, C. L., E. W. Thompson, et al. (2007). "Mesenchymal to epithelial transition in development and disease." Cells Tissues Organs 185(1-3): 7-19.
- Chambers, K. F., J. R. Bacon, et al. (2009). "Gene expression profile of primary prostate epithelial and stromal cells in response to sulforaphane or iberin exposure." Prostate.
- Charafe-Jauffret, E., C. Ginestier, et al. (2006). "Gene expression profiling of breast cell lines identifies potential new basal markers." Oncogene 25(15): 2273-84.
- Charafe-Jauffret, E., F. Monville, et al. (2007). "Moesin expression is a marker of basal breast carcinomas." Int J Cancer 121(8): 1779-85.

- Chen JS, C.-S. E., Suzuki T, Neale GA, Mihara K, Pui CH, Campana D. (2001). "Identification of novel markers for monitoring minimal residual disease in acute lymphoblastic leukaemia." Blood 97(7): 2115-20.
- Cheng, L., T. D. Jones, et al. (2005). "Molecular genetic evidence for a common clonal origin of urinary bladder small cell carcinoma and coexisting urothelial carcinoma." Am J Pathol 166(5): 1533-9.
- Cheng, L., A. L. Weaver, et al. (2000). "Predicting the survival of bladder carcinoma patients treated with radical cystectomy." Cancer 88(10): 2326-32.
- Chevallier, J. M. (1994). "[The bladder. Surgical anatomy. Cystectomy]." Soins Chir(159): 41-3.
- Chico, D. E. and I. Listowsky (2005). "Diverse expression profiles of glutathione-S-transferase subunits in mammalian urinary bladders." Arch Biochem Biophys 435(1): 56-64.
- Chiquet-Ehrismann, R. (2004). "Tenascins." Int J Biochem Cell Biol 36(6): 986-90.
- Christofori, G. (2006). "New signals from the invasive front." Nature 441(7092): 444-50.
- Christoph, F., S. Hinz, et al. (2007). "A gene expression profile of tumor suppressor genes commonly methylated in bladder cancer." J Cancer Res Clin Oncol 133(6): 343-9.
- Chu, M. L., K. Mann, et al. (1987). "Characterization of three constituent chains of collagen type VI by peptide sequences and cDNA clones." Eur J Biochem 168(2): 309-17.
- Chuaqui, R. F., R. F. Bonner, et al. (2002). "Post-analysis follow-up and validation of microarray experiments." Nat Genet 32 Suppl: 509-14.
- Cutroneo, K. R., S. L. White, et al. (2007). "Phenotypic expression of human hepatoma cells in culture." J Cell Biochem 100(5): 1081-5.
- Cutroneo, K. R., S. L. White, et al. (2007). "Therapies for bleomycin induced lung fibrosis through regulation of TGF-beta1 induced collagen gene expression." J Cell Physiol 211(3): 585-9.
- Dalbagni, G., E. Genega, et al. (2001). "Cystectomy for bladder cancer: a contemporary series." J Urol 165(4): 1111-6.
- Day, I. N., X. H. Chen, et al. (2004). "Late life metabolic syndrome, early growth, and common polymorphism in the growth hormone and placental lactogen gene cluster." J Clin Endocrinol Metab 89(11): 5569-76.
- de Vicente, J. C., M. F. Fresno, et al. (2005). "Expression and clinical significance of matrix metalloproteinase-2 and matrix metalloproteinase-9 in oral squamous cell carcinoma." Oral Oncol 41(3): 283-93.
- Deryugina, E. I., G. X. Luo, et al. (1997). "Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2." Anticancer Res 17(5A): 3201-10.
- Di Carlo, A., D. Terracciano, et al. (2006). "Urinary gelatinase activities (matrix metalloproteinases 2 and 9) in human bladder tumors." Oncol Rep 15(5): 1321-6.
- Do, R., R. S. Kiss, et al. (2009). "Squalene synthase: a critical enzyme in the cholesterol biosynthesis pathway." Clin Genet 75(1): 19-29.
- Donaldson, M. S. (2004). "Nutrition and cancer: a review of the evidence for an anti-cancer diet." Nutr J 3: 19.
- Engel, L. S., E. Taioli, et al. (2002). "Pooled analysis and meta-analysis of glutathione S-transferase M1 and bladder cancer: a HuGE review." Am J Epidemiol 156(2): 95-109.
- Erickson, H. P. (1993). "Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions." Curr Opin Cell Biol 5(5): 869-76.

- Fahling, M., A. Perlewitz, et al. (2004). "Regulation of collagen prolyl 4-hydroxylase and matrix metalloproteinases in fibrosarcoma cells by hypoxia." Comp Biochem Physiol C Toxicol Pharmacol 139(1-3): 119-26.
- Fatemi, S. H., T. J. Reutiman, et al. (2008). "Phosphodiesterase-4A expression is reduced in cerebella of patients with bipolar disorder." Psychiatr Genet 18(6): 282-8.
- Friedrich, M. G., D. J. Weisenberger, et al. (2004). "Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients." Clin Cancer Res 10(22): 7457-65.
- Fujita, A., J. R. Sato, et al. (2008). "Identification of COL6A1 as a differentially expressed gene in human astrocytomas." Genet Mol Res 7(2): 371-8.
- Gallagher, E. P., J. L. Gardner, et al. (2006). "Several glutathione S-transferase isozymes that protect against oxidative injury are expressed in human liver mitochondria." Biochem Pharmacol 71(11): 1619-28.
- Galoian, K. A., N. Garamszegi, et al. (2007). "Molecular mechanism of tenascin-C action on matrix metalloproteinase-1 invasive potential." Exp Biol Med (Maywood) 232(4): 515-22.
- Garcia-Closas, M., N. Malats, et al. (2005). "NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses." Lancet 366(9486): 649-59.
- Garcia-Closas, R., M. Garcia-Closas, et al. (2007). "Food, nutrient and heterocyclic amine intake and the risk of bladder cancer." Eur J Cancer.
- Gaspar, A. V., A. Al-Janobi, et al. (2005). "Glutathione S-transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli." Am J Clin Nutr 82(6): 1283-91.
- Gerdes, A. M. (2002). "[Cancer genetics. A review of oncological molecular biology seen in relation to the human genome]." Ugeskr Laeger 164(22): 2865-71.
- Golka, K., A. W. Rettenmeier, et al. (2006). "[The causes of urinary bladder cancer and possibilities of prevention.]." Urologe A 45(3): 361-368.
- Golka, K., T. Schmidt, et al. (2008). "The influence of polymorphisms of glutathione S-transferases M1 and M3 on the development of human urothelial cancer." J Toxicol Environ Health A 71(13-14): 881-6.
- Golka, K., T. Seidel, et al. (2005). "[Occupational and non-occupational risk factors in bladder cancer patients in an industrialized area located in former East-Germany]." Aktuelle Urol 36(5): 417-22.
- Gontero, P., S. Banisadr, et al. (2004). "Metastasis markers in bladder cancer: a review of the literature and clinical considerations." Eur Urol 46(3): 296-311.
- Gontero, P., G. Casetta, et al. (2004). "Phase II study to investigate the ablative efficacy of intravesical administration of gemcitabine in intermediate-risk superficial bladder cancer (SBC)." Eur Urol 46(3): 339-43.
- Goto, S., M. Kawakatsu, et al. (2009). "Glutathione S-transferase pi localizes in mitochondria and protects against oxidative stress." Free Radic Biol Med 46(10): 1392-403.
- Grossman, H. B., R. B. Natale, et al. (2003). "Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer." N Engl J Med 349(9): 859-66.
- Grossman, H. B., M. Soloway, et al. (2006). "Surveillance for recurrent bladder cancer using a point-of-care proteomic assay." JAMA 295(3): 299-305.

- Hall, C. L., J. Dai, et al. (2006). "Type I collagen receptor (alpha 2 beta 1) signaling promotes the growth of human prostate cancer cells within the bone." Cancer Res 66(17): 8648-54.
- Hart, I. R. and A. Saini (1992). "Biology of tumour metastasis." Lancet 339(8807): 1453-7.
- Hauptmann, S., D. Budianto, et al. (2003). "Adhesion and migration of HRT-18 colorectal carcinoma cells on extracellular matrix components typical for the desmoplastic stroma of colorectal adenocarcinomas." Oncology 65(2): 174-81.
- Hayes, J. D., M. O. Kelleher, et al. (2008). "The cancer chemopreventive actions of phytochemicals derived from glucosinolates." Eur J Nutr 47 Suppl 2: 73-88.
- Hazar, B., G. Polat, et al. (2004). "Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in Hodgkin's and non-Hodgkin's lymphoma." Int J Clin Pract 58(2): 139-43.
- Hofbauer, K. H., B. Gess, et al. (2003). "Oxygen tension regulates the expression of a group of procollagen hydroxylases." Eur J Biochem 270(22): 4515-22.
- Holting, T., A. Zielke, et al. (1994). "Transforming growth factor-beta 1 is a negative regulator for differentiated thyroid cancer: studies of growth, migration, invasion, and adhesion of cultured follicular and papillary thyroid cancer cell lines." J Clin Endocrinol Metab 79(3): 806-13.
- Howell, S. J. and K. J. Doane (1998). "Type VI collagen increases cell survival and prevents anti-beta 1 integrin-mediated apoptosis." Exp Cell Res 241(1): 230-41.
- <http://www.bdbeurope.com>. (2009). "BD biosciences." 2009.
- Huber, M. A., N. Kraut, et al. (2005). "Molecular requirements for epithelial-mesenchymal transition during tumor progression." Curr Opin Cell Biol 17(5): 548-58.
- Hugo, H., M. L. Ackland, et al. (2007). "Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression." J Cell Physiol 213(2): 374-83.
- Hung, R. J., P. Boffetta, et al. (2004). "GST, NAT, SULT1A1, CYP1B1 genetic polymorphisms, interactions with environmental exposures and bladder cancer risk in a high-risk population." Int J Cancer 110(4): 598-604.
- Hurst, R., R. M. Elliott, et al. (2008). "Se-methylselenocysteine alters collagen gene and protein expression in human prostate cells." Cancer Lett 269(1): 117-26.
- Hwa, J. S., H. J. Park, et al. (2005). "Identification of proteins differentially expressed in the conventional renal cell carcinoma by proteomic analysis." J Korean Med Sci 20(3): 450-5.
- Hwang, E. S. and E. H. Jeffery (2005). "Induction of quinone reductase by sulforaphane and sulforaphane N-acetylcysteine conjugate in murine hepatoma cells." J Med Food 8(2): 198-203.
- Hwang, E. S. and H. J. Lee (2006). "Allyl isothiocyanate and its N-acetylcysteine conjugate suppress metastasis via inhibition of invasion, migration, and matrix metalloproteinase-2/-9 activities in SK-Hep 1 human hepatoma cells." Exp Biol Med (Maywood) 231(4): 421-30.
- Hwang, E. S. and H. J. Lee (2006). "Phenylethyl isothiocyanate and its N-acetylcysteine conjugate suppress the metastasis of SK-Hep1 human hepatoma cells." J Nutr Biochem 17(12): 837-46.
- Imoto, I., H. Izumi, et al. (2006). "Frequent silencing of the candidate tumor suppressor PCDH20 by epigenetic mechanism in non-small-cell lung cancers." Cancer Res 66(9): 4617-26.
- Iyengar, P., T. P. Combs, et al. (2003). "Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic

- transcriptional programs and proto-oncogene stabilization." Oncogene 22(41): 6408-23.
- Iyengar, P., V. Espina, et al. (2005). "Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment." J Clin Invest 115(5): 1163-76.
- Jeronimo, C., G. Varzim, et al. (2002). "I105V polymorphism and promoter methylation of the GSTP1 gene in prostate adenocarcinoma." Cancer Epidemiol Biomarkers Prev 11(5): 445-50.
- Jiang, Z. Q., C. Chen, et al. (2003). "Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers." Life Sci 72(20): 2243-53.
- Jichlinski, P., L. Guillou, et al. (2003). "Hexyl aminolevulinate fluorescence cystoscopy: new diagnostic tool for photodiagnosis of superficial bladder cancer--a multicenter study." J Urol 170(1): 226-9.
- Jocham, D., H. Stepp, et al. (2007). "Photodynamic Diagnosis in Urology: State-of-the-Art." Eur Urol.
- Jones, F. S. and P. L. Jones (2000). "The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling." Dev Dyn 218(2): 235-59.
- Jones, S. B. and J. D. Brooks (2006). "Modest induction of phase 2 enzyme activity in the F-344 rat prostate." BMC Cancer 6(1): 62.
- Jones, T. D., M. Wang, et al. (2005). "Molecular evidence supporting field effect in urothelial carcinogenesis." Clin Cancer Res 11(18): 6512-9.
- Joseph, M. A., K. B. Moysich, et al. (2004). "Cruciferous vegetables, genetic polymorphisms in glutathione S-transferases M1 and T1, and prostate cancer risk." Nutr Cancer 50(2): 206-13.
- Jourenkova-Mironova, N., A. Voho, et al. (1999). "Glutathione S-transferase GSTM3 and GSTP1 genotypes and larynx cancer risk." Cancer Epidemiol Biomarkers Prev 8(2): 185-8.
- Juge, N., R. F. Mithen, et al. (2007). "Molecular basis for chemoprevention by sulforaphane: a comprehensive review." Cell Mol Life Sci 64(9): 1105-27.
- Kallergi, G., S. Agelaki, et al. (2007). "Activation of FAK/PI3K/Rac1 signaling controls actin reorganization and inhibits cell motility in human cancer cells." Cell Physiol Biochem 20(6): 977-86.
- Kammerer, R. A., T. Schulthess, et al. (1998). "Tenascin-C hexabrachion assembly is a sequential two-step process initiated by coiled-coil alpha-helices." J Biol Chem 273(17): 10602-8.
- Kaur, G., M. Roberti, et al. (2007). "Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol." Thromb Res 119(2): 247-56.
- Kausch, I., C. Doehn, et al. (2006). "Recent improvements in the detection and treatment of nonmuscle-invasive bladder cancer." Expert Rev Anticancer Ther 6(9): 1301-11.
- Kesarwani, P., R. Singh, et al. (2008). "Association of GSTM3 intron 6 variant with cigarette smoking, tobacco chewing and alcohol as modifier factors for prostate cancer risk." Arch Toxicol.
- Keum, Y. S., W. S. Jeong, et al. (2004). "Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms." Mutat Res 555(1-2): 191-202.
- Keum, Y. S., W. S. Jeong, et al. (2005). "Chemopreventive functions of isothiocyanates." Drug News Perspect 18(7): 445-51.

- Kiemeny, L. A. (2008). "Hereditary bladder cancer." Scand J Urol Nephrol Suppl(218): 110-5.
- Kim, J., H. S. Lee, et al. (2005). "Silencing and CpG island methylation of GSTP1 is rare in ordinary gastric carcinomas but common in Epstein-Barr virus-associated gastric carcinomas." Anticancer Res 25(6B): 4013-9.
- Kim, M. K. and J. H. Park (2009). "Conference on "Multidisciplinary approaches to nutritional problems". Symposium on "Nutrition and health". Cruciferous vegetable intake and the risk of human cancer: epidemiological evidence." Proc Nutr Soc 68(1): 103-10.
- Kim, W. J. and Y. J. Kim (2009). "Epigenetic biomarkers in urothelial bladder cancer." Expert Rev Mol Diagn 9(3): 259-69.
- Kirkali, Z., T. Chan, et al. (2005). "Bladder cancer: epidemiology, staging and grading, and diagnosis." Urology 66(6 Suppl 1): 4-34.
- Konig-Greger, D., H. Riechelmann, et al. (2004). "Genotype and phenotype of glutathione-S-transferase in patients with head and neck carcinoma." Otolaryngol Head Neck Surg 130(6): 718-25.
- Kopp, M. U., K. H. Winterhalter, et al. (1997). "DNA methylation accounts for the inhibition of collagen VI expression in transformed fibroblasts." Eur J Biochem 249(2): 489-96.
- Lamm, D. L. (2000). "Efficacy and safety of bacille Calmette-Guerin immunotherapy in superficial bladder cancer." Clin Infect Dis 31 Suppl 3: S86-90.
- Lampe, A. K. and K. M. Bushby (2005). "Collagen VI related muscle disorders." J Med Genet 42(9): 673-85.
- Larsson, S. C., S. O. Andersson, et al. (2008). "Fruit and vegetable consumption and risk of bladder cancer: a prospective cohort study." Cancer Epidemiol Biomarkers Prev 17(9): 2519-22.
- LC, C. (1998). "Decreased incidence of prostate cancer with selenium supplementation." Br J Urol 81: 730-734.
- Le Clainche C, S. D., Ferrari A, Klingauf M, Grohmanova K, Veligodskiy A, Didry D, Le D, Egile C, Carlier MF, Kroschewski R. (2007). "IQGAP1 stimulates actin assembly through the N-WASP-Arp2/3 pathway." J Biol Chem 282(1): 426-35.
- Lee, J. M., S. Dedhar, et al. (2006). "The epithelial-mesenchymal transition: new insights in signaling, development, and disease." J Cell Biol 172(7): 973-81.
- Lerner S P, S. C. N., Schoenberg M P (2005). Textbook of bladder cancer, Taylor & Francis Ltd
- Li, C. and W. Hung Wong (2001). "Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application." Genome Biol 2(8): RESEARCH0032.
- Li, C. and W. H. Wong (2001). "Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection." Proc Natl Acad Sci U S A 98(1): 31-6.
- Li, H. C., D. C. Cao, et al. (2004). "Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma." Breast Cancer Res Treat 88(1): 75-85.
- Libra, M., A. Scalisi, et al. (2009). "Uterine cervical carcinoma: role of matrix metalloproteinases (review)." Int J Oncol 34(4): 897-903.
- Liotta, L. A., K. Tryggvason, et al. (1980). "Metastatic potential correlates with enzymatic degradation of basement membrane collagen." Nature 284(5751): 67-8.

- Lodygin, D., A. Epanchintsev, et al. (2005). "Functional epigenomics identifies genes frequently silenced in prostate cancer." Cancer Res 65(10): 4218-27.
- Lopez-Velez, M., F. Martinez-Martinez, et al. (2003). "The study of phenolic compounds as natural antioxidants in wine." Crit Rev Food Sci Nutr 43(3): 233-44.
- Lotan, Y. and C. G. Roehrborn (2003). "Sensitivity and specificity of commonly available bladder tumor markers versus cytology: results of a comprehensive literature review and meta-analyses." Urology 61(1): 109-18; discussion 118.
- Mannervik, B. (1985). "The isoenzymes of glutathione transferase." Adv Enzymol Relat Areas Mol Biol 57: 357-417.
- Matias-Roman, S., B. G. Galvez, et al. (2005). "Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium." Blood 105(10): 3956-64.
- Matsui, H., K. Kubochi, et al. (1999). "Collagen biosynthesis in gastric cancer: immunohistochemical analysis of prolyl 4-hydroxylase." J Surg Oncol 70(4): 239-46.
- Matusheski, N. V. and E. H. Jeffery (2001). "Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile." J Agric Food Chem 49(12): 5743-9.
- Michaud, D. S., D. Spiegelman, et al. (1999). "Fruit and vegetable intake and incidence of bladder cancer in a male prospective cohort." J Natl Cancer Inst 91(7): 605-13.
- Mighell, A. J., J. Thompson, et al. (1997). "Human tenascin-C: identification of a novel type III repeat in oral cancer and of novel splice variants in normal, malignant and reactive oral mucosae." Int J Cancer 72(2): 236-40.
- Miller, Y. E., J. D. Minna, et al. (1989). "Lack of expression of aminoacylase-1 in small cell lung cancer. Evidence for inactivation of genes encoded by chromosome 3p." J Clin Invest 83(6): 2120-4.
- Mithen, R. (2001). "Glucosinolates and their degradation products." Advances in botanical research(35): 213-262.
- Mohammed, A., Z. Khan, et al. (2008). "Biological markers in the diagnosis of recurrent bladder cancer: an overview." Expert Rev Mol Diagn 8(1): 63-72.
- Mori, K., H. Enokida, et al. (2009). "CpG hypermethylation of collagen type I alpha 2 contributes to proliferation and migration activity of human bladder cancer." Int J Oncol 34(6): 1593-602.
- Munday, R. and C. M. Munday (2002). "Selective induction of phase II enzymes in the urinary bladder of rats by allyl isothiocyanate, a compound derived from Brassica vegetables." Nutr Cancer 44(1): 52-9.
- Munday, R. and C. M. Munday (2004). "Induction of phase II detoxification enzymes in rats by plant-derived isothiocyanates: comparison of allyl isothiocyanate with sulforaphane and related compounds." J Agric Food Chem 52(7): 1867-71.
- Munday, R., Y. Zhang, et al. (2006). "Evaluation of isothiocyanates as potent inducers of carcinogen-detoxifying enzymes in the urinary bladder: critical nature of in vivo bioassay." Nutr Cancer 54(2): 223-31.
- Munday, R., Y. Zhang, et al. (2008). "Structure-activity relationships and organ specificity in the induction of GST and NQO1 by alkyl-aryl isothiocyanates." Pharm Res 25(9): 2164-70.
- Mungan, N. A., K. K. Aben, et al. (2000). "Gender differences in stage-adjusted bladder cancer survival." Urology 55(6): 876-80.

- Nabeshima, K., T. Inoue, et al. (2002). "Matrix metalloproteinases in tumor invasion: role for cell migration." Pathol Int 52(4): 255-64.
- Nakata, S., J. Sato, et al. (1996). "Correlation analysis between bladder cancer and cigarette smoking in various countries." Tohoku J Exp Med 178(2): 169-76.
- Nakayama, M., C. J. Bennett, et al. (2003). "Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection." Am J Pathol 163(3): 923-33.
- Nakayama, M., M. L. Gonzalgo, et al. (2004). "GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer." J Cell Biochem 91(3): 540-52.
- Novin, M. G. and M. Nouri (2007). "Effect of matrigel on function and morphology of human endometrial epithelial cell in vitro." Iran Biomed J 11(2): 87-94.
- Ohno, Y. and K. Aoki (1980). "Epidemiology of urinary bladder cancer: international variation in mortality." Nagoya J Med Sci 43(1-2): 41-64.
- Okamura, N., Y. Mori, et al. (1996). "[Experimental studies on the cell adhesion molecule E-cadherin and in vitro invasion of endometrial carcinoma cell lines]." Nippon Sanka Fujinka Gakkai Zasshi 48(5): 335-42.
- Oosterlinck, W. (2004). "Guidelines on diagnosis and treatment of superficial bladder cancer." Minerva Urol Nefrol 56(1): 65-72.
- Oosterlinck, W., B. Lobel, et al. (2002). "[EAU Recommendations 2001. "Guidelines on bladder cancer"]." Prog Urol 12(5): 1161-3.
- Oosterlinck, W., B. Lobel, et al. (2002). "Guidelines on bladder cancer." Eur Urol 41(2): 105-12.
- Oosterlinck, W., E. Solsona, et al. (2005). "Low-grade Ta (noninvasive) urothelial carcinoma of the bladder." Urology 66(6 Suppl 1): 75-89.
- Orend, G. and R. Chiquet-Ehrismann (2000). "Adhesion modulation by antiadhesive molecules of the extracellular matrix." Exp Cell Res 261(1): 104-10.
- Orlichenko, L. S. and D. C. Radisky (2008). "Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development." Clin Exp Metastasis 25(6): 593-600.
- Osmanov Iu, I., N. N. Petrovichev, et al. (2005). "[Immunohistochemical characterization of biphasic synovial sarcomas]." Vopr Onkol 51(1): 81-5.
- Park, O. J. and Y. J. Surh (2004). "Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies." Toxicol Lett 150(1): 43-56.
- Parsons, C. L. (1986). "Bladder surface glycosaminoglycan: efficient mechanism of environmental adaptation." Urology 27(2 Suppl): 9-14.
- Parsons, C. L., C. W. Stauffer, et al. (1988). "Reversible inactivation of bladder surface glycosaminoglycan antibacterial activity by protamine sulfate." Infect Immun 56(5): 1341-3.
- Patel, S. P., M. Hotston, et al. (2005). "The protective effects of green tea in prostate cancer." BJU Int 96(9): 1212-4.
- pathology2.jhu.edu/bladder_cancer (2007). "Bladder Cancer."
- Patrikainen, L., K. Porvari, et al. (2007). "Expression profiling of PC-3 cell line variants and comparison of MIC-1 transcript levels in benign and malignant prostate." Eur J Clin Invest 37(2): 126-33.
- Pelucchi, C., C. Bosetti, et al. (2006). "Mechanisms of disease: The epidemiology of bladder cancer." Nat Clin Pract Urol 3(6): 327-40.

- Peng, D. F., M. Razvi, et al. (2009). "DNA hypermethylation regulates the expression of members of the Mu-class glutathione S-transferases and glutathione peroxidases in Barrett's adenocarcinoma." Gut 58(1): 5-15.
- Petri, N., C. Tannergren, et al. (2003). "Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo." Drug Metab Dispos 31(6): 805-13.
- Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.
- Pirila, E., N. S. Ramamurthy, et al. (2003). "Gelatinase A (MMP-2), collagenase-2 (MMP-8), and laminin-5 gamma2-chain expression in murine inflammatory bowel disease (ulcerative colitis)." Dig Dis Sci 48(1): 93-8.
- Pirila, E., A. Sharabi, et al. (2003). "Matrix metalloproteinases process the laminin-5 gamma 2-chain and regulate epithelial cell migration." Biochem Biophys Res Commun 303(4): 1012-7.
- Pljesa-Ercegovac, M., A. Savic-Radojevic, et al. (2009). "Enhanced GSTP1 expression in transitional cell carcinoma of urinary bladder is associated with altered apoptotic pathways." Urol Oncol.
- Prols, F., A. Hartner, et al. (1999). "Mesangial cells and their adhesive properties." Exp Nephrol 7(2): 137-46.
- Przybylo, J. A. and D. C. Radisky (2007). "Matrix metalloproteinase-induced epithelial-mesenchymal transition: tumor progression at Snail's pace." Int J Biochem Cell Biol 39(6): 1082-8.
- Qiu, W., B. Zhou, et al. (2007). "The induction of growth arrest DNA damage-inducible gene 45 beta in human hepatoma cell lines by S-adenosylmethionine." Am J Pathol 171(1): 287-96.
- Ramesh, N. and R. Geha (2008). "Recent advances in the biology of WASP and WIP." Immunol Res.
- Ramos-Gomez, M., M. K. Kwak, et al. (2001). "Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice." Proc Natl Acad Sci U S A 98(6): 3410-5.
- RefSeq.
- Renard, I., S. Joniau, et al. (2009). "Identification and Validation of the Methylated TWIST1 and NID2 Genes through Real-Time Methylation-Specific Polymerase Chain Reaction Assays for the Noninvasive Detection of Primary Bladder Cancer in Urine Samples." Eur Urol.
- Reszka, E., W. Wasowicz, et al. (2006). "Genetic polymorphism of xenobiotic metabolising enzymes, diet and cancer susceptibility." Br J Nutr 96(4): 609-19.
- Reszka, E., W. Wasowicz, et al. (2007). "Antioxidant defense markers modulated by glutathione S-transferase genetic polymorphism: results of lung cancer case-control study." Genes Nutr 2(3): 287-94.
- Rodriguez, L. G., X. Wu, et al. (2005). "Wound-healing assay." Methods Mol Biol 294: 23-9.
- Rose, P., Q. Huang, et al. (2005). "Broccoli and watercress suppress matrix metalloproteinase-9 activity and invasiveness of human MDA-MB-231 breast cancer cells." Toxicol Appl Pharmacol 209(2): 105-13.
- Rosenbaum, E., M. O. Hoque, et al. (2005). "Promoter hypermethylation as an independent prognostic factor for relapse in patients with prostate cancer following radical prostatectomy." Clin Cancer Res 11(23): 8321-5.

- Roy, R., G. Louis, et al. (2008). "Tumor-specific urinary matrix metalloproteinase fingerprinting: identification of high molecular weight urinary matrix metalloproteinase species." Clin Cancer Res 14(20): 6610-7.
- Ruhl, M., M. Johannsen, et al. (1999). "Soluble collagen VI induces tyrosine phosphorylation of paxillin and focal adhesion kinase and activates the MAP kinase erk2 in fibroblasts." Exp Cell Res 250(2): 548-57.
- Rungapamestry, V., A. J. Duncan, et al. (2006). "Changes in glucosinolate concentrations, myrosinase activity, and production of metabolites of glucosinolates in cabbage (*Brassica oleracea* Var. capitata) cooked for different durations." J Agric Food Chem 54(20): 7628-34.
- Rungapamestry, V., A. J. Duncan, et al. (2007). "Effect of cooking brassica vegetables on the subsequent hydrolysis and metabolic fate of glucosinolates." Proc Nutr Soc 66(1): 69-81.
- Sanchez-Carbayo, M. and C. Cordon-Cardo (2007). "Molecular alterations associated with bladder cancer progression." Semin Oncol 34(2): 75-84.
- Sangar, V. K., N. Ragavan, et al. (2005). "The economic consequences of prostate and bladder cancer in the UK." BJU Int 95(1): 59-63.
- Santin, A. D., F. Zhan, et al. (2004). "Gene expression profiles in primary ovarian serous papillary tumors and normal ovarian epithelium: identification of candidate molecular markers for ovarian cancer diagnosis and therapy." Int J Cancer 112(1): 14-25.
- Sanyal, S., F. Festa, et al. (2004). "Polymorphisms in DNA repair and metabolic genes in bladder cancer." Carcinogenesis 25(5): 729-34.
- Sanyal, S., C. Ryk, et al. (2007). "Polymorphisms in NQO1 and the clinical course of urinary bladder neoplasms." Scand J Urol Nephrol 41(3): 182-90.
- Sasse, J. and S. R. Gallagher (2008). "Detection of proteins on blot transfer membranes." Curr Protoc Immunol Chapter 8: Unit 8 10B.
- Savic-Radojevic, A., J. Mimic-Oka, et al. (2007). "Glutathione S-Transferase-P1 Expression Correlates with Increased Antioxidant Capacity in Transitional Cell Carcinoma of the Urinary Bladder." Eur Urol.
- Schenkman, E. and D. L. Lamm (2004). "Superficial bladder cancer therapy." ScientificWorldJournal 4 Suppl 1: 387-99.
- Shah, F. D., S. N. Shukla, et al. (2009). "Clinical significance of matrix metalloproteinase 2 and 9 in breast cancer." Indian J Cancer 46(3): 194-202.
- Shapiro, T. A., J. W. Fahey, et al. (2006). "Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study." Nutr Cancer 55(1): 53-62.
- Sherman-Baust, C. A., A. T. Weeraratna, et al. (2003). "Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells." Cancer Cell 3(4): 377-86.
- Simic, T., J. Mimic-Oka, et al. (2005). "Glutathione S-transferase T1-1 activity upregulated in transitional cell carcinoma of urinary bladder." Urology 65(5): 1035-40.
- Siri, A., B. Carnemolla, et al. (1991). "Human tenascin: primary structure, pre-mRNA splicing patterns and localization of the epitopes recognized by two monoclonal antibodies." Nucleic Acids Res 19(3): 525-31.
- Slaughter, D. P., H. W. Southwick, et al. (1953). "Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin." Cancer 6(5): 963-8.

- Song, L. and P. J. Thornalley (2007). "Effect of storage, processing and cooking on glucosinolate content of Brassica vegetables." Food Chem Toxicol 45(2): 216-24.
- Spector, D., Q. Yang, et al. (2007). "High urea and creatinine concentrations and urea transporter B in mammalian urinary tract tissues." AMERICAN JOURNAL OF PHYSIOLOGY-RENAL PHYSIOLOGY 292(1): F467-F474.
- Sriramarao, P. and M. A. Bourdon (1993). "A novel tenascin type III repeat is part of a complex of tenascin mRNA alternative splices." Nucleic Acids Res 21(1): 163-8.
- Statistics, G. C. (2004). "Bladder Cancer statistics in the UK " www.cancer.gov/statistics; www.cancer.gov/statistics.
- Svehlikova, V., S. Wang, et al. (2004). "Interactions between sulforaphane and apigenin in the induction of UGT1A1 and GSTA1 in CaCo-2 cells." Carcinogenesis 25(9): 1629-37.
- Tada, Y., M. Wada, et al. (2002). "The association of death-associated protein kinase hypermethylation with early recurrence in superficial bladder cancers." Cancer Res 62(14): 4048-53.
- Takahashi, Y., S. D. Hursting, et al. (2006). "Genistein affects androgen-responsive genes through both androgen- and estrogen-induced signaling pathways." Mol Carcinog 45(1): 18-25.
- Takiuchi, H., M. Kanokogi, et al. (1994). "[Expression of integrin molecule in urological tumor cell lines by using RT-PCR method]." Nippon Hinyokika Gakkai Zasshi 85(4): 584-8.
- Tang, L., G. Li, et al. (2006). "The principal urinary metabolites of dietary isothiocyanates, N-acetylcysteine conjugates, elicit the same anti-proliferative response as their parent compounds in human bladder cancer cells." Anticancer Drugs 17(3): 297-305.
- Tang, L. and Y. Zhang (2004). "Dietary isothiocyanates inhibit the growth of human bladder carcinoma cells." J Nutr 134(8): 2004-10.
- Tang, L. and Y. Zhang (2004). "Isothiocyanates in the chemoprevention of bladder cancer." Curr Drug Metab 5(2): 193-201.
- Tang, L. and Y. Zhang (2005). "Mitochondria are the primary target in isothiocyanate-induced apoptosis in human bladder cancer cells." Mol Cancer Ther 4(8): 1250-9.
- Thejass, P. and G. Kuttan (2006). "Antimetastatic activity of Sulforaphane." Life Sci 78(26): 3043-50.
- Thimmulappa, R. K., K. H. Mai, et al. (2002). "Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray." Cancer Res 62(18): 5196-203.
- Thompson, E. W. and E. D. Williams (2008). "EMT and MET in carcinoma--clinical observations, regulatory pathways and new models." Clin Exp Metastasis 25(6): 591-2.
- Tijhuis, M. J., M. H. Visser, et al. (2006). "Glutathione S-transferase phenotypes in relation to genetic variation and fruit and vegetable consumption in an endoscopy-based population." Carcinogenesis.
- Traka, M., A. V. Gasper, et al. (2005). "Transcriptome analysis of human colon Caco-2 cells exposed to sulforaphane." J Nutr 135(8): 1865-72.
- Tse, J. C. and R. Kalluri (2007). "Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment." J Cell Biochem 101(4): 816-29.
- Tsunoda, T., H. Inada, et al. (2003). "Involvement of large tenascin-C splice variants in breast cancer progression." Am J Pathol 162(6): 1857-67.

- Valgimigli, L. and R. Iori (2009). "Antioxidant and pro-oxidant capacities of ITCs." Environ Mol Mutagen 50(3): 222-37.
- van der Horst, C. and K. P. Junemann (2004). "[The aging bladder. anatomy and physiology]." Urologe A 43(5): 521-6.
- van der Meijden, A. P., R. Sylvester, et al. (2005). "EAU guidelines on the diagnosis and treatment of urothelial carcinoma in situ." Eur Urol 48(3): 363-71.
- Varma, R. R., S. M. Hector, et al. (2005). "Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10." Oncol Rep 14(4): 925-32.
- Vasala, K., P. Kuvaja, et al. (2008). "Low Circulating Levels of ProMMP-2 Are Associated with Adverse Prognosis in Bladder Cancer." Tumour Biol 29(5): 279-286.
- Vasala, K., P. Paakko, et al. (2003). "Matrix metalloproteinase-2 immunoreactive protein as a prognostic marker in bladder cancer." Urology 62(5): 952-7.
- Vila de Muga, S., P. Timpson, et al. (2009). "Annexin A6 inhibits Ras signalling in breast cancer cells." Oncogene 28(3): 363-77.
- Villar, J., M. I. Arenas, et al. (2007). "PCPH/ENTPD5 expression enhances the invasiveness of human prostate cancer cells by a protein kinase C delta-dependent mechanism." Cancer Res 67(22): 10859-68.
- Voho, A., O. Impivaara, et al. (2006). "Distribution of glutathione S-transferase M1, P1 and T1 genotypes in different age-groups of Finns without diagnosed cancer." Cancer Detect Prev 30(2): 144-51.
- Vrabie, C. D., A. Petrescu, et al. (2007). "Molecular changes in superficial bladder cancer." Rom J Morphol Embryol 48(2): 131-8.
- Wallerand, H., G. Robert, et al. (2009). "The epithelial-mesenchymal transition-inducing factor TWIST is an attractive target in advanced and/or metastatic bladder and prostate cancers()." Urol Oncol.
- Wittstock, U. and B. A. Halkier (2002). "Glucosinolate research in the Arabidopsis era." Trends Plant Sci 7(6): 263-70.
- www.cancer.gov/cancertopics (2007). "Bladder Cancer." National Cancer Institute.
- Xu, K., S. Hou, et al. (2002). "Prognostic value of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in bladder carcinoma." Chin Med J (Engl) 115(5): 743-5.
- Yamamoto, Y. and M. Negishi (2008). "The antiapoptotic factor growth arrest and DNA-damage-inducible 45 beta regulates the nuclear receptor constitutive active/androstane receptor-mediated transcription." Drug Metab Dispos 36(7): 1189-93.
- Yanagisawa, N., L. Geironson, et al. (2005). "Expression of matrix metalloproteinase-2, -7 and -9 on human colon, liver and bile duct cell lines by enteric and gastric Helicobacter species." FEMS Immunol Med Microbiol 44(2): 197-204.
- Yancik, R. (2005). "Population aging and cancer: a cross-national concern." Cancer J 11(6): 437-41.
- Ye, L., A. T. Dinkova-Kostova, et al. (2002). "Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans." Clin Chim Acta 316(1-2): 43-53.
- Ying, H., Y. Yu, et al. (2001). "Antisense of ATP synthase subunit e inhibits the growth of human hepatocellular carcinoma cells." Oncol Res 12(11-12): 485-90.
- Yoshida, T., E. Yoshimura, et al. (1999). "Involvement of tenascin-C in proliferation and migration of laryngeal carcinoma cells." Virchows Arch 435(5): 496-500.

- Yuan, Y., Z. R. Qian, et al. (2008). "Reduction of GSTP1 expression by DNA methylation correlates with clinicopathological features in pituitary adenomas." Mod Pathol 21(7): 856-65.
- Zeltner, R., K. F. Hilgers, et al. (2008). "A promoter polymorphism of the alpha 8 integrin gene and the progression of autosomal-dominant polycystic kidney disease." Nephron Clin Pract 108(3): c169-75.
- Zhang, Y. (2004). "Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action." Mutat Res 555(1-2): 173-90.
- Zhang, Y., R. Munday, et al. (2006). "Induction of GST and NQO1 in cultured bladder cells and in the urinary bladders of rats by an extract of broccoli (*Brassica oleracea italica*) sprouts." J Agric Food Chem 54(25): 9370-6.
- Zhang, Y., R. Munday, et al. (2006). "Induction of GST and NQO1 in Cultured Bladder Cells and in the Urinary Bladders of Rats by an Extract of Broccoli (*Brassica oleracea italica*) Sprouts." J Agric Food Chem 54(25): 9370-9376.
- Zhang, Y., P. Talalay, et al. (1992). "A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure." Proc Natl Acad Sci U S A 89(6): 2399-403.
- Zhang Y, T. P. C. R. (1994). " Anticarcinogenic activities of organic ITC." 54: 1976-1981.
- Zhao, H., J. Lin, et al. (2007). "Dietary isothiocyanates, GSTM1, GSTT1, NAT2 polymorphisms and bladder cancer risk." Int J Cancer.
- Zhu, H., Z. Jia, et al. (2009). "Cruciferous dithiolethione-mediated coordinated induction of total cellular and mitochondrial antioxidants and phase 2 enzymes in human primary cardiomyocytes: cytoprotection against oxidative/electrophilic stress and Doxorubicin toxicity." Exp Biol Med (Maywood) 234(4): 418-29.
- Zhu, T. (2003). "Global analysis of gene expression using GeneChip microarrays." Curr Opin Plant Biol 6(5): 418-25.
- Zielke, A., S. Hoffmann, et al. (1999). "Pleiotropic effects of thyroid stimulating hormone in a differentiated thyroid cancer cell line. Studies on proliferation, thyroglobulin secretion, adhesion, migration and invasion." Exp Clin Endocrinol Diabetes 107(6): 361-9.