The association of novel polymorphisms with Stress Fracture Injury in Elite Athletes: Further insight into the SFEA cohort.

Abstract

Objective: To determine, in conjunction with a wider investigation, whether 11 genetic variants in the vicinity vitamin D, collagen and *Wnt* signalling pathways were associated with stress fracture injury in the Stress Fracture Elite Athlete (SFEA) cohort.

Design: Genotype-phenotype association study.

Method: Self-reported stress fracture history and demographic data were recorded in 518 elite athletes, 449 male and 69 female (mean age 24.2±5.5 y) from the SFEA cohort. Elite athletes were assigned to two groups based on history stress fracture injury. Data were analysed for the whole cohort and sub-stratified in male only and multiple stress fracture cases. Genotype was determined using a proprietary fluorescence-based competitive allele-specific polymerase chain reaction assay.

Results: *SOST* SNP rs1877632 and *VDR* SNPs rs10735810 and rs731236 were associated with stress fracture ($p<0.05$). In the whole cohort, rs1877632 heterozygotes and homozygotes of the rare allele combined made up 59% of stress fracture sufferers in comparison to 46% in the non-stress fracture group ($p=0.05$). In the multiple stress fracture cohort, homozygotes of the rare allele of rs10735810 and rs731236 showed an association with stress fracture when compared to those homozygotes for the common allele combined with heterozygotes ($p= 0.03$; $p=0.01$). No significant associations were shown in the other SNPs analysed ($p>0.05$).

Conclusions: These data suggest an important role for *SOST* SNP rs1877632 and VDR SNPs rs10735810 and rs731236 in the pathophysiology of stress fracture. This might be due to the

role of the SNPs in the regulation of bone remodelling and adaptation to mechanical loading, with potential implications for the prevention and treatment of stress fracture.

Keywords

Bone; Genetics; bone remodelling; *SOST*; *Wnt* Signalling.

Introduction

Stress fracture injuries are caused by mechanical loading that is applied in a rhythmic, repeated, sub-threshold manner (1), although the exact pathophysiology is not fully understood (2). The high volume, intensity and type of training that is required to be successful in elite sport makes athletes in body weight loaded sports particularly susceptible to sustaining this type of injury (3).

Stress fracture injuries account for 0.7%-20% of all athletic sports injuries (Bennell et al., 1997; Fredericson et al., 2006), cause significant discomfort, result in a prolonged loss of training time (Ranson et al., 2010) and can have a significant detrimental financial effect on the athlete and/or the club/organisation. The pathophysiology of stress fracture is complex (4) including, but not limited to risk factors such as: the female athlete triad (Barrack et al 2014), unaccustomed or excessive exercise (5), nutritional deficiencies (6), previous stress fracture (Tenforde et al., 2013) and abnormal bone mineral density (BMD) (7). To further compound the complexly related to the cause of injury, an individual's genotype has also been associated with an increased susceptibility to stress fracture injury (8;9).

Initial analysis of genetic associations with stress fracture prevalence in the SFEA cohort has shown that single-nucleotide polymorphisms (SNPs) located in the vicinity of components of the *RANK/RANKL/OPG* signalling pathway, and the *P2X7* receptor are associated with stress fracture injuries in elite athletes and military recruits (10;11). Other SNPs, which are thought

to control other bone regulatory pathways, in the vicinity of *VDR* (12), *GC* (13), *COL1A1* (14) and *Wnt* (15) have been associated with bone phenotypes, but any association with stress fracture in athletes is yet to be shown. SNPs in the vicinity of genes in the *Wnt* signalling pathway are particularly strong candidates to be associated with stress fracture injury in an athletic population, due to their suggested role in the regulation of bone formation, and mechanotransduction (16). While VDR SNPs have been shown to regulate of vitamin status, protein-protein interactions and mediate cell transcriptional factors (17).

Therefore, the aim of the present study was determine whether 11 SNPs in the vicinity vitamin D, collagen and Wnt signalling pathways were associated with stress fracture injury in the Stress Fracture Elite Athlete (SFEA) cohort.

Method

A sample of 518 male (n=449) and female (n=69) elite athletes (table 1) were recruited to form the Stress Fracture in Elite Athletes (SFEA) cohort. Participating elite athletes competed in various sports including, football (n = 218), cricket (n = 156), track and field (n = 67, running events (n = 62), rowing (n = 13), boxing (n = 2), tennis (n = 12), hockey (n = 26) and gymnastics (n = 7), with each sport having both stress fracture Cases and non-stress fracture Control participants. Elite athletes were mainly white Caucasian (83.2% in the stress fracture Cases and 79.9% in the non-stress fracture Controls). Professional athletes were classified as elite due to their full time participation in sport; nonprofessional athletes were classified as elite if they regularly competed at international or national level. Each participant completed a statement of informed consent and a health status questionnaire, which was followed by an athletic status questionnaire detailing age and playing position if applicable. Participants with stress fracture injuries confirmed by medical imaging (*e.g.*, magnetic resonance imaging or computed tomography), were classified as cases, while those who had never experienced a

stress fracture injury, or reported symptoms of stress fracture, were classified as controls. Athletes that reported to have suffered a stress fracture injury without imaging confirmation and those who had experienced symptoms of stress fracture injury were withdrawn from the analysis (n=17). Ethical approval was granted by the Nottingham Trent University Ethical Review Committee, and each participant provided written informed consent prior to their involvement in the study.

Sub-classifications

Athletes were recruited from a range of sports (football, cricket, track and field, field hockey, gymnastics, rowing and boxing) and ranged from elite national class to Olympic medallists. Males, and individuals with multiple stress fractures were analysed in separate sub-classifications due to the size of the cohort (males n=449) and the greater genetic component that may be present in cases of multiple stress fracture injury.

Procedures

Genotyping

Saliva samples were collected, and genomic DNA was extracted with Norgen saliva collection and extraction kits (Norgen Biotek Corp., Saliva DNA Collection Kit, Thorold, Canada). All procedures were conducted in accordance with manufacturers guidelines.

SNPs were selected on the basis of their association with BMD and fragility fracture (12, 13, 14, 15, 17, 20, 23, 26, 28) and samples were genotyped by LGC genomics (Herts, UK), who were blinded to the clinical status (case or control) of the genotyped individuals, using proprietary fluorescence-based competitive allele-specific polymerase chain reaction assay.

Statistical analysis

Statistical analyses were performed using statistical package SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Student's t test was used for analysis of descriptive variables. Pearson's chi-squared ($\chi 2$) test was used to assess associations in genotype frequencies and to assess the observed frequency of each genotype with what would be expected in accordance with Hardy-Weinberg equilibrium. The Benjamini and Hochberg false discovery rate test was applied in order to account for multiple comparisons. Acceptable level of significance was classified as $p<0.05$.

Results

All SNPs were in accordance with Hardy Weinberg equilibrium (HWE; Supplementary table 1.) and none of the SNPs are in linkage equilibrium.

Of the 11 SNPs investigated, three were significantly associated with stress fracture injury in one or more of the classifications (Table 2). Significant associations ($p<0.05$) with stress fracture injury were shown with *SOST* and *VDR* alleles. No differences were seen in the other SNPs investigated ($p>0.05$).

Wnt Signalling

SOST SNP rs1877632 heterozygotes were associated with stress fracture risk in the whole cohort, and cases of multiple stress fracture, when compared to homozygotes of the common allele ($p<0.05$; Table 3). Associations were also shown when heterozygotes were combined with homozygotes of the rare allele and compared to homozygotes of the common allele in the same classification ($p<0.05$). The frequency of the rare allele was greater in stress fracture

sufferers in the whole cohort and cases of multiple stress fracture in comparison to non-stress fracture Control groups ($p<0.05$). No associations were shown when stress fracture occurrence was assessed with either *LRP5* SNP rs3736228 or *Wnt16* SNP rs3801387 genotypes ($p>0.05$).

Vitamin D SNPs

An association between *VDR* SNP rs10735810 and increased occurrence of multiple stress fracture injury was shown in homozygotes of the rare f allele, when compared to homozygotes of the common allele combined with heterozygotes ($p<0.05$; Table 3). Stress fracture occurrence was associated with the frequency of the rare allele in cases of multiple stress fracture and in male athletes ($p<0.05$). Those with at least one copy of the rare allele of rs731236 had a greater stress fracture occurrence in cases of multiple stress fractures ($p<0.05$). No significant associations were shown between *VDR* SNPs rs1544410 and rs79752321, GC SNPs rs7041and rs4588, CTR SNP rs1801197 and COL1A1 SNP rs1800012 and stress fracture occurrence ($p>0.05$).

After correcting for multiple comparisons, using the Benjamini and Hochberg false discovery rate test, none of the associations above remained significant.

Discussion

The present study shows that three novel SNPs, in close proximity to *SOST* and *VDR* genes, were associated with stress fracture injury in elite athletes. The sclerostin encoding *SOST* SNP rs1877632, suggested to have a role in the functioning of the *Wnt* signalling pathway (15), was associated with stress fracture injury in elite athletes, and in cases of multiple stress

fracture. The *Wnt* signalling pathway is a predominant regulator of bone metabolism, having a role in the mediation of the differentiation and longevity of osteoblasts, thus positively affecting bone formation (18). The purported role of *VDR* SNPs in the mediation of vitamin D status (17) and transcription activation characteristics of the *VDR* protein (19) may be causative factors in the aetiology of stress fracture injury.

The current study's finding, shows for the first time that *SOST* SNP rs1877632 is associated with stress fracture injury and is in accordance with previous research showing associations between *SOST* SNPs and bone phenotypes including, sclerosteosis (20) and BMD at the lumbar spine (15) and femoral neck (17). That said, the A allele was associated with increased stress fracture risk in the present study, whereas previous research (15) has shown that A allele homozygotes were associated with increased BMD at the lumbar spine in a large cohort of elderly male participants. Dissimilarity in phenotypic and lifestyle characteristics of elite athletes and elderly males make it difficult to directly compare the findings; moreover a reduction in bone formation as a result of ageing has recently been suggested to be due to insufficient *Wnt* activity in response to mechanical loading (20), thus explaining a potential mechanistic reason for these contrasting associations. The mechanisms underlying the increased stress fracture risk in those with the variant A allele are not known, although allelic variation in *SOST* SNPs has been associated with serum sclerostin levels (21) and *SOST* null mice have increased bone formation compared to their wild-type counterparts (23). It is possible that the rare allele of rs1877632 down regulates sclerostin expression, and, as sclerostin inhibits *Wnt* signalling (24), this could result in an increased susceptibility to stress fracture injury due to a reduction in bone formation.

The elite athletes in the present study were all competing in weight bearing sports, which has been associated with high levels of sclerostin (25). Greater sclerostin concentrations are possibly due to the increased number of sclerostin expressing osteocytes located within an athletes' bone mineral (26) compared to a non-athlete, suggesting there may be a mechanostransductive element of sclerostin mediation (26). Increased sclerostin levels are thought to act through a negative feedback loop to limit 'excessive' increases in bone formation as the result of high volume loading (26). Currently, the effect of repeated mechanical loading has on *SOST* expression has been equivocal, but recently it has been shown that osteocyte *SOST* expression is down regulated during exercise (27). The effects of habitual high volume and high magnitude mechanical loading, resulting in transient increases in sclerostin concentrations (25), combined with the SNP rs1877632 genotype, may be the reason for the increase in stress fracture susceptibility.

VDR SNPs rs731236 and rs10735810 were significantly associated with stress fracture injury in the whole cohort, in males, and in cases of multiple stress fracture. The present data in elite athletes showing the variant allele of rs10735810 to be associated with stress fracture injury is in line with previous research in military personnel (9). The confirmation of this SNPs role in stress fracture injury in a separate population that experience different exercise demands, may underline its important role in bone homeostasis and suggests the need for further investigation into the role of this SNPs in the repair and maintenance of bone tissue.

The variant allele of rs731236 was associated with stress fracture injury in cases of multiple stress fractures. Previous studies have shown the rare allele of rs731236 to be associated with decreased bone phenotypes consisting mainly of trabecular bone (12). Despite showing some

agreement with previous research, the role of the *VDR* gene in bone homeostasis and stress fracture prevalence is contentious due to seemingly divergent findings. The absence of any *VDR* SNPs consistently associated with bone phenotypes in GWAS studies suggest the *VDR* SNPs could be a proxy for other functional SNPs, and/or the associations are restricted to certain populations (28). Also, the majority of the candidate gene studies focusing on *VDR* can be characterised by relatively small cohorts, which is particularly the case in those investigating the association with stress fracture prevalence (n=64, 9; n=192, 8), increasing the likelihood of false positives. Although the mechanisms by which *VDR* SNPs influence stress fracture risk remain unclear, the binding of the VDR to $1,25(OH)_2D$ induces absorption of calcium and phosphate for bone mineralisation and homeostatic metabolism (27). Mutations in *VDR* cause vitamin D-resistant rickets, characterised by increased risk of fracture and genu varum (Kristjansson et al., 1993), due to insufficient absorption of calcium and phosphate by the intestine rather than a direct influence on $1,25(OH)_2D_3$ concentrations (Ralston and de Crombrugghe, 2006). McClung and Karl (2010) suggest that allelic variation in polymorphisms within the *VDR* gene can increase the concentration of $1,25(OH)_2D$, which may facilitate bone health and therefore reduce the risk of bone injury. *VDR* null mice have low bone mass characterised by hypocalcaemia, as well as elevated $1,25(OH)_2D_3$ levels, while *VDR* activity has been shown to influence RANKL expression (29), thus offering possible mechanistic explanations for the associations shown.

The number of SNPs showing an association with stress fracture injury in the SFEA cohort (10;11) and in previous research (8;9) highlights the likely complexity of stress fracture aetiology. Alterations in bone remodelling, and mechanotransductive responses to mechanical loading are two plausible explanations for the associations. GWAS have shown two main biological pathways to be related to bone mineral density and fragility fracture (28);

the *RANK/RANKL/OPG* signalling pathway and the *Wnt*-signalling pathway. The majority of SNPs associated with stress fracture prevalence in the present study are either directly related to or have a downstream influence on these pathways, adding substantiation to their role as major regulatory pathways in bone health.

Although the SFEA cohort is the largest to be studied for genetic associations with stress fracture prevalence to date, it is not without its limitations. Whilst heterogeneity in ethnicity, sport type, training loads/stresses and environmental factors (*e.g.*, nutrition/energy availability) are acknowledged as variable factors in the present study, these factors are currently unavoidable given the low number of elite athletes available to participate in such studies and the difficulty in recruiting participants due to perceived disruption of training schedules. Investigations into large numbers of single sport athletes is recommended to confirm our findings, however divergent playing styles and positions with sports make this a challenging undertaking. After correcting for multiple comparisons none of the findings remained significant. The SNPs were not randomly selected, however, and were selected based on existing evidence of a bone phenotypic association with known mechanisms supporting their potential role in stress fracture injury and therefore it is unlikely the findings occurred by chance. The conservative nature and problems with conducting multiple comparison testing (30) increase the chances of a type two error occurring. Further studies are needed to establish the underpinning mechanisms that explain how SNPs are associated with stress fracture injury as it is not clear how allelic variations influence bone adaptations and subsequently escalate stress fracture risk.

In conclusion, SNPs in the vicinity of *VDR* and the *Wnt* signalling pathways are associated with increased stress fracture prevalence in elite athletes. These data suggest an important

role for SNPs in stress fracture susceptibility, possibility through the regulation of bone adaptation to mechanical loading.

Practical implications

- Elite athletes with particular genetic variances may have an increased susceptibility to stress fracture injury.
- Information related to individual variance in *SOST* and *VDR* SNPs may be of use to sport and exercise practitioners in the multifaceted management, treatment and prevention of stress fracture injury.
- The findings suggest that genetic regulators of bone adaptation to mechanical loading are key to stress fracture injury pathophysiology. This should be considered when managing an athlete's training load.

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Table 1. Athlete characteristics for stress fracture and non-stress fracture groups.

Characteristics	Stress fracture (n=125)	Non-stress fracture (n=376)	*P*-value
Age (y)	27.7±7.5	24.4±5.4	<0.01*
Age at stress fracture (y)	19.9±3.9	-	
Height (m)	1.82±0.10	1.81±0.08	0.45
Body Mass(kg)	77.3±14.5	77.8±10.5	0.72
BMI	23.2±2.7	23.7±2.2	0.07
Age at elite (y)	18.2+4.2	17±2.2	<0.01*
Current Training (h/wk)	20±11.3	18.2±10.1	0.12
Current alcohol consumption (units/wk)	5.2±6.9	4.1±6.1	0.15

* was used to denote significance.

Table 2. Association of SNPs with stress fracture injury in elite athletes for the whole cohort, males and multiple stress fractures. (Gene) = closest gene; (p) = p value; (Homo) = homozygote for the variant allele; (Combined with Heterozygote) = homozygote for the variant allele combine with heterozygote; (AF) = allele frequency.

			Whole cohort (n = 518). *P* values			Males (n = 449). *P* values			Multiple Stress Fractures (n= 49). *P* values		
RS Number	Location	Gene	Homo	Combined with Heterozygote	AF	Homo	Combined with Heterozygote	AF	Homo	Combined with Heterozygote	AF
rs1544410	12q13.11	*VDR*	0.46	0.27	0.44	0.39	0.26	0.51	0.10	0.11	0.47
rs731236	12q13.11	*VDR*	0.50	0.36	0.61	0.45	0.53	0.97	0.01*	0.21	0.21
rs7975232	12q13.11	*VDR*	0.62	0.54	0.92	0.39	0.28	0.64	0.50	0.97	0.52
rs10735810	12q13.11	*VDR*	0.17	0.20	0.03*	0.35	0.62	0.21	0.01*	0.00*	0.02*
rs7041	4q12-q13	*GC*	0.50	0.99	0.84	0.71	0.84	0.74	0.53	0.32	0.21
rs4588	4q12-q14	*GC*	0.31	0.22	0.39	0.65	0.09	0.18	0.88	0.88	0.95
rs1801197	7q21.3	*CTR*	0.72	0.74	0.48	0.57	0.53	0.30	0.99	0.88	0.87
rs1800012	17q21.33	*COL1A1*	0.38	0.93	0.57	0.67	0.96	0.81	0.33	0.61	0.94
rs3801387	7q31.31	*WNT16*	0.30	0.45	0.14	0.30	0.29	0.09	0.62	0.40	0.31
rs1877632	17q11.2	*SOST*	0.05*	0.02*	0.04*	0.28	0.12	0.11	0.05*	0.02*	0.05*
rs3736228	11q13.4	*LRP5*	0.46	0.27	0.44	0.82	0.73	0.75	0.10	0.11	0.47

* depicts significant differences ($p<0.05$).

Table 3. Distribution of genotypes between stress fracture and non-stress fracture participants for SNPs showing significant associations in the whole cohort, males only or multiple stress fracture.

SNP	Stress fracture N	Non-stress fracture N	X^2 P value		Stress fracture N	Non-stress fracture N	X^2 P value
Whole cohort							
VDR rs10735810							
FF	49	169		F	148	483	
Ff	50	145		f	90	221	
ff	20	38		Total	238	704	0.3
Total	119	352	0.17				
VDR rs731236							
tt	13	46		t	87	260	
Tt	61	168		T	141	452	
TT	40	142		Total	228	712	0.61
Total	114	356	0.50				
SOST rs1877632							
CC	50	191		C	159	513	
TC	59	131		T	85	207	
TT	13	38		Total	244	720	0.04*
Total	122	360	0.05*				
Male							
VDR rs10735810							
FF	42	150		F	120	429	
Ff	36	129		f	66	195	
ff	15	33		Total	186	624	0.21
Total	93	312	0.35				
VDR rs731236							
tt	9	43		t	66	235	
Tt	48	149		T	112	401	
TT	32	126		Total	178	636	0.97
Total	89	318	0.45				
SOST rs1877632							
CC	43	172		C	128	458	
TC	42	114		T	64	180	
TT	11	33		Total	192	638	0.11
Total	96	319	0.28				
Multiple stress fractures							
VDR rs10735810							
FF	14	169		F	49	483	
Ff	21	145		f	45	221	
ff	12	38		Total	94	704	0.02*
Total	47	352	0.01*				
VDR rs731236							
tt	3	46		t	37	260	
Tt	31	168		T	49	452	
TT	9	142		Total	86	712	0.21

	Total	43	356	0.01*				
SOST rs1877632								
	CC	15	175		C	58	493	
	TC	28	143		T	40	229	
	TT	6	43		Total	98	722	0.05*
	Total	49	361	0.05*				

* depicts significant differences ($p<0.05$). Values have not been adjusted for multiple comparisons.