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Origins and functional consequences of somatic mitochondrial DNA mutations in human cancer

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2 **human cancer**

3

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49 **ABSTRACT**

50 Recent sequencing studies have extensively explored the somatic alterations present in the

51 nuclear genomes of cancers. Although mitochondria control energy metabolism and apoptosis,

52 the origins and impact of cancer-associated mutations in mitochondrial DNA (mtDNA) are

53 unclear. Here, we analysed somatic alterations in mtDNA from 1,675 tumors across 31

54 histologies. We identified 1,907 somatic substitutions, which exhibited dramatic replicative

55 strand bias, predominantly C>T and A>G on the mitochondrial heavy strand. This strand-

56 asymmetric signature differs from those found in nuclear cancer genomes but matches the

57 inferred germline process shaping primate mtDNA sequence content. Numbers of mtDNA

58 mutations showed considerable heterogeneity across tumor types. Missense mutations were

59 selectively neutral and often gradually drifted towards homoplasmy over time. In contrast,

60 mutations resulting in protein truncation undergo negative selection and were almost exclusively

61 heteroplasmic. Our findings indicate that the endogenous mutational mechanism has far greater

62 impact than any other external mutagens in mitochondria, and is fundamentally linked to mtDNA

63 replication.

64

INTRODUCTION

All cancers result from somatic mutations in their genomes. Beyond the ~3,200Mb of nuclear genomic DNA, human cells have hundreds to thousands of mitochondria present in every cell, each carrying one or a few copies of the 16,569bp circular mitochondrial genomes (Koppenol et al., 2011; Legros et al., 2004; Smeitink et al., 2001). In addition to their role in cellular energy balance through oxidative phosphorylation, mitochondria are involved in many essential cellular functions including modulation of oxidation-reduction status, contribution to cytosolic biosynthetic precursors and initiation of apoptosis. Mitochondria in eukaryotic cells evolved by endosymbiosis from a free-living α-proteobacterium (Gray et al., 1999). Over two billion years of co-evolution, many ancestral mitochondrial genes have transferred to the nucleus (Calvo and Mootha, 2010; Falkenberg et al., 2007; Wallace, 2012). What remains in the mitochondrial genome is distinctive for the striking asymmetry between the two complementary mtDNA strands in terms of nucleotide content and gene distribution (Andrews et al., 1999). The heavy (H) strand is guanine-rich (C/G=0.4) and is the template from which most mitochondrial proteins (12 out of 13) are transcribed, whereas only one protein-coding gene, *MT-ND6*, is transcribed from the correspondingly cytosine-rich light (L) strand.

Mutations in the mitochondrial genome cause inherited disease (Chinnery, 1993), with a maternal inheritance pattern because only eggs contribute mitochondria to the zygote. The penetrance of inherited mitochondrial disease is determined stochastically by both the random assortment of mutated versus wild-type mitochondrial genomes during meiosis and random drift during the early cell divisions after fertilization. In cancer, the role of somatically acquired mtDNA mutations is controversial. Although cancer-specific mutations have been previously

88 reported (Brandon et al., 2006; Chatterjee et al., 2006; He et al., 2010; Larman et al., 2012;

89 Polyak et al., 1998), the limited sample size or poor sensitivity of capillary sequencing for

90 heteroplasmic mutations have not allowed a comprehensive analysis of the mutational signatures

91 of mitochondrial mutations nor their likely functional significance. It has long been proposed that

92 mitochondria might contribute to cancer development given their fundamental importance to

93 cellular biology (Wallace, 2012). Previous reports suggested mitochondrial somatic mutations

94 might be under positive selection and thus contribute to cancer development, but the small

95 numbers of reported mutations render this conclusion uncertain (Brandon et al., 2006; Chatterjee

96 et al., 2006; Larman et al., 2012; Schon et al., 2012). Nonetheless, the hypothesis of functionally

97 relevant mitochondrial mutations is an appealing one because cancer cells have greatly increased

98 energy demands over normal cells, and demonstrate a switch from aerobic glycolysis in

99 mitochondria to lactic acid fermentation in the cytosol (the Warburg effect) (Hanahan and

100 Weinberg, 2011; Koppenol et al., 2011).

101

102 In each cell cycle, the replicating genome is at risk of *de novo* mutations, which can promote the

103 development of cancer. These mutations may be generated by intrinsic cellular errors during

104 DNA replication or repair or through exposure to mutagens, such as reactive oxygen species,

105 tobacco smoke and ultraviolet light (Pleasance et al., 2010a; Pleasance et al., 2010b). Recently,

106 >20 mutational signatures operative in cancers have been identified in the nuclear genome

107 (Alexandrov et al., 2013). Whether any of these mutational processes also affect the

108 mitochondrial genome has not been studied. Furthermore, whether there are mtDNA-specific

109 mutational processes in somatic cells remains unclear, although the many unique features of

110 mtDNA replication and repair, coupled with the high concentration of reactive oxygen species

111 generated by the electron transport chain, could be associated with distinctive mutation

112 signatures.

113

114 In this study, we compare 1,675 cancer and paired-normal mtDNA sequences across 31 tumor

115 types using massively parallel DNA sequencing technologies to obtain a systematic and unbiased

116 catalogue of somatic mitochondrial mutations. We find that mtDNA mutations are almost

117 exclusively the product of a mutational process that is specific to mitochondria and probably

118 linked to the unique mechanism of genome replication these organelles employ. We find no

119 evidence for positive selection of mitochondrial mutations during oncogenesis, suggesting that

120 they confer no clonal advantage on the nascent cancer cells.

121

RESULTS

mtDNA Sequencing and Mutation Calling

We extracted the mtDNA sequences from 704 whole-genome and 971 whole-exome sequencing data generated on primary cancers and compared them with mtDNA sequences from their matched normal samples. Given the abundance of mtDNA per cancer cell, a standard coverage of 30-40x in the nuclear genome provides significantly greater coverage of the mitochondrial genome (average read-depth = 7901.0x), enabling accurate identification of somatic mutations including rare heteroplasmic variants. We also assessed whether whole exome sequencing could be used to identify mtDNA mutations from off-target reads derived from the mitochondrial genome. We found an average read-depth of 92.1x across the mitochondrial genome in exome studies. From 139 samples in which we had both exome and whole genome sequencing data, the overall read depths correlated strongly (R^2=0.59, Figure 1-figure supplement 1) as did variant allele fractions for mtDNA somatic mutations (R^2=0.97, Figure 1-figure supplement 2). Validation experiments suggested the sensitivity of whole-exome sequencing for detection of mtDNA somatic mutations to be 71.4% compared to whole-genome sequencing (Figure 1-figure supplement 3 and Materials and Methods, "Off-target mtDNA reads in whole-exome sequencing" and "DNA cross-contamination").

To reduce potential false-positive calls of mtDNA somatic mutations, we only report variants called with an allele fraction of >3%. This eliminates the risk of miscalls due to mtDNA-derived pseudogenes in the nucleus (NuMTs) because mtDNA copy numbers are 100-1000 times higher than nuclear genomes in human somatic cells and the sequence homology between mtDNA and NuMTs presented in the human reference genome is generally <95% (in 96 out of 101 NuMTs

145 with length greater than 300bp). Furthermore, pairwise comparison between cancer and matched

146 normal mtDNAs from the same individual further minimizes the contamination of NuMTs in the

147 mutation calling.

148

149 **The Catalog of mtDNA Somatic Mutations**

150 In total, 1,675 tumor-normal pairs across 31 tumor types were analysed (Table 1 and

151 Supplementary file 1). For 61 of these patients, we had sequencing data available from multiple

152 sites of the primary cancer, several time-points or matched primary cancers and metastases (a

153 total of 73 such cancer samples), allowing us to study the timing of mtDNA mutations in cancer

154 evolution (Supplementary file 1). We identified 1,907 somatic mtDNA substitutions (Figure 1

155 and Supplementary file 2). In contrast to inherited polymorphisms (n=38,706, available at

156 Supplementary file 2), which were almost always homoplasmic in both the cancer and

157 counterpart normal, the variant allele fractions (VAFs) of these somatic substitutions were highly

158 variable in the cancer, ranging from our detection threshold (3%) to homoplasmy (100%). Of

159 these 1,907 somatic substitutions, 1,209 (63.4%) were not registered in the databases of mtDNA

160 common polymorphism (Ingman and Gyllensten, 2006; Levin et al., 2013). In comparison, when

161 we examined substitutions found in both the tumor and the normal samples from a patient, only

162 21 (0.05%) were not registered in the polymorphism databases, a significantly different fraction

163 from the tumor-only variants ($p<10^{-10}$; chi-squared test). We found 595 (31.2%) recurrent

164 mutations that can be collapsed onto 246 mtDNA positions, which is a 6.9-fold higher level of

165 recurrence than expected by chance ($p<10^{-10}$). This suggests that the generation or fixation of

166 mtDNA mutations is not random, but influenced by factors such as the underlying mutational

167 process or positive selection.

168

169 Of the 1,675 cancer samples, 976 (58.3%) harbored at least one somatic substitution and 521

170 (31.1%) had multiple substitutions, ranging from 2 to 7 (Figure 2A). In those with multiple

171 substitutions, 72 pairs of mutations were sufficiently close to phase (Nik-Zainal et al., 2012b)

172 such that we could determine whether they were linked on the same mtDNA genome or were on

173 different copies. We found that 45 (62.5%) pairs of mutations were linked on the same mtDNA

174 genome (Supplementary file 3 and Figure 2-figure supplement 1). Furthermore, of these linked

175 mutations, 33 showed a clear temporal order: that is, one mutation was demonstrably subclonal

176 to the other. This is rather unexpected, since each somatic cell has 100-1,000 copies of the

177 mitochondrial genome, and we might anticipate that random mutations would, on average, affect

178 different copies. That many pairs of mutations are phased on the same mtDNA genome and yet

179 show a clear subclonal relationship suggests that they occur sufficiently separated in time to

180 allow the mitochondrial genome carrying the earlier mutation to drift towards a substantial

181 fraction of all genomes in that cell before the second mutation occurs, consistent with a previous

182 report (De Alwis et al., 2009).

183

184 The number of somatic mtDNA substitutions varied significantly according to tumor type

185 ($p=4.4\times10^{-52}$) after correcting for confounding variables such as sequencing coverage: gastric,

186 hepatocellular, prostate and colorectal cancers had the highest numbers of mtDNA substitutions

187 (Figure 2B). In contrast, hematologic cancers (acute lymphoblastic leukaemia,

188 myeloproliferative disease and myelodysplastic syndrome) had fewer mutations. Several possible

189 explanations could underpin these differences across tumor types. It could be that the mutation

190 rates differ across cell lineages; it could be that selection pressures shape the number of

191 mutations; or the number of mtDNA genome generations could differ across cell lineages. Of

192 these explanations, we believe that the second is unlikely because, as we shall see, positive

193 selection is not a major component of mitochondrial mutations. Interestingly, we find a positive

194 correlation between the number of mtDNA somatic mutations and age at diagnosis in breast

195 cancers (p=0.0004; Figure 2C), in keeping with the idea that the number of mitochondrial

196 generations is linked to mutation burden. The mutational burden of an established cancer

197 represents the accumulated variation acquired in the lineage of cell divisions from fertilized egg

198 to transformed cell, and will include events acquired in normal development and homeostasis as

199 well as those acquired during tumorigenesis (Stratton et al., 2009). Interestingly, mtDNA

200 mutations have been found at high rates in normal colonic crypt cells (Ericson et al., 2012;

201 Taylor et al., 2003). Given that we find high burdens of mutations in colonic tumors as well, the

202 differences we see across tumor types may arise from pre- or post-transformation differences in

203 mtDNA burden across tissues.

204

205 **Extracting mtDNA Mutational Signatures**

206 With respect to signatures of somatic substitutions, C>T and T>C transitions constituted 90.9%

207 of all the 1,907 substitutions (Figure 1) among the six classes of possible base substitutions. To

208 characterize this aggregated signature of mtDNA cancer specific mutations in more detail, we

209 looked for the presence of mtDNA strand bias between the complementary H and L strands of

210 mtDNA. The two main substitution classes showed an extreme level of mtDNA strand bias.

211 84.1% of the C>T transitions were on the H strand. This level of strand bias occurred despite the

212 fact that cytosine is 2.4-fold less common on the H than the L strand, so the C>T substitution

213 rate is 12.6-fold higher on the H strand. By contrast, 76.8% of the T>C transitions were on the L

214 strand despite its lower thymine content (1.3-fold less than the H strand). This implies that the

215 T>C mutation rate on the L strand is 4.2-fold higher than on the H strand.

216

217 We then examined the sequence context in which these mutations occurred by examining the

218 bases immediately 5' and 3' to the mutated bases. This generates 96 possible mutation classes

219 (the 6 substitution classes multiplied by the 16 combinations of immediate 5' and 3'

220 nucleotides). Both C>T and T>C mutations showed highly distinctive sequence contexts. $C_H > T_H$

221 substitutions (i.e. C>T mutations on the H strand) were enriched for the Np$\underline{C}$pG trinucleotide

222 context (8- to 15-fold more frequent than expected by chance; Figure 3A). By contrast, $T_L > C_L$

223 substitutions (i.e. T>C mutations on the L strand) showed 5- to 8-fold enrichment in Np$\underline{T}$pC.

224 This strand-asymmetric mutational signature is not similar to any of the 21 cancer associated

225 mutational signatures recently identified from the nuclear DNA of 30 different cancer types

226 (Alexandrov et al., 2013).

227

228 Of the 18 tumor types that presented at least 25 mtDNA somatic substitutions in this study, the

229 mutational signatures were broadly consistent across tumor types (Figure 3B), with the exception

230 that multiple myeloma had a somewhat higher rate of $T_H > C_H$ changes than other histologies

231 (p=8.1×10^{-6}). Thus, in contrast to the mutational signatures found in nuclear genomes, where

232 there is striking heterogeneity both across tumor types and across individuals within a tumor type

233 (Alexandrov et al., 2013), the mutational profile in the mitochondrial genome of somatic cells is

234 remarkably homogeneous.

235

Replication-coupled Mutational Process in Mitochondria

The major known cause of mutational strand bias in nuclear DNA is transcription-coupled nucleotide excision repair, where DNA lesions on the transcribed (non-coding) strand are more frequently repaired (Alexandrov et al., 2013). However, we find that the strand bias always favors $C_H > T_H$ and $T_L > C_L$ whether the gene is transcribed from the H strand or from the L strand (Figure 3-figure supplement 1). This is not compatible with transcription-coupled repair, for which the direction of strand bias is fundamentally dictated by which strand is transcribed.

Instead, the mtDNA mutational strand bias reported here appears to be driven by differences in replication between the two strands. mtDNA replication harbors substantial strand asymmetry between the H and L strands: mtDNA replication initiates from an origin of replication (O_H) in the D-Loop, with the nascent H and the L strand replicating as leading and lagging strand, respectively (Clayton, 1982; Falkenberg et al., 2007; Holt and Reyes, 2012). We observed that C>T substitutions were prevalent in the leading (heavy) strand, whereas T>C substitutions were found in the lagging (light) strand (Figure 1). Remarkably, this strand bias was reversed in the D-Loop itself (Figures 1 and 3C), further suggesting that the mtDNA somatic mutations are replication-coupled: according to a recently proposed bidirectional model of mtDNA replication (Holt and Reyes, 2012; Yasukawa et al., 2006; Yasukawa et al., 2005), mtDNA replication is also able to initiate from the so-called Ori-b site, typically located around genomic position 16,197 and proceeds on both strands away from the origin (Figure 1). Replication of the nascent H strand continues unimpeded like the traditional model, but the nascent L strand terminates at the so-called O_H site, typically around mtDNA position 191bp. Under this model, then, the

258 leading and lagging strand are reversed in the few hundred base-pairs of the D-Loop, which is

259 consistent with the reversed mutational signature in this region (Figures 1 and 3C).

260

Equivalent Mutational Signature during Human mtDNA Evolution

262 It is not entirely straightforward to infer the mutational signatures operating on the mitochondrial

263 genome in the germline. *De novo* mutations are generally rare and often discovered because they

264 cause disease; distinguishing the ancestral base and the derived base is challenging for single

265 nucleotide polymorphisms; and comparative mtDNA genomics across species extends over

266 considerable evolutionary time. In contrast, because ancestral and derived states are defined for

267 tumor-normal pairs, a much clearer picture emerges of the somatic mtDNA mutation signature.

268 We therefore assessed whether the signature that emerges for somatic mitochondrial mutations

269 could extend to explain sequence composition of the human mtDNA genome.

270

271 It appears that the mutational mechanism which has generated the $C_H>T_H$ and $T_L>C_L$ signature in

272 cancer mtDNA is equivalent to the one that has been operating during evolution of human

273 germline mtDNA (Nikolaou and Almirantis, 2006). This manifests as the depletion of certain

274 codons in the reference human mtDNA sequence through the action of the $C_H>T_H$ and $T_L>C_L$

275 mutational process over time (Figure 4A). For example, the GC<u>G</u> triplet codon (Alanine) appears

276 to have been replaced by its synonymous GC<u>A</u> codon (due to $C_H>T_H$ ($G_L>A_L$)), with the former

277 being 15.8-fold less frequently observed in the 12 mtDNA protein-coding genes that are

278 transcribed from the H strand (and encoded on the L strand). All 32 synonymous codon pairs

279 present the same tendency. Consistent with this interpretation, the gene transcribed from the L

280   strand (*MT-ND6*) demonstrates the opposite direction of skew. Further analyses of mtDNA

281   codon usage from seven animal species suggest that the $C_H > T_H$ and $T_L > C_L$ mutational pressure

282   may be characteristic of vertebrates, and primates in particular (Figure 4-figure supplement 1).

283

284   To quantify whether the somatic mutational signature we have defined can fully explain the

285   trinucleotide frequency of human mtDNA, we performed evolutionary simulations. First, we

286   simulated the evolution of a random DNA sequence under the mutational signature described

287   here. By mutational pressure alone, the random sequence starts losing certain hyper-mutable

288   trinucleotides until eventually reaching a stationary sequence composition. The actual sequence

289   composition of the human mitochondrial genome strongly resembles this stationary distribution

290   (Pearson's r=0.83; p<0.0001; Figure 4-figure supplement 2). In a second simulation, a random

291   sequence encoding the exact amino acid sequence of the reference mitochondrial genome was

292   evolved by synonymous mutations under the observed mtDNA signature until reaching a

293   stationary sequence composition (mutation-selection equilibrium). These simulations also

294   eventually approximate the observed human mitochondrial genome (Pearson's r=0.96, p<0.0001;

295   Figure 4B). These analyses strongly suggest that the mitochondrial mutation signature observed

296   in cancer cells closely reflects the mutation signature active in the germline, which has

297   continuously shaped the mitochondrial genome during human evolution.

298

299   **Negative Selection on Truncating mtDNA Mutations and tRNA Anticodons**

300   Next, we assessed the functional impact of somatic mtDNA mutations. Of the 1,907

301   substitutions, 1,153 (60.5%) were in the 13 protein-coding genes. These include 63 nonsense, 4

302   stop-lost, 878 missense and 208 silent substitutions (Supplementary file 2). In addition, out of

303   251 indels we observed, 110 occurred within protein-coding genes (Supplementary file 2). Of the

304   missense substitutions, 245 (27.9%) were recurrent, affecting 107 distinct mtDNA sites.

305   Although this very high level of mutation clustering could, at first sight, be interpreted as

306   evidence for positive selection, we found that silent substitutions were also frequently recurrent

307   (28 recurrent variants in 13 mtDNA sites), with no substantial difference in recurrence rates

308   between silent and missense mutations (p=0.19; Figure 5-figure supplement 1). We believe this

309   recurrence to be the consequence of a high mtDNA mutation rate with restricted mutational

310   signature ($C_H > T_H$ and $T_L > C_L$). Independently recurring mutations in human germline mtDNA

311   are well described across human evolution (Levin et al., 2013).

312

313   The ratio of somatic missense to silent substitutions (Rms:s) is apparently higher (4.2, 878/208)

314   than that observed for cancer-associated somatic mutations in nuclear DNA (generally around

315   2:1 to 3:1 across tumor types (Greenman et al., 2007; Nik-Zainal et al., 2012a). At face value,

316   this again could be interpreted as evidence for positive selection. However, as described above,

317   the somatic mtDNA mutational signature shows extreme strand asymmetry and the same

318   mutational signature has been operative in the germline over evolutionary time. Thus, the

319   dominant mutational signature has already acted on potentially synonymous sites in the

320   mitochondrial genome (Figure 4A), meaning that any new somatic changes are much less likely

321   to be silent. In keeping with this, a dN/dS ratio (Materials and Methods) calculated taking into

322   account both the mutational signature and the mtDNA codon usage revealed that missense

323   mutations accumulate at a frequency very close to that expected under neutrality (dN/dS=1.21;

324   95% confidence interval, 1.015 - 1.434; p=0.031). This indicates that despite the apparent high

325 ratio of missense to silent mutations, the vast majority of mtDNA mutations are passengers with

326 no convincing evidence suggesting the existence of driver mitochondrial DNA mutations.

327 Additional gene-by-gene analysis further revealed that no single gene had a higher than expected

328 rate of missense or nonsense mutations (Supplementary file 4).

329

330 For nonsense substitutions and frameshift indels, we observe a somewhat different picture.

331 Taking into account the mutation signature and amino acid composition of the mitochondrial

332 genome, the overall ratio of nonsense mutations to silent mutations is exactly that expected by

333 chance (dNonsense/dS=1.004; 95% confidence interval, 0.699-1.443; p=0.98). However, while

334 missense and silent substitutions exhibited equivalent variant allele fractions (average VAFs;

335 40.1% and 40.9%, respectively; p=0.8), nonsense substitutions presented significantly lower

336 VAFs (average 26.4%; p=6x10^{-5}), as did frameshift indels (average 25.0%; p=2x10^{-3}; Figure

337 5A). Taken together, these data suggest that nonsense mutations occur at the expected rate given

338 the underlying mutational process. However, while silent and missense substitutions frequently

339 achieve high allele fractions in tumor cells due to the effects of random drift, there are

340 significantly greater constraints on mitochondrial genomes carrying protein-inactivating

341 mutations. The inference here is that cancer cells carrying such deleterious mutations at or near

342 homoplasmy are at a selective disadvantage, and hence do not contribute to clonal expansions,

343 underlining the importance of functional mitochondria to cancer cells. The extent of such

344 disadvantage may vary according to tumor type: for example colorectal cancers show less

345 negative selection compared to breast cancers (p=0.028; Figure 5-figure supplement 2).

346

347   We found 171 mtDNA substitutions in mitochondrial tRNA sequences, which is very similar to

348   the expected number (168.2, p=0.82) from the mutational signature. Interestingly, none of the

349   substitutions was located in the trinucleotide anticodon site of the tRNA (expected number=7.6,

350   p=0.006). This suggests mutations in tRNA anticodons confer a similar selective disadvantage as

351   protein-truncating mutations, presumably because such mutations would lead to systematic

352   erroneous aminoacylation of nascent proteins during translation of the relevant codon.

353

354   Next, we assessed whether any specific somatic mutations showed evidence of positive selection.

355   Out of the 1,907 somatic substitutions, 16 (0.8%) overlapped with known disease-associated

356   mtDNA mutations, such as mutations frequently detected in MELAS (Mitochondrial

357   Encephalomyopathy, lactic acidosis, and stroke-like episodes) and LHON (Leber hereditary

358   optic neuropathy) (Supplementary file 2). In addition, ten mutations within mitochondrial

359   protein-coding, tRNA and rRNA genes showed significantly higher recurrent rate than expected

360   from background mutational signature (Supplementary file 5). However, it remains unclear

361   whether this high recurrence reflects positive selection, because any factors not included in our

362   background model of the mutational process, such as local mutation hotspots, could also explain

363   a mild excess of mutations at a given nucleotide.

364

mtDNA Mutations across Tumor Evolution

365

366   We investigated whether somatic mtDNA mutations are more likely to become homoplasmic

367   later in tumor evolution by assessing paired cancer samples, either primary and metastasis

368   (breast, colorectal and prostate) or primary and relapse (myeloma) (Figure 5B and

369   Supplementary file 1). As mentioned earlier, 73 late (metastasis or relapse) cancer samples were

370   sequenced in addition to the primary tissues. Among the mtDNA mutations identified in either of

371   the paired cancer samples, a number of different patterns were observed. There were mutations at

372   high VAF in the primary not found in the metastasis (n=49); mutations in the metastasis not

373   found in the primary (n=49); and shared mutations (n=71) at high or low VAF, sometimes with

374   evidence for drift (VAF difference > 0.2) between the two samples (n=25). These data,

375   particularly the mutations found in the metastasis only, suggest that mitochondrial mutations can

376   occur throughout the time course of tumor evolution, and still drift to homoplasmy with

377   appreciable frequency, as suggested previously (Coller et al., 2001). To assess the plausibility of

378   this conclusion, we modeled the dynamics of mtDNA mutations based on a few simplifying

379   assumptions (Materials and Methods, Evolutionary dynamics of neutral mitochondrial

380   mutations). We find that the expected number of neutral mitochondrial mutations drifting to

381   homoplasmy increases linearly with mutation rate and number of cell divisions. Based on a

382   mutation rate of 10^{-7}/base-pair/generation (Coller et al., 2001; Hudson and Chinnery, 2006), this

383   leads to an average ~1 homoplasmic mutation for every 1,000 cell generations.

384

385   **Origins of mtDNA somatic mutations**

386   We also explored whether the mutational forces that are so critical to shaping the nuclear

387   genome during tumor evolution could affect the mitochondrial genome. In cancers associated

388   with exogenous mutagens, such as tobacco-associated lung cancer and ultraviolet light-

389   associated melanomas, we found no evidence of the mutational signatures characteristic of these

390   carcinogens among the mtDNA mutations (Figure 5C, Figure 5-figure supplement 3). Moreover,

391   *BRCA1* and *BRCA2* mutations showed no evident influence on mitochondrial genomes in breast

392   cancer (Figure 5C), in contrast to their effects on nuclear genomes exhibiting an even

393   distribution of mutations across all trinucleotide contexts (Alexandrov et al., 2013; Nik-Zainal et

394   al., 2012a). Taken together, it appears that the primary mtDNA mutational process is endogenous

395   to mitochondria and is very different to those operating in nuclear DNA. It is surprising that the

396   endogenous mutational process has far greater impact than any external forces, as the

397   physicochemical interactions of ultraviolet light or the chemicals in cigarette smoke with DNA

398   should be similar in both genomes. The simulations described above suggest the major

399   explanation to be that the endogenous mutation rate is several orders of magnitude greater than

400   that expected for exogenous carcinogens, thus swamping any signal.

401

DISCUSSION

In theory, there are two potential sources of the mtDNA variants we observed in cancer tissues: (1) somatically acquired, or *de novo*, mutations accumulated during the cancer clone's lineage of cell divisions from the fertilized egg or (2) low-level heteroplasmic mtDNA present in the oocyte (therefore maternally inherited) amplified in cancer but lost from normal tissue by random drift (Freyer et al., 2012; He et al., 2010; Payne et al., 2013). We believe the majority of the variants we find are genuinely acquired somatically. First, of the 45 pairs of somatic mutations phased together on the same copy of the mtDNA genome, at least 33 (73.3%) showed a clear subclonal relationship and therefore their occurrence is separated in time, or apparently somatic. Secondly, 63.4% of our substitutions were not previously reported as germline polymorphisms. This is a much higher rate than reported for equivalent analyses on heteroplasmic variants in non-cancer samples (8/37; 21.6%) (Li et al., 2010), although methodological differences may somewhat contribute to this apparent difference (Avital et al., 2012; Goto et al., 2011). Thirdly, if the variants were due to inherited, low-level heteroplasmy, we would not expect to see such variation across tissue types, since all tissue types derive from the fertilized egg. It is difficult to distinguish whether the variants we observe occur before or after the initiating driver mutations that herald tumorigenesis, but our analysis of paired samples does suggest that they can occur both early and late. Given the homogeneity of the mutational signature across tumor types and its inferred resemblance to the germline mtDNA mutational process, we would hypothesise that new mutations occur at a fairly constant and high rate per mitochondrial genome replication throughout all cell divisions.

424   On the basis of the mutational signature observed here, somatic substitutions are unlikely to be

425   attributable to reactive oxygen species (ROS), as previous reports have suggested (Larman et al.,

426   2012; Polyak et al., 1998). Guanine oxidation by ROS predominantly causes G:C>T:A

427   transversion (Delaney et al., 2012; Thilly, 2003), which constitute only 4.0% of the mutations in

428   our data (Figure 5C). Instead, we propose three replication-coupled mechanisms that can explain

429   the strand asymmetric $C_H>T_H$ and $T_L>C_L$ mutational signature and define a model of the mtDNA

430   mutational process (Figure 5D). First, the parent H strand, displaced and single-stranded during

431   mtDNA replication (Holt and Reyes, 2012), could be more prone to cytosine deamination

432   (generating $C_H>T_H$) and/or adenine deamination (Faith and Pollock, 2003; Lindahl, 1993;

433   Saccone et al., 1999) (generating $T_L>C_L$). Secondly, endogenous mtDNA polymerase (*POLG*)

434   replication errors (Zheng et al., 2006) (which show the pattern of C>T and A>G substitutions)

435   could be preferentially generated on the leading strand (Pavlov et al., 2002). Thirdly, there may

436   be differences between the efficiency of repair between the leading and lagging strand (Pavlov et

437   al., 2003). Further, the mutation pattern reported here is consistent with the hypothesized

438   bidirectional initiation of mtDNA genome replication (Holt and Reyes, 2012; Yasukawa et al.,

439   2006; Yasukawa et al., 2005).

440

441   It appears that most of the mtDNA missense mutations we observe become fixed in tumor

442   progenitor cells without distinct physiological advantage. All the statistical testing performed in

443   this study – variant allele fraction comparison across different categories of somatic mutations,

444   number of recurrent mutations and dN/dS ratio – suggest that mtDNA somatic substitutions

445   accumulate largely neutrally. This is not different from previous observations in nuclear

446   genomes: of the thousands of somatic mutations found in a cancer genome, many fewer than a

447   hundred are believed to confer a selective advantage to the cancer cell (Stratton et al., 2009). In

448   contrast, protein-truncating mutations showed evidence of negative selection, at the level of

449   constraints on the allele fraction achieved. The implication of this is that the inactivating

450   mutations occur at an appreciable rate, but the fraction of mitochondrial genomes per cell

451   carrying these variants cannot increase beyond a certain limit without impairing the selective

452   fitness of that cell. Having a sizable number of mitochondria with fully intact proteome remains

453   critical to the fitness of a cancer cell.

454

MATERIALS AND METHODS

Sequencing data

All the sequences were generated by Illumina platforms (either Genome Analyzer or HiSeq 2000). With respect to TCGA data, we downloaded aligned bam files through UCSC CGHub (http://cghub.ucsc.edu). Sequencing reads were aligned on the human reference genome build 37 (GRCh37) and human reference mtDNA sequence (revised Cambridge reference sequence, rCRS (Andrews et al., 1999)), mainly by BWA alignment tool. Samtools (Li and Durbin, 2009) and Varscan2 (Koboldt et al., 2012) were used for manipulating sequence reads and for calling somatic mutations, respectively. Sequence data have been deposited in the European Genome-phenome Archive (EGA; https://www.ebi.ac.uk/ega/home ; study accession # EGAS00001000968; dataset accession numbers EGAD00001001014 for primary samples and EGAD00001001015 for metastatic samples). Sample accession numbers are available in the Supplementary file 6.

Off-target mtDNA reads in whole-exome sequencing

Most of the currently available whole-exome capture kits, including Agilent Technologies SureSelect Human All Exon 50Mb (Agilent Technologies Inc.) used mostly in this study, do not target mtDNA genes (Falk et al., 2012). However, because of the abundance of mtDNA in human cells (100-100,000 copies per cell), it is expected that a number of mtDNA fragments could be off-target captured. We checked whether the amount of off-target mtDNA reads was sufficient for mtDNA variant detection. Whole-exome sequencing (normal samples) generated by CGP (n=855), WUGSC (Washington University Genome Sequencing Center; n=140) and BCM (Baylor College of Medicine; n=85) contained ~100 off-target mtDNA reads per 1M autosomal reads (Figure 1-figure supplement 4). We concluded these could be sufficient for the downstream analyses, because ordinary 10Gb whole-exome data would provide ~60x read-depth for mtDNA here. However, whole-exome data sequenced by BI (Broad Institute; n=436) included far less, ~3 off-target mtDNA per 1M autosomal reads, which would show ~2x mtDNA read-depth per 10Gb exome sequencing (Figure 1-figure supplement 4). It may be due to "improved" exome-capture protocols by BI to increase the DNA-capture efficiency and on-target rate (Fisher et al., 2011). Therefore, we did not include whole-exome data sequenced from BI for further analysis.

139 samples were sequenced by both whole-genome and whole-exome sequencing. From these, we compared the amount of off-target mtDNA reads from whole-exome sequencing with that of whole-genome sequencing. It showed clear positive linear correlation (Figure 1-figure supplement 1).

DNA cross-contamination

Given the abundance of mtDNA in the cancer cells, 1-214x coverage cancer whole nuclear genome sequencing provides extensive coverage of mtDNA (average read-depth = 7901.0x; table S2) enabling accurate identification of somatic mutations, even if heteroplasmic. Whole exome sequencing data were also included because off-target reads provided sufficient coverage (average read-depth = 92.1x) to analyze mtDNA mutations.

491

492 This high coverage of mtDNA, especially from whole-genome sequencing, permitted us to identify heteroplasmic

493 variants (our detection threshold was 3%; see "Variant calling" for more details). However, because sample-swaps

494 and/or DNA cross-contaminations would definitely generate false-positive somatic variants, we filtered out

495 suspicious DNA samples as described below.

496

497 1) Major sample-swaps

498 A subset of tumor and normal sequencing pairs, of which the nuclear genotypes were not matching with each other,

499 were removed from further analyses. We randomly selected 320 common single-nucleotide polymorphism sites on

500 the 22 human autosomes, of which the minor allele frequency is ~50% (45-55%) according to The 1000 Genomes

501 Project (Genomes Project et al., 2010). Of the 320 sites, homozygous positions in normal tissues (where showed >90%

502 variant allele fraction (VAF) with bases Q score>20) were compared with the corresponding genotypes in the

503 counterpart cancer. Sample pairs were removed if the genotype mismatch rate was greater than 0.1 ($\frac{Nhet+Nwt}{Nhom+Nhet+Nwt}$;

504 N_{het}, number of heterozygote positions; N_{hom}, number of homozygote positions; N_{wt}, number of wildtype

505 positions)(Figure 1-figure supplement 5A). We note 0 is expected for the rate when genotyping is perfect and

506 sample pairs are from the same individual. By contrast, 0.5 is expected when samples were from different

507 individuals.

508

509 2) Minor cross-contamination

510 We estimated DNA cross-contamination levels with the VAF of autosomal homozygous SNPs genotyped from the

511 common (population minor allele frequency ~50%) SNP sites. Theoretically, if there is no sequencing (and mapping)

512 error, all the homozygote SNP sites in pure samples should present 100% VAFs. However, when samples are

513 contaminated, corresponding VAFs are reduced because the contaminant has only a ~25% of chance of having

514 homozygote SNPs on the same site. Therefore, minor contamination levels (C) of each cancer sequencing data were

515 estimated as below:

516

$$C = 2 \times \frac{\sum(RCwt) - Ne}{\sum(RDhom) - Ne}$$

517

518 , where RD_{hom} is sequencing read-depth, RC_{wt} is readcount of wildtype alleles and Ne is number of sequencing

519 errors on each autosomal homozygote SNP site. For high accuracy, we only counted base with sufficient quality

520 score (Q>20). In order to estimate Ne, we assumed a conservative rate (sequencing error rate = 0.001). We

521 considered sites covered by at least 10 reads and 90% VAF (Figure 1-figure supplement 5B). 95% confidence

522 intervals of cross-contamination levels were calculated using binomial distribution.

523 In order to clear somatic variants, here we made the very conservative assumption that somatic variants present in

524 excess of 5-times of the 95% upper limit of C levels were true somatic rather than false positives by low-level of

525 cross-contamination.

526

527 3) Germline polymorphisms and back mutations

528 We further checked samples for contamination using known mtDNA polymorphisms. Because human mtDNA is

529 small (16,569 bp) and extensively explored previously, most of germline mtDNA polymorphisms are already known.

530 For example, 97.7% of the 39,036 inherited substitutions were known polymorphisms in the mtDB database

531 (Ingman and Gyllensten, 2006). Therefore, when a tumor sample is contaminated by other samples, many somatic-

532 like mtDNA substitutions by contaminants are likely to be overlapped with known mtDNA polymorphisms.

533 At the same time, low-level contamination would generate excessive back mutations, which appeared to reverse

534 germline common polymorphisms into wildtype alleles. Taken together, both the number of somatic substitutions

535 known in mtDB and number of back mutations can be good indicators for mtDNA cross-contamination. Therefore,

536 we filtered out tumor tissues with ≥ 3 known potentially somatic mutations or with ≥ 2 back mutations from the

537 further analyses (Figure 1-figure supplement 5A and B).

538

539

540 **Variant calling**

541 We extracted mtDNA reads using Samtools (Li and Durbin, 2009). We used VarScan2 (Koboldt et al., 2012) for

542 initial variant calling with a few options (--strand-filter 1 (mismatches should be reported by both forward and

543 reverse reads), --min-var-freq 0.03 (minimum VAF 3%), --min-avg-qual 20 (minimum base quality 20), min-

544 coverage 3 and --min-reads2 2). With respect to the --strand-filter, it generally removes variant when >90% of

545 mismatches are reported from either of the H or the L mtDNA strand. However, where only reads with a specific

546 orientation are could be aligned dominantly (i.e. in both extreme region of mitochondrial reference genome; only L

547 strand reads could be aligned on the 5' extreme of mtDNA), we compared strand bias between "perfect matches" (#

548 perfect matches from L strand reads / total # perfect matches) and mismatches (# mismatches from L strand reads /

549 total # mismatches). If the difference between those two bias < 0.1, the mutations were rescued. Of the 1,907

550 mutations, 54 (2.8%) were rescued accordingly.

551

552 Putative somatic variants called by VarScan2 were further filtered using criteria shown below.

553

554   (1) At least 4 unique reads supporting variants AND all variant reads at least 20 phred-scale sequencing quality

555   score (Q 20 = 1% sequencing error rate) AND at least 3% variant allele fractions (VAFs).

556   a. Regardless of in WGS and in WES, the ≥4 mismatches and the ≥3% VAF criteria must be

557   satisfied simultaneously.

558   b. However, in WGS, the minimum number of reads (n=4) criterion is not essential, because the

559   ≥3% VAF criterion is much more stringent (3% VAF request at least 240 mismatches (>>4)

560   given mtDNA coverage is ~8,000 for WGS).

561   c. In WES, the ≥3% VAF criterion is relatively less important than in WGS, because the ≥4

562   mismatches criterion is more stringent. For example, 4 mismatches in 90x (WXS average)

563   coverage region (VAF=4.4%) automatically fulfill the ≥3% VAF criterion. For less covered

564   regions (i.e. <40x coverage; n=285 out of total 1,907 substitutions), the VAF criterion

565   becomes less important, because 4 mismatches would generate ≥10% VAF, much higher

566   than the minimum threshold (i.e. 3%). As results, we are missing lower heteroplasmic

567   variants (i.e. variants with 3%-10% heteroplasmic levels) from low coverage samples

568   (mostly by WXS). The lower sensitivity of WXS is also confirmed in our validation study (See

569   "Validation of somatic variants" below).

570 (2) There is no minimum threshold for total coverage (# perfect matches + # mismatches).

571 (3) To increase sensitivity for detecting mutations, we rescued mutations with 3 unique variant reads (with at

572   least 20 phred-scale sequencing quality score) when VAFs is ≥20%. Of 1,907 somatic substitutions, 32

573   (1.7%) were rescued accordingly.

574 (4) All somatic variants presenting with VAFs lower than our very conservative threshold for minor cross-

575   contamination (5-times 95% upper limit of contamination levels for each tumor sample, see above "Minor

576   cross-contamination of DNA samples") were removed. When we could not estimate cross-contamination

577   levels because of low sequencing depth of coverage (for nuclear genome), a conservative criterion (10%

578   contamination level threshold) was explicitly used.

579 (5) Substitutions were further visually inspected using IGV (Thorvaldsdottir et al., 2013). Thirteen frequent

580   false positive variants (shown below) by misalignment due to extensive level of homopolymers in rCRS

581   and due to sequencing error in the reference mtDNA genome (3107N, see Mitomap

582   (http://www.mitomap.org/bin/view.pl/MITOMAP/CambridgeReanalysis) for more information) were

583   explicitly removed:

584

585   1) misalignment due to ACCCCCCCTCCCCC (rCRS 302-315)

586   A302C, C309T, C311T, C312T, C313T, G316C

587   2) misalignment due to GCACACACACACC (rCRS 513-525)

588   C514A, A515G, A523C, C524G

589   3) misalignment due to 3107N in rCRS (ACNTT, rCRS 3105-3109)

590   C3106A, T3109C, C3110A

591

592 We compared our variant calls with common inherited mtDNA polymorphisms deposited in the mtDB database as

593 of 24. Jul. 2013 (Ingman and Gyllensten, 2006). Gene annotation of somatic variants was done using custom script

594 based on human mtDNA gene information (Ruiz-Pesini et al., 2007).

595

596 **Validation of somatic variants**

597 To validate the sensitivity and specificity of variant calling in this study, 19 tumor and normal pairs (which were

598 originally whole-genome sequenced) were whole-exome sequenced and mtDNA variants were assessed

599 independently. Among the 28 somatic substitutions originally detected from the 19 tumor-normal whole-genome

600 sequencing pairs, 20 (71.4%) were called as somatic (Figure 1-figure supplement 3). In addition, 5 (17.9%)
601 presented evidence of variant reads in the validation set, although it was filtered out because of its low read-depth of
602 coverage in exome sequencings (showed 2-5 variant reads). Moreover, because 3 remaining sites were not
603 sufficiently covered in the validation set to call somatic variants, these could not be evidence of the inaccuracy of
604 whole-genome sequencing data, therefore not considered in the accuracy validation. Taken together, all the 25
605 somatic substitutions by whole-genome sequencing were highly likely to be true positives, therefore we concluded it
606 provided ~100% accuracy in the mtDNA somatic substitution assessment. Actually, the high accuracy of whole-
607 genome sequencing is very likely and what we expect, because it provides extensive coverage of mtDNA (average
608 read-depth > 7,500x), ~3% heteroplasmic variants would present >200 variant reads.

609 By contrast, the validation set (whole-exome sequencing) called 21 somatic substitutions. Of these, 20 were
610 common with whole-genome sequencing, and one was incorrectly called as somatic though it was actually germline
611 substitutions in the whole-genome sequencing data. In addition, as mentioned above, the validation set missed 8
612 somatic substitutions called by whole-genome sequencing. Six out of eight undercalls (75%) were low
613 heteroplasmic substitutions in whole-genome sequencing, ranging from 3.36% to 8.68%. Based on these data, we
614 suggest 71.4% sensitivity (20/28) and 95.2% specificity (20/21) for exome-sequencing in detecting upto 3%
615 heteroplasmic somatic mtDNA substitutions in cancer.

616 We further checked the correlation of heteroplasmy level between the 20 mtDNA somatic mutations called both
617 whole-genome and whole-exome sequencing. It showed great linear relationship (R^2=0.97, Figure 1-figure
618 supplement 2), further suggesting whole-exome sequencing data is appropriate for accurate detection of mtDNA
619 somatic mutations.

620

Substitution phasing

622 We phased 72 somatic substitution pairs, which arose in a single cancer sample and which located sufficiently close
623 (from 10bp to ~500bp) therefore both sites could be sequenced by same sequence fragments (Supplementary file 3
624 and Figure 2-figure supplement 1). We classified them as 'different strand', 'co-clonal' and 'sub-clonal' using
625 criteria as follow:

626

627 Different strand: the two somatic substitutions are obligate on different strands. Reads that report
628 wildtype1(wt)-substitution2(subs) and subs1-wt2, but subs1-subs2, are observed.
629 Co-clonal: reads reporting wt1-wt2 and subs1-subs2 are only observed.
630 Subclonal: One substitution is subclonal to the other, but the two are definitely phased. Reads subs1-subs2
631 and either subs1-wt2 or wt1-subs2 are observed.

632

Tumor type and mtDNA somatic substitutions

634 To understand the relationship between tumor types and number of mtDNA mutations, Poisson regression and
635 ANOVA was applied to our dataset using R software (http://www.r-project.org).

636

637   Fit1 <- glm(N_{sub} ~ Cov_T + Cov_N, family=poisson())

638   Fit2 <- glm(N_{sub} ~ Cov_T + Cov_N + t, family=poisson())

639   anova(Fit1, Fit2, test="Chisq")

640

641   , where N_{sub} is number of mtDNA substitutions of each sample, Cov_T and Cov_N are coverage of tumor and normal

642   mtDNA, respectively, (if Cov is >200, we replaced it by 200), t is tumor types.

643

644   **Age and mtDNA somatic substitutions**

645   Poisson regression was applied to our breast cancer dataset.

646

647   Fit1 <- glm(N_{sub} ~ Cov_T + Cov_N + a, family=poisson())

648

649   , where N_{sub} is number of mtDNA substitutions of each sample, Cov_T and Cov_N are coverage of tumor and normal

650   mtDNA, respectively, (if Cov is >200, we replaced it by 200), a is age at diagnosis. P-value in estimation of a was

651   shown in the manuscript.

652

653   **Mutational signature and strand bias**

654   Different mutational processes generate different combinations of mutation types, termed "signatures" (Nik-Zainal

655   et al., 2012a). For example, ultraviolet (UV) light and tobacco smoking (polycyclic aromatic hydrocarbons)

656   frequently generate C>T transitions and G>T transversions on non-transcribed (coding) strands in melanoma and

657   lung cancers, respectively (Pleasance et al., 2010a; Pleasance et al., 2010b). To understand the mutational processes

658   influencing cancer mtDNA, we correlated the 1,907 mtDNA substitutions with 21 cancer specific mutational

659   signatures in the nuclear DNA recently identified (Alexandrov et al., 2013). However, none of the signature could

660   explain the highly unique mtDNA substitutions.

661

662   Mutational signature and strand bias was assessed as described in our previous reports (Alexandrov et al., 2013).

663   Briefly, The immediate 5′ and 3′ sequence context was extracted from rCRS. Substitution rate for each trinucleotide

664   context was calculated with the number of substitution normalized by the frequency of the trinucleotide context

665   observed in the rCRS, in the L and H strand, respectively. For analyses of substitutions falling in the mtDNA genes

666   (13 protein-coding and 22 tRNA genes), transcribed/non-transcribed strand was also considered for comparison.

667   In order to prove the strand bias is not transcription but replication-coupled, we checked strand biases of

668   polymorphisms in the 12 L strand protein-coding genes, 1 H strand protein-coding gene (*MT-ND6*) and/or 22 tRNAs

669   (Figure 3 – supplement1). For this specific purpose, we did not consider the sequence context (immediate 5' and 3'

670   bases) because it over-classifies mutations (i.e. the number of mutation classes (n=96) is larger than that of

671   mutations). In other words, 12 classes of substitutions (six classes of possible base substitutions (C>A, C>G, C>T,

672   T>A, T>C, T>G) x two strands (L and H strands)) were considered. Substitution rates are ratio between observed

673   and expected numbers (H_0=same mutation rate for all substitution classes) for each substitution class. In order to

674 understand which model (replicative or transcriptional strand) is appropriate to explain the strand-bias, chi-square

675 tests were used between numbers of observed mutations for each class and expected ones under the background

676 signature.

677

678 **mtDNA codon usage**

679 We counted the codon frequencies in 13 mtDNA protein-coding genes. Because 12 L strand protein-coding genes

680 and 1 H strand gene (*MT-ND6*) are under opposite mutational pressure (T>C and G>A for L strand genes; A>G and

681 C>T for *MT-ND6*), we separated L and H strand genes for this analysis. T>C skew and G>A skew were calculated

682 as shown below, to understand the $T_L>C_L$ and $C_H>T_H$ (equivalent to $G_L>A_L$) substitutions during the evolution of

683 human mtDNA:

684

$$T > C_{skew} = \frac{N_C - N_T}{N_C + N_T} \quad and \quad G > A_{skew} = \frac{N_A - N_G}{N_A + N_G}$$

685

686 , where N_A, N_C, N_G and N_T are number of A, C, G, and T base in the 3rd position of triplet codons in mtDNA genes,

687 respectively.

688 For the assessment of mtDNA codon usage of other animal species, we analyzed the mtDNA sequence of *C. elegans*

689 (accession# NC_001328), *D. melanogaster* (accession# NC_001709), *D. rerio* (accession# NC_002333), *X. laevis*

690 (accession# NC_001573), *M. musculus* (accession# EU450583), *G. Domesticus* (accession # NC_235570), and *P.*

691 *troglodytes* (NC_001643). We considered only L strand mtDNA genes in the cross-species analysis.

692

693 **Recurrent substitutions**

694 To compare the number of recurrent substitutions between silent and missense substitutions, we randomly selected

695 100 substitutions each from 198 silent substitutions in the 3rd base of triplet codons, 440 missense substitutions in

696 the 1st base of triplet codons, and 405 missense substitutions in the 2nd base of triplet codons. We counted numbers

697 of recurrent substitutions in each group. This was iterated 300 times independently. ANOVA testing was applied to

698 determine the difference between the three groups (Figure 5-figure supplement 1).

699

700 **dN/dS ratio**

701 To estimate dN/dS values for missense mutations (w_{mis}), we used an adaptation of the method described previously

702 (Greenman et al., 2006). Briefly, the rate of mutations is modeled as a Poisson process, with a rate given by a

703 product of the mutation rate and the impact of selection. To obtain accurate estimates of dN/dS we used two separate

704 models, one using 12 single-nucleotide substitution rates and a more complex one accounting for any context-

705 dependence effect by 1-nucleotide upstream and downstream using 192 substitution rates. For example in the 12-

706 rate model, the expected number of A>C mutations ($\lambda_{A>C}$) would is modeled as

707

708 $\lambda_{syn,A>C} = r_{A>C} * L_{syn,A>C}$

709 $\lambda_{mis,A>C} = r_{A>C} * w_{mis} * L_{mis,A>C}$

710

711 , being $L_{syn,A>C}$ and $L_{mis,A>C}$ the number of sites that can suffer a synonymous and missense A>C mutation,

712 respectively, which are calculated for any particular sequence. The likelihood of observing the number of missense

713 A>C mutations ($N_{mis,A>C}$) given the expected $\lambda_{mis,A>C}$ is then calculated as:

714

715 Lik = Poisson($N_{mis,A>C} | r_{A>C}, w_{mis}$)

716

717 and the likelihood of the entire model is the product of all individual likelihoods. W_{mis} is fixed to be equal in all 12

718 (or 192) equations describing each substitution type and a hill-climbing algorithm is used to find the maximum-

719 likelihood estimates for all rate and selection parameters. Likelihood Ratio Tests are then used to test deviations

720 from neutrality ($w_{mis} = 1$). The dN/dS ratio reported in the main text corresponds to the full context-dependent model

721 with 192 substitution rates. This method allows quantifying the strength of selection avoiding the confounding effect

722 of gene length, sequence composition, different rates of each substitution type and context-dependent mutagenesis.

723

724 **Short indels**

725 Along with the 1,907 somatic mtDNA substitutions, we identified 109 and 142 somatic short insertions and

726 deletions, respectively, from the 1,675 cancer mtDNA sequences using Varscan2 (Supplementary file 2).

727

728 **Evolutionary dynamics of neutral mitochondrial mutations**

729 We model the evolutionary dynamics of mitochondrial mutations under random drift and derive a simple equation

730 for the expected number of homoplasmic mutations. There exist multiple levels at which mitochondrial mutations

731 evolve: within mitochondria, in the cytoplasm and on the cellular level (Rand, 2011). Here we focus on the

732 dynamics in a single cell, which represents the founder of the last clonal expansion in the tumor cell population. The

733 cellular dynamics during a clonal expansion are difficult to describe analytically, but it is important to realize that

734 mutations a clonal expansion preserves the allele frequencies of neutral variants and that mutations that occur after

735 the expansion are unlikely to contribute to measurable allele frequencies, as the population becomes large.

736

737 We model the evolutionary dynamics of mitochondrial mutations in the cytoplasm of a single cell by a Wright-

738 Fisher process (Wright, 1931), in which the number of mitochondria in a subsequent generation is a binomial sample

739 of the mitochondria in the previous generation. The number of mitochondria M is kept fixed. The marginal allele

740 frequency X of a single site has two absorbing boundaries $X=0$ and $X=M$ (homoplasmy) and the probability of

741 fixation of an allele at frequency X by neutral drift is $\rho = X/M$ (Wright, 1931). Note that this process leads, on the

742 population level, to a dichotomization of heteroplasmic variants to either go extinct or become homoplasmic and

743 fixate in a cell.

744

745 Mutations on any of L (=16,569nt) sites in the mitochondrial genome are assumed to occur at a uniform rate μ per

746 nucleotide per cell division, which is of order 10^{-7}, based on a human inter-generational comparison (Coller et al.,

747 2001). Hence the rate of neutral evolution is simply $\mu L M / M = \mu L$ (Kimura, 1984). Lastly, the expected time to

748 fixation in the Wright-Fisher process is $t = 2M$. Putting these things together, the expected number of mutant

749 alleles N in a cell initially without any mitochondrial mutations after T generation is

750

751 $E[N] = \mu L (T - 2M)$

752

753 This equation predicts a linear accumulation of neutral mutations over time, with a delay imposed by number

754 of mitochondrial copies. A similar behavior has been reported using numerical simulations (Coller et al.,

755 2001). When also considering heteroplasmic mutations, the expected number of alterations may be slightly

756 higher.

757

758 To check whether our model yields the correct behaviour, we use the following numbers: The observed order

759 of magnitude of mitochondrial mutations per patient was N=1. The sequencing coverage on the mitochondrial

760 genome indicates that there we of order M=100 mitochondrial genome copies present per cancer cell. The

761 expected number of mutations per cell division is $\mu L = 1.6 \times 10^{-3}$, it therefore requires around 1000 cell

762 generations T to accumulate on average one homoplasmic mutation. This number of generations appears

763 realistic for regenerating tissues. As expected, epithelial cancers had among the highest observed number of

764 mitochondrial mutations, while hematopoietic cancers typically had lower numbers.

765

766 **Statistical testing**

767 Statistical testing was performed using R software. All p-values were calculated by two-tailed testing. Figures were

768 generated using R and Microsoft Excel software.

769

770

771

772

COMPETING INTERESTS

The authors declare that they have no conflict of interest with this manuscript.

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FIGURE LEGENDS AND TABLES

Figure 1. Mitochondrial somatic substitutions identified from 1,675 Tumor-Normal pairs. mtDNA genes and intergenic regions are shown. The strand of genes is shown based on mtDNA strand containing equivalent sequences of transcribed RNA. Substitution categories (silent, non-silent (missense and nonsense), non-coding (tRNA and rRNA) and intergenic) are shown by shapes of each substitution. Six classes of substitutions are presented color-coded. The substitutions on the H, and L strand (when six substitutional class were considered) are shown outside and inside of mtDNA genes, respectively. Vertical axes for H and L strand substitutions represent the VAF of each variant.

Figure 2. mtDNA somatic substitutions of human cancer. (A) Number of somatic substitutions in a tumor sample. **(B)** Average number of somatic substitutions per sample across 31 tumor types. **(C)** Age of diagnosis and number of mtDNA somatic substitutions in breast cancers.

Figure 3. Replicative strand bias for mtDNA somatic substitutions. (A) Replicative strand-specific substitution rate (# of observed / # of expected) by 96 trinucleotide context. Substitutions in a specific mtDNA segment (from Ori-b to O_H) are not included, because they present a different substitutional signature. **(B)** Mutational signature across tumor types. Eighteen tumor types, which include at least 25 mtDNA mutations, were shown. **(C)** Inverted substitution signature in the Ori-b - O_H.

Figure 4. Mutational signature similar to processes shaping human mtDNA sequence over evolutionary time **(A)** triplet codon depletion in human mtDNA by equivalent ($C_H>T_H$ and $T_L>C_L$) mutational pressure. Relative frequency of each triplet codon within synonymous pairs (NNT-NNC or NNA-NNG) is shown by color. The arrows beside the box highlight the T>C (red) and G>A (blue) substitutional pressures on the L strand in germline mtDNA **(B)** Correlation of triplet codon frequencies between from observed and from simulated evolutions of a random sequence mtDNA by the mtDNA somatic mutational signature with constraining mitochondrial protein sequences.

964

965

966 **Figure 5. Selection and mutational process for mtDNA somatic substitutions. (A)** Truncating

967 mutations (nonsense substitutions and frame-shifting (FS) coding indels) present significantly

968 lower VAF. **(B)** Change of VAF of mtDNA somatic mutation between primary and metastatic

969 (or late) cancer tissues. **(C)** Mutational signature for mtDNA across various tumor types. None of

970 the three highlighted mechanisms or nuclear DNA double-strand breaks repair mechanism

971 (*BRCA*) match with the mtDNA mutational signature. * Only substitutions in protein-coding

972 genes considered. **(D)** A proposed model of mtDNA mutational process.

973

974

975 **Figure Supplements Legends**

976

977 **Figure 1-figure supplement 1. Correlation in amount of mtDNA reads between whole-**

978 **genome and whole-exome sequencing.** 139 DNA samples, either from tumors or bloods,

979 sequenced by whole-genome sequencing were additionally sequenced by whole-exome

980 sequencing. We compared the amount of mtDNA reads between whole-genome and whole-

981 exome sequencing. As shown in this figure, we found strong positive correlation. * CGP; Cancer

982 Genome Project, Wellcome Trust Sanger Institute, WUGSC; Washington University Genome

983 Sequencing Center

984

985 **Figure 1-figure supplement 2. Correlation of heteroplasmy levels between whole-genome**

986 **and whole-exome sequencing.** To validate the sensitivity and specificity of variant calling in

987 this study, 19 tumor and normal pairs (which were originally whole-genome sequenced) were

988 whole-exome sequenced and mtDNA variants were assessed independently. We correlated the

989 heteroplasmic levels of 20 mutations detected in common.

990

991 **Figure 1-figure supplement 3. Validation of mtDNA somatic substitutions.**

992

993 **Figure 1-figure supplement 4. Amount of off-target mtDNA reads across four sequencing**

994 **centers. *** CGP; Cancer Genome Project, Wellcome Trust Sanger Institute (n = 855), WUGSC;

995 Washington University Genome Sequencing Center (n=140), BCM; Baylor College of Medicine

996 (n=85)**,** BI; Broad Institute (n=435)

997

998 **Figure 1-figure supplement 5. Filtering samples of potential DNA contaminations. (A)** A

999 histogram presenting potential sample-swaps in tumor-sample pairs. **(B)** A histogram presenting

1000 potential minor DNA cross-contamination in tumor samples. Cross-contamination levels were

1001 considered in filtering substitutions (see "Minor cross-contamination of DNA samples" section

1002 in Materials and Methods). **(C)** Histograms showing number of somatic substitutions

1003 overlapping with known inherited polymorphisms and **(D)** number of back mutations.

1004

1005 **Figure 2-figure supplement 1. VAFs of phased somatic mtDNA substitutions.**

1006 This figure presents VAF pairs between co-clonal, subclonal and different strand mtDNA

1007 substitutions. We expect similar VAFs for co-clonal pairs; lower VAF in sub-clonal mutations

1008 compared to clonal ones; and sum of a VAF pair is equal or less than 1.0.

1009

1010 **Figure 3-figure supplement 1. Replicative strand bias observed in mtDNA substitutions. (A)**

1011 Mutational signature of mtDNA somatic substitutions on the 12 L strand genes by replicative

1012 strand (L/H strand). It agrees very well with the background mutational signature. (Chi-square

1013 p=0.99999) **(B)** Mutational signature of mtDNA somatic substitutions on the H strand gene (*MT-*

1014 *ND6*) by replicative strand. It is very close to the background very close to the expected

1015 background signature (Chi-square p=0.027). If we consider signature by transcriptional strand,

1016 the signature difference is very clear (Chi-square p=1×10^{-21}). These suggest the strand bias not to

1017 be transcription-coupled, but replication coupled. **(C)** Mutational spectrum of mtDNA somatic

1018 substitutions on the 22 tRNA genes by replicative strand. Again, it agrees very well with the

1019 background mutational signature. (Chi-square p=0.71) **(D)** Mutational spectrum of mtDNA

1020 somatic substitutions on the 22 tRNA genes by non-transcribed (coding) and transcribed (non-

1021 coding) strand. Strand bias was greatly subsided because somatic substitutions on 14 L strand

1022 and 8 H strand tRNAs neutralize the strand bias ($C_H>T_H$ and $T_L>C_L$) each other. As a result, this

1023 signature of tRNA mutations by transcriptional strand is significantly different from the

1024 background one (Chi-square p=3.3×10^{-12}). Taken all together, we concluded that the cause of

1025 strand bias is not transcription-coupled but is replicative.

1026

1027 **Figure 4-figure supplement 1. TC and GA skew for L strand mtDNA genes across 8 animal**
1028 **species.** *C.elegans* (a nematode) and *D. melanogaster* (fruit fly) mtDNA appears to have
1029 $G_L \ll A_L$ (due to $C_H > T_H$ mutational pressure) and $C_L \gg T_L$ (due to $C_L > T_L$ mutational pressure) in
1030 the 3rd base of triplet codon in L strand genes. Therefore they seem to have predominant C>T
1031 mutational pressure without strand bias. *D. rerio* (zebrafish), *X. laevis* (frog) and *M. musculus*
1032 (mouse) presents $G_L \ll A_L$ (due to $C_H > T_H$ mutational pressure), but similar number of C_L and T_L.
1033 Therefore, mtDNA of these sequences is thought to have $C_H > T_H$, with strand bias. The existence
1034 of $T_L > C_L$ is not clear. Finally, mtDNA of *H. sapiens*, *P. troglodytes* (Chimpanzee) and *G.*
1035 *domesticus* (Chicken) shows clear $C_H > T_H$ and $T_L > C_L$ as mentioned in the main manuscript.
1036 Interestingly, $T_L > C_L$ seems to be slightly stronger in the mitochondria of Chicken than that of
1037 human (or Chimp). We suggest there would be some differences in the mechanism of mtDNA
1038 replication across the evolution tree.
1039
1040 **Figure 4-figure supplement 2. Correlation of triplet codon frequencies between from**
1041 **observed and from simulated evolutions under the mtDNA somatic mutational signature.**
1042
1043 **Figure 5-figure supplement 1. Number of recurrent substitutions between silent and**
1044 **missense substitutions.** 100 sites were randomly selected from silent substitutions (at 3rd base of
1045 triplet codon) and missense substitutions (at 1st and 2nd base of triplet codon). No significant
1046 difference was observed among these three groups.
1047
1048 **Figure 5-figure supplement 2. Comparison of VAF of protein-truncating mutations**
1049 **(nonsense substitution and indels) across tumor types.** Four tumor types with more than 10
1050 protein-truncating mutations are shown. Fisher's exact were applied between breast and other
1051 tissue types.
1052
1053 **Figure 5-figure supplement 3. Negligible impacts of external mutagens (UV and tobacco**
1054 **smoking) to the somatic mtDNA mutations.** No evidence of UV and tobacco smoking was
1055 identified even in melanoma and lung cancers, respectively. (Left) We compared the proportion
1056 of C>T (and G>A) substitutions in the CpC (GpG) context (mutational signature for
1057 UV(Alexandrov et al., 2013)) between melanomas and breast cancers (controls). Because UV

1058   shows trivial impact to the nuclear DNA somatic mutations of breast cancers (Alexandrov et al.,

1059   2013), the vast majority of mtDNA C>T substitutions in the CpC context from breast cancers

1060   were not generated by UV. (Right) We compared the proportion of C>A (G>T) substitutions

1061   between lung and breast (control) cancers. C>A (G>T) substitutions are dominantly generated by

1062   tobacco smoking. Like UV, the impact of tobacco smoking to the somatic mutations of breast

1063   cancers is trivial (Alexandrov et al., 2013).

1064

1065 **Legends of Supplementary Files**

1066

1067 **Supplementary file 1. Sequencing information of 1,675 tumor-normal pairs.**

1068

1069 **Supplementary file 2. Catalogues of somatic mutations (substitutions and indels) and**

1070 **inherited polymorphisms identified in this study.**

1071

1072 **Supplementary file 3. List of phased somatic substitutions.**

1073

1074 **Supplementary file 4. dN/dS for 13 protein-coding genes in mitochondria.**

1075

1076 **Supplementary file 5. List of somatic substitution with higher recurrent rate than expected.**

1077

1078 **Supplementary file 6. Data accession numbers.**

1079

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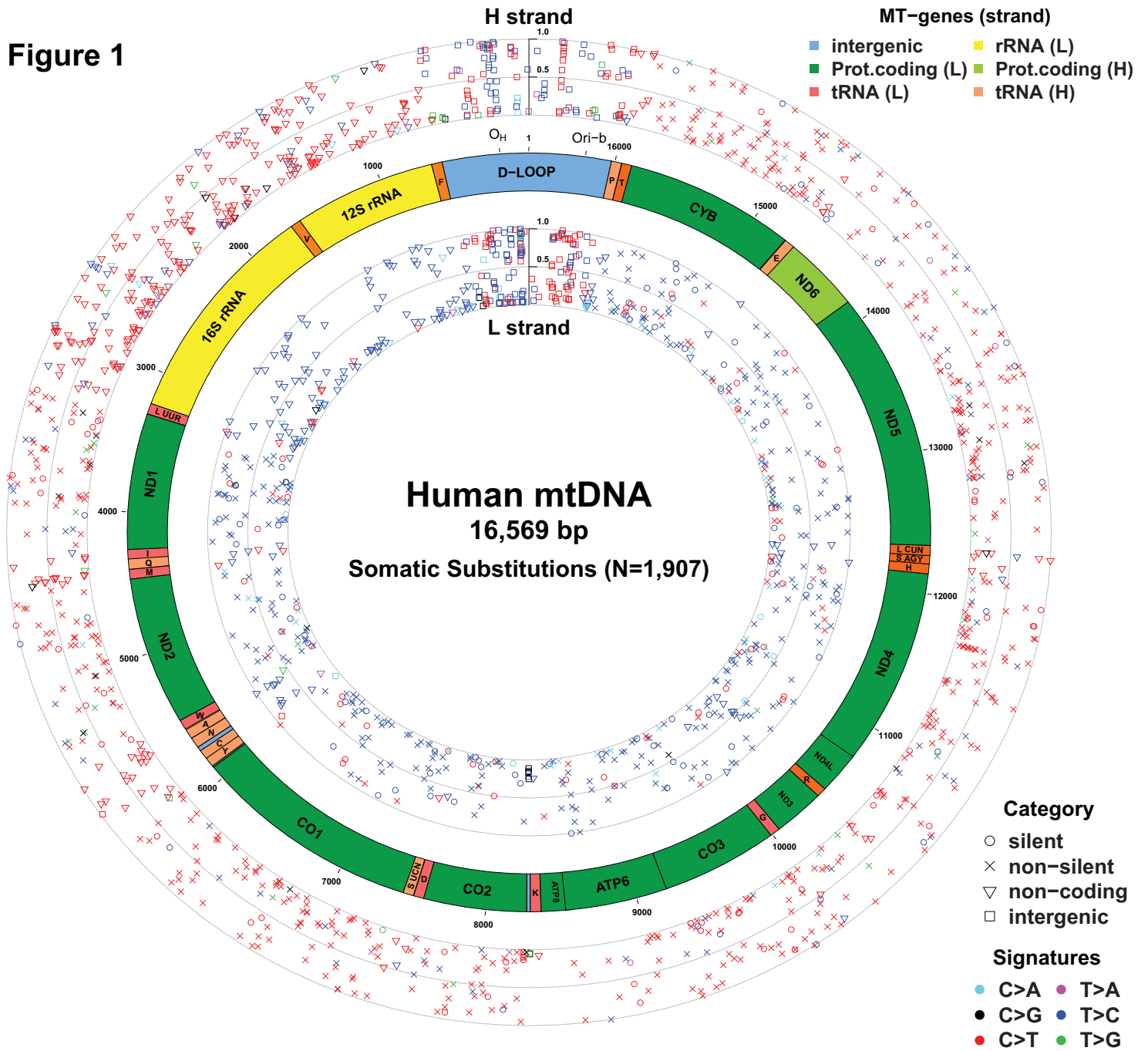
Table 1. Summary statistics of mtDNA sequence data

	WGS	WXS	Average mt RD (WGS)	Average mt RD (WXS)	Total		WGS	WXS	Average mt RD (WGS)	Average mt RD (WXS)	Total
Breast	284	98	11,594.3	52.7	382	Meningioma	0	12	-	42.5	12
Colorectal	1	75	34,916.9	276.6	76	Ependymoma	1	9	10,323.7	52.7	10
Lung	60	0	2,798.1	-	60						
Prostate	80	0	17,810.6	-	80	MPD	12	138	1,517.0	10.9	150
Hepatocellular	0	47	-	205.8	47	MDS	3	75	5,648.7	44.5	78
Melanoma	13	13	513.9	353.5	26	ALL	64	6	886.6	35.9	70
Gastric	0	13	-	184.1	13	CLL	6	0	5,002.2	-	6
Cholangiocarcinoma	0	8	-	143.9	8	AML	1	6	6,783.6	27.4	7
Mesothelioma	0	6	-	106.3	6	Multiple myeloma	0	69	-	43.2	69
Bladder	54	0	646.2	-	54	AMKL	0	9	-	24.2	9
Renal	0	23	-	35.4	23	Lymphoma	0	4	-	99.5	4
Ovarian	0	38	-	58.9	38						
Uterine	27	23	736.0	149.5	50	Osteosarcoma	38	90	9,525.5	119.2	128
Cervical	0	52	-	85.2	52	Chondrosarcoma	0	47	-	99.1	47
Adenoid cystic ca.	1	60	714.7	75.6	61	Ewing sarcoma	0	27	-	69.5	27
Head&Neck	43	3	1,369.1	18.8	46	Kaposi sarcoma	0	9	-	181.0	9
						Chordoma	16	11	1,240.0	82.1	27
Total; 31 cancer types							704	971			1,675

1085   WGS, whole-genome sequencing; WXS, whole-exome sequencing; mt RD, mitochondrial read-depth; MPD,

1086   myeloproliferative disease; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukaemia; CLL, chronic

1087   lymphoblastic leukaemia; AML, acute myeloid leukaemia; AMKL, acute megakaryoblastic leukaemia.
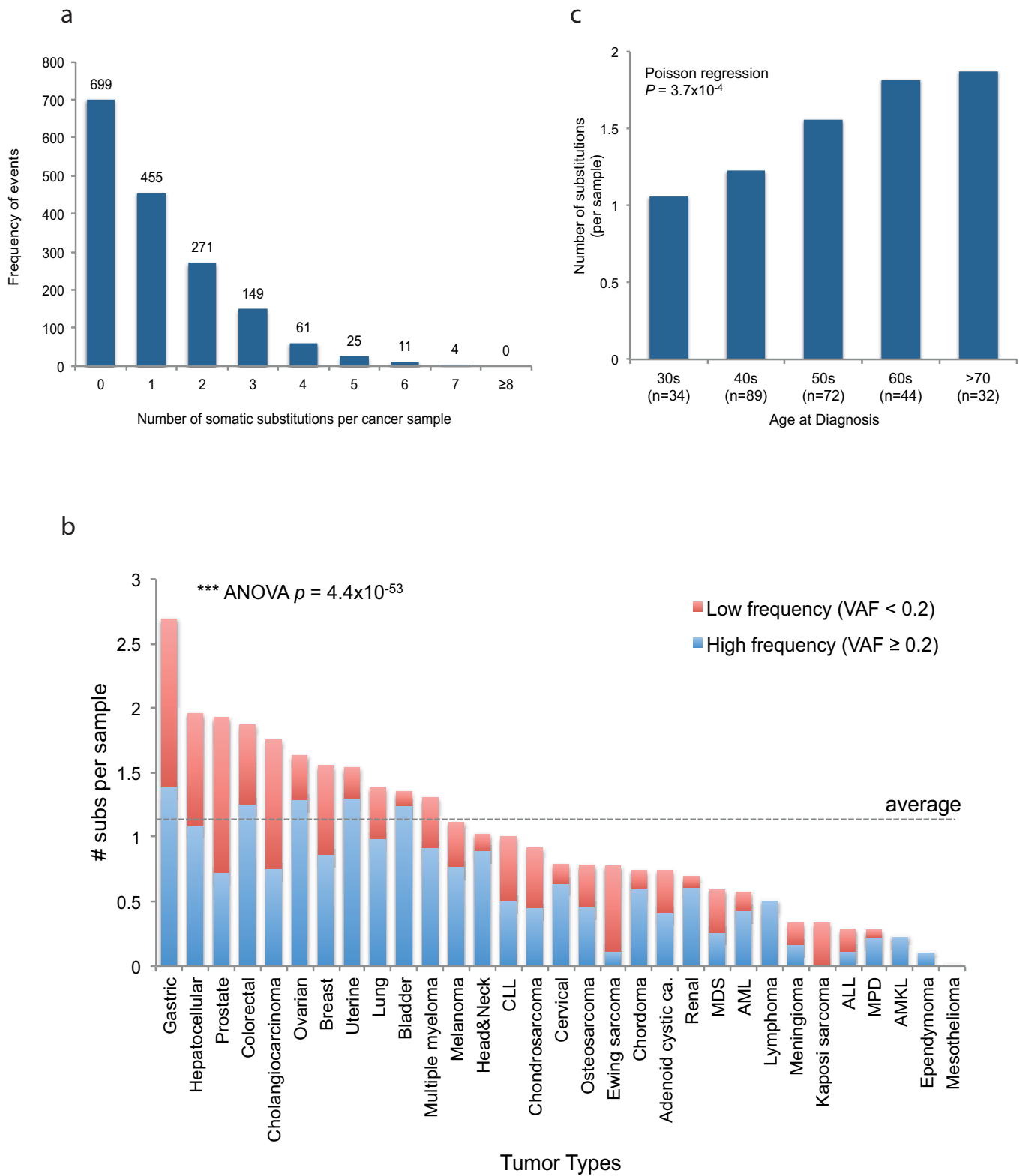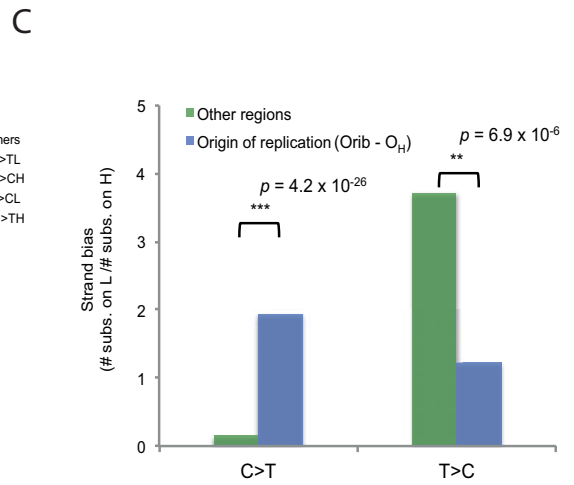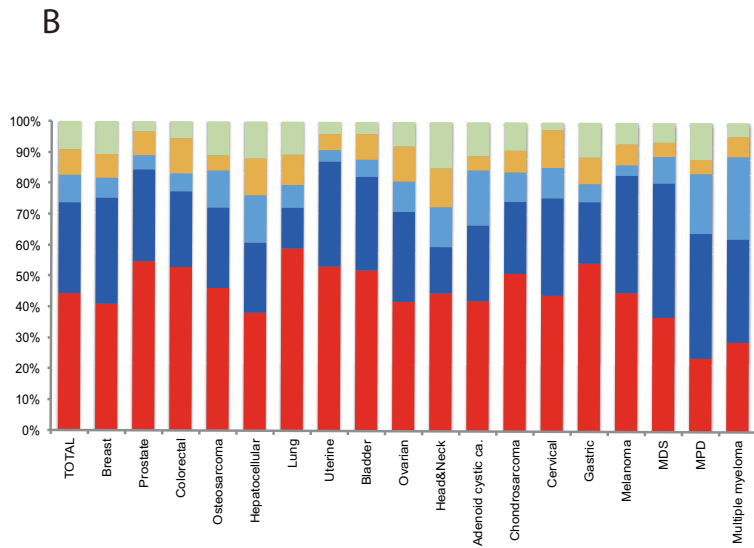
Figure 1

Human mtDNA
16,569 bp
Somatic Substitutions (N=1,907)

H strand

L strand

MT−genes (strand)
- intergenic
- Prot.coding (L)
- tRNA (L)
- rRNA (L)
- Prot.coding (H)
- tRNA (H)

Category
- ○ silent
- × non−silent
- ▽ non−coding
- □ intergenic

Signatures
- C>A
- C>G
- C>T
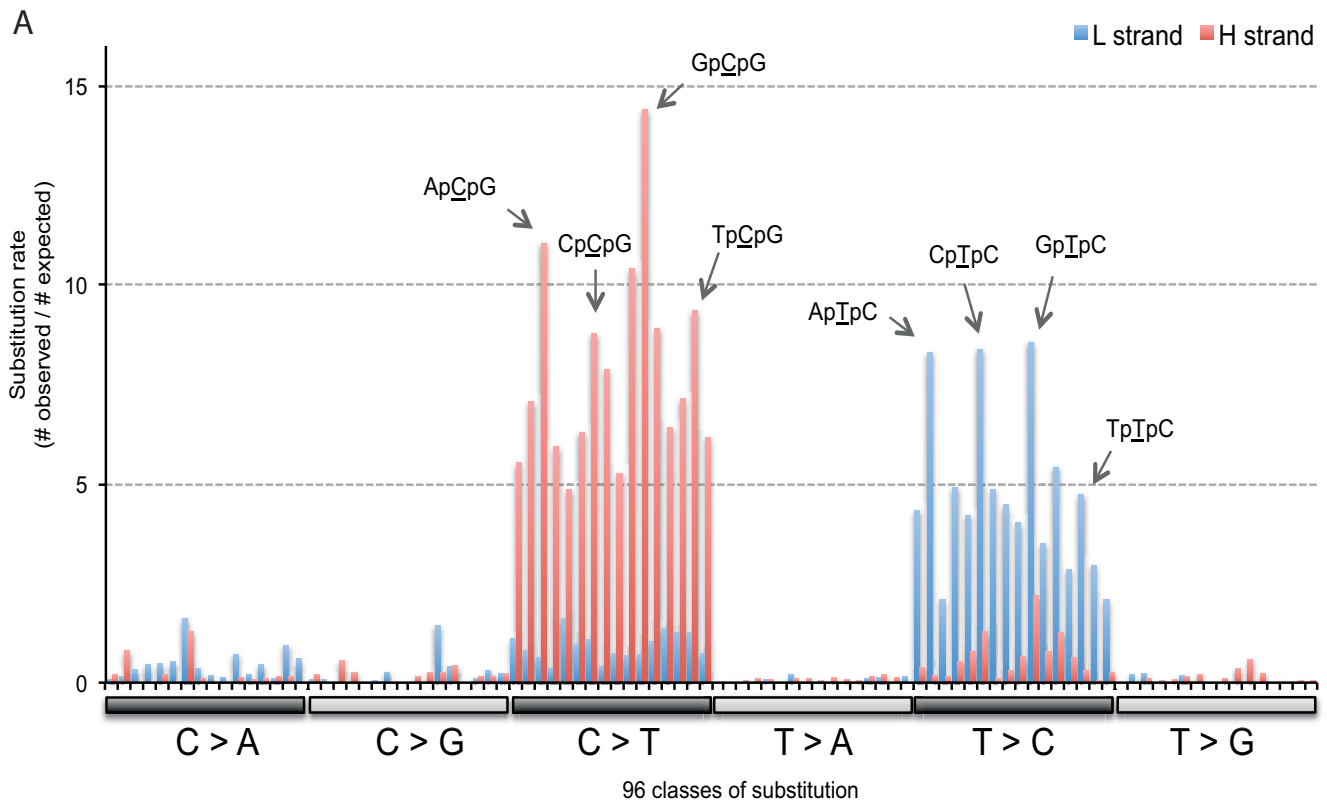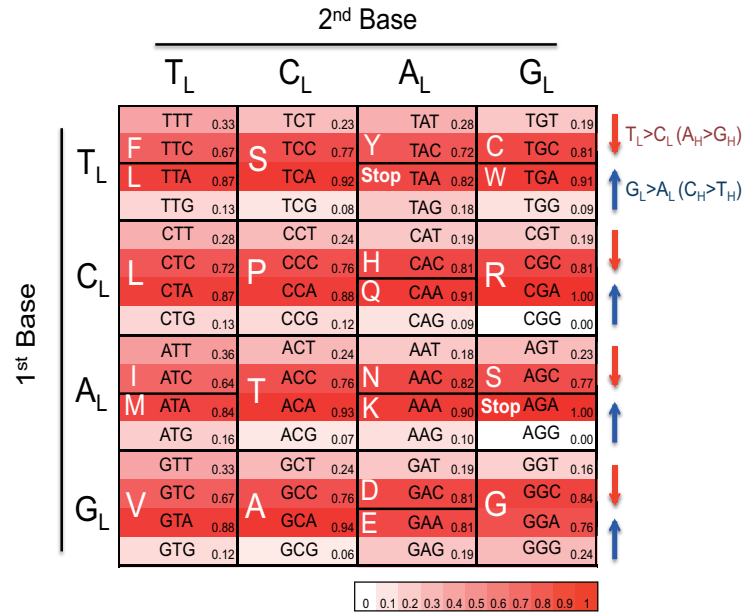- T>A
- T>C
- T>G

Figure 2

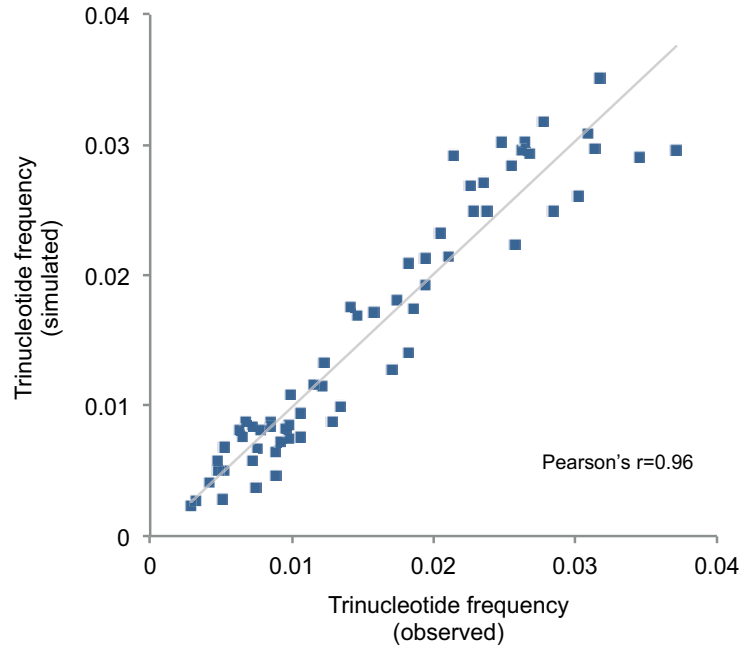Figure 3

A

B

Figure 4

Figure 5

Participants in ICGC Breast Cancer Group who supplied or verified samples

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Ethical approval references

Genome Analysis of myeloid and lymphoid malignancies (10/H0306/40)
Genomic Analysis of Mesothelioma (11/EE/0444)
Myeloid and lymphoid cancer genome analysis (07/S1402/90)
The Treatment of Down Syndrome Children with Acute Myeloid Leukemia and Myelodysplastic Syndrome(AAML0431)
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PREDICT-Cohort (09/H0801/96)
ICGC Prostate (Evaluation of biomarkers in urological diseases) (LREC 03/018)
ICGC Prostate (779) (Prostate Complex CRUK Sample Cohort) (MREC/01/4/061)
ICGC Prostate (Tissue collection at radical prostatectomy) (CRE-2011.373)
Somatic molecular genetics of human cancers, melanoma and myeloma (Dana Farber Cancer Institute)(08/H0308/303)
Breast Cancer Genome Analysis for the International Cancer Genome Consortium Working Group (09/H0306/36)
Genome analysis of tumours of the bone (09/H0308/165)

Author Contributions

Y.S.J., M.R.S and P.J.C conceived and designed this study. Y.S.J and M.G performed analysis of the sequence data. Y.S.J and L.B.A analyzed mutational signature. I.M investigated dN/dS ratio of mtDNA substitutions. A.P.B and J.W.T provided bioinformatics support for sequencing data acquisition. M.G. performed the simulation study on evolutionary dynamics. S.N.-Z., M.R., H.R.D., E.P., G.G., A.S., N.B., S.B., P.S.T., J.N., C.E.M., G.S.V., A.R.G., M.-Q.D., A.U., J.E.P., B.T.T., N.M., M.G., P.V., A.K.E.-N., T.S., V.P.C., R.G., J.A.T., D.N.H., D.M., C.F.P., A.Y.W., H.W., D.B., R.E., C.C., D.N., T.V., W.B.I., G.S.B., A.M.F., P.A.F., A.G.L., P.F.C., U.M., contributed samples and scientific advice. Y.S.J, M.G., I.M., M.R.S and P.J.C wrote the manuscript. M.R.S and P.J.C directed the overall research.