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Comparative Analysis of Germline and Somatic Micro-lesion Mutational Spectra in 17 Human Tumour Suppressor Genes

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Abstract

Mutations associated with tumorigenesis may either arise somatically or can be inherited through the germline. In this study, we performed a comparison of somatic, germline and shared (found in both soma and germline) mutational spectra for 17 human tumour suppressor genes which included missense single base-pair substitutions and micro-deletions/micro-insertions. Somatic and germline mutational spectra were similar in relation to C.G>T.A transitions but differed with respect to the frequency of A.T>G.C, A.T>T.A and C.G>A.T substitutions. Shared missense mutations were characterised by higher mutability rates, greater physicochemical differences between wild-type and mutant amino acid residues, and a tendency to occur in evolutionarily conserved amino acid residues and within CpG/CpHpG oligonucleotides. Mononucleotide runs (≥ 4 bp) were identified as hotspots for shared micro-deletions/micro-insertions. Both germline and somatic micro-deletions/micro-insertions were found to be significantly overrepresented within the 'indel hotspot' motif, GTAAGT. Using a naïve Bayes' classifier trained to discriminate between somatic, recurrent somatic, germline, shared and recurrent shared missense mutations, 63.1% of mutations in our dataset were correctly recognized. Using this classifier to analyse an independent dataset of probable driver mutations, we concluded that ~50% of these somatic missense mutations possess features consistent with their being either shared or recurrent, suggesting that a disproportionate number of such lesions are likely to be drivers of tumorigenesis.

Key Words: germline and somatic mutational spectra; tumour suppressor genes; recurrent mutation; mutation hotspot; non-B DNA; driver mutations

Introduction

A major distinction to be made between somatic and germline mutations is that the former occur during mitotic cell cycles whereas the latter are generally meiotic in origin. In addition, whilst somatic cancer-causing gene lesions come to clinical attention by conferring a growth advantage upon the affected cells or tissue, germ-line gene mutations causing inherited disease normally come to attention by conferring a disadvantage upon the individual, usually through haploinsufficiency. Finally, whereas inherited disease usually implies only one or two pathological mutations at a specific locus, cancer is often characterized by multiple somatic mutations distributed genome-wide. Those somatic mutations which confer a growth advantage on the cells in which they occur, which are positively selected for in the emerging tumour mass and which have therefore been causally implicated in tumorigenesis, are termed 'driver' mutations [Stratton *et al.*, 2009]. By contrast, those mutations which do not confer any growth advantage and have not been subject to selection during tumorigenesis, are termed 'passenger' mutations [Stratton *et al.*, 2009]. Such passenger mutations may arise at high frequency as a consequence either of increased genomic instability or simply due to the considerable number of cell divisions required to convert a single transformed cell into a clinically detectable tumour [Lengauer *et al.*, 1998; Boland and Ricciardiello, 1999; Simpson 2008; Parmigiani *et al.* 2009; Stratton *et al.*, 2009].

Despite these basic differences, the mutational spectra (and hence the underlying mutational mechanisms) associated with single base-pair substitutions [Krawczak *et al.*, 1995; Schmutte and Jones, 1998; Cole *et al.*, 2008; Lobo *et al.*, 2009], micro-deletions and micro-insertions [Jego *et al.*, 1993; Greenblatt *et al.* 1996] and gross gene rearrangements [Oldenburg *et al.*, 2000; Kolomietz *et al.*, 2002] in specific genes often appear to exhibit marked similarities between the germline and the soma. Further, certain triplet repeats associated with a number of inherited human conditions are known to be unstable in both the germline and somatic tissues, a finding

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3 which serves to explain not only the phenomenon of genetic anticipation characteristic of these
4 disorders but also their inherent inter-individual clinical variability [Giovannone et al., 1997;
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6 Leeflang et al., 1999; Martorell et al., 2000; Sharma et al., 2002; Pollard et al., 2004]. However,
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8 by contrast, highly variable human minisatellites can display markedly different degrees of
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10 instability between the soma and the germline [Buard et al., 2000; Stead and Jeffreys, 2000;
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12 Shanks et al., 2008]. These studies notwithstanding, few attempts have so far been made to
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14 compare the nature, location and relative frequency of germline and somatic mutations.
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20 Human cancer genes usually harbour either somatic or germline mutations [Goode et al., 2002;
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22 Futreal et al., 2004; Vogelstein and Kinzler, 2004]. There is, however, one category of cancer
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24 gene, broadly termed tumour suppressors, that by virtue of their being mutated in both the
25
26 germline and the soma, provides us with an ideal model system to compare somatic vs. germline
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28 mutational spectra [Futreal et al., 2004]. Tumour suppressor genes, defined as “genes that sustain
29
30 loss-of-function mutations in the development of cancer” [Haber and Harlow, 1997], are
31
32 involved in the regulation of a diverse array of different cellular functions including cell cycle
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34 checkpoint control, detection and repair of DNA damage, protein ubiquitination and degradation,
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36 mitogenic signalling, cell specification, differentiation and migration, and tumour angiogenesis
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38 [Sherr, 2004]. They encode proteins with a regulatory role in cell cycle progression (e.g. Rb),
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40 DNA-binding transcription factors (e.g. p53) and inhibitors of cyclin-dependent kinases required
41
42 for cell cycle progression (e.g. p16). In inherited cancer syndromes, the mutational inactivation
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44 of both tumour suppressor alleles is required to change the phenotype of the cell. This ‘two hit
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46 hypothesis’ provides the basis for our mechanistic understanding of tumour suppressor gene
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48 mutagenesis: a first (inherited) mutation in one tumour suppressor allele is followed by the
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50 somatic loss of the remaining wild-type allele via a number of different mutational mechanisms
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52 [Knudson, 2001]. Whereas the inherited lesion is usually fairly subtle, the second (somatic) hit
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54 may also involve the deletional loss of the entire gene or even a substantial portion of the
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3 chromosome involved. Alternatively, both 'hits' may constitute somatic mutations: whatever the
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5 actual mechanism, the end result is the same – the loss or inactivation of both gene copies. Some
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7 interplay may however occur between the soma and the germline in that the location of the
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9 germline mutation can in some instances influence the nature, frequency and location of the
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11 subsequent somatic mutation [Lamlum et al., 1999; Groves et al., 2002; Latchford et al., 2007;
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13 Dallosso et al., 2009].
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17 Tumour suppressor genes are often somatically inactivated by mutational mechanisms that are
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19 almost exclusively confined to the soma and which are found only infrequently in the germline
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21 (e.g. gross mutations characterized by loss of heterozygosity, epi-mutations such as methylation-
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23 mediated promoter inactivation, and micro-lesions within highly repetitive sequence elements
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25 that are consequent to microsatellite instability). However, a typical spectrum of somatic
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27 mutations associated with tumorigenesis may also include gross rearrangements, copy number
28
29 variation, and various types of micro-lesion (e.g. micro-deletions, micro-insertions and indels)
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31 including single base-pair substitutions [Loeb and Harris, 2008; Stratton et al., 2009]. Although
32
33 the somatic micro-lesions are often quite similar to their germline counterparts, few studies of
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35 tumour suppressor genes have so far attempted to compare and contrast germline and somatic
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37 mutational spectra with respect to these relatively subtle types of mutation. However, such
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39 studies have indicated that germline and somatic micro-lesions can display remarkable
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41 similarities in terms of mutation type, location and relative frequency of occurrence, and hence
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43 by inference the putative underlying mechanisms of mutagenesis [Marshall et al., 1997; Ali et
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45 al., 1999; Gallou et al., 1999; Richter et al., 2003; Upadhyaya et al., 2004; Glazko et al., 2004;
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47 Tartaglia et al., 2006; Baser et al., 2006; Upadhyaya et al., 2008].
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55 We attempt here a first formal comparison between germline and somatic micro-lesion
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57 mutational spectra for a total of 17 different human tumour suppressor genes [*APC* (MIM#
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59 611731), *ATM* (MIM# 607585), *BRCA1* (MIM# 113705), *BRCA2* (MIM# 600185), *CDHI*
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3 (MIM# 192090), *CDKN2A* (MIM# 600160), *NF1* (MIM# 162200), *NF2* (MIM# 607379),
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5 *PTCH1* (MIM# 601309), *PTEN* (MIM# 601728), *RBI* (MIM# 180200), *STK11* (MIM# 602216),
6
7 *TP53* (MIM# 191170), *TSC1* (MIM# 605284), *TSC2* (MIM# 191092), *VHL* (MIM# 608537) and
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9 *WT1* (MIM# 607102)].
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15 **Materials and Methods**

16 *Sources of germline and somatic mutation data*

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18 Data on germline and somatic micro-lesions (viz. missense mutations, micro-deletions and
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20 micro-insertions involving ≤ 20 bp) were collated for 17 different human tumour suppressor
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22 genes. Germline mutation data were obtained from the Human Gene Mutation Database
23
24 [HGMD; <http://www.hgmd.org>; Stenson et al., 2009]. Somatic mutation data were compiled
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26 from a number of different sources including online somatic mutational databases viz. *Catalogue*
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28 *of Somatic Mutations in Cancer* (<http://www.sanger.ac.uk/genetics/CGP/cosmic>; *RBI* and
29
30 *PTEN*), the *Breast Cancer Information Core* (<http://research.nhgri.nih.gov/bic>; *BRCA1*), the *RBI*
31
32 *Gene Mutation Database* (<http://www.verandi.de/joomla>; *RBI*), the *International NF2 Mutation*
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34 *Database* (<http://www.hgmd.cf.ac.uk/nf2>; *NF2*), the *CDKN2A Database*
35
36 (<https://biodesktop.uvm.edu/perl/p16>; *CDKN2A*) and the *IARC TP53 Mutation Database*
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38 (<http://www-p53.iarc.fr>; *TP53*), the *VHL Mutations Database* (<http://www.umd.be/VHL/>), and
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40 data privately communicated by Eamonn Maher (*VHL*) and Gareth Evans (*NF2*). Additional
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42 somatic mutation data [for *APC*, *ATM*, *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTCH1*, *STK11*, *TSC1*,
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44 *TSC2* and *WT1*] were obtained by searching PubMed.
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53 To be regarded as *bona fide* somatic mutations, and therefore suitable for inclusion in this
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55 analysis, reported lesions had to have been shown not only to be present in a tumour tissue but
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57 also to be absent from a non-tumour tissue (usually blood) from the same patient. Hence,
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59 mutational data derived from ‘sporadic’ patients were not included unless a non-tumour tissue
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3 had also been examined in order to exclude the possibility that the lesions detected were
4 constitutional in origin. Depending upon the number of independent occurrences, f , of a given
5 somatic or shared mutation described in the literature, these mutation types were further
6 subdivided into two categories: *recurrent mutations* ($f > 1$) and *non-recurrent mutations* ($f = 1$). At
7 the time this study was initiated (October 2006), the number of available germline and somatic
8 missense mutations for each of the 17 studied tumour suppressor genes were as listed in Table 1.
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18 The analysis reported here focussed exclusively on missense mutations and micro-deletions/
19 micro-insertions. Nonsense mutations in tumour suppressor genes have already been addressed
20 in the context of a general meta-analysis of this type of lesion [Mort et al., 2008]. Indels
21 (representing a combination of micro-deletion and micro-insertion) were excluded from the
22 analysis owing to their paucity.
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32 *Control datasets of potential mutations*

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34 For every tumour suppressor gene examined, all possible single base-pair substitutions in the
35 gene coding sequence that (i) could potentially have given rise to a missense mutation and (ii)
36 were not already included in either of the corresponding observed somatic and/or germline
37 mutational spectra, were generated. These 'potential missense mutations' were used as a control
38 dataset.
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46 For each tumour suppressor gene, a matching control dataset of 'potential micro-deletions' was
47 also generated by randomly selecting a first breakpoint and then choosing the length of the
48 simulated micro-deletion (and therefore, the position of the second breakpoint) by reference to
49 the probability distribution calculated for micro-deletions (from 1 bp to 20 bp) observed in the
50 corresponding dataset of mutations. A matching dataset of micro-insertions was generated in
51 similar fashion, with the sites of insertion being randomly selected. Since some of the micro-
52 deletion/micro-insertion breakpoints occurred within an intron, extended cDNA sequences
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3 comprising exons and additional flanking intron sequences were used to generate corresponding
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5 control datasets.
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10 *Grantham scores*

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12 The ‘Grantham score’ or ‘Grantham difference’ [Grantham, 1974] measures the chemical
13
14 difference between wild-type and mutated amino acid residues in terms of their side chain
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16 composition (i.e. the weight ratio of non-carbon components in end-groups or rings to carbons in
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18 side chains), polarity (i.e. basic, acidic or nonpolar depending upon side chain charge) and
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20 molecular volume.
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25 On average, the physicochemical differences manifested by orthologous amino acid
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27 substitutions that have accumulated over evolutionary time will tend to be relatively small. By
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29 contrast, disease-causing substitutions are expected to exhibit higher Grantham scores, indicative
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31 of more dramatic physicochemical differences between the wild-type and mutated amino acid
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33 residues [Krawczak et al., 1998]. The values tabulated by Grantham [1974] were used in this
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35 study to calculate a median Grantham score for each set of missense mutations for each tumour
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37 suppressor gene.
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43 *Degree of evolutionary conservation*

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45 Amino acid residues that are highly conserved in orthologous proteins frequently represent sites
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47 of structural or functional importance. Hence, such highly conserved amino acid residues/protein
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49 regions often constitute hotspots for observed pathological mutations as a consequence of
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51 phenotype selection (rather than intrinsic mutability). To assess the degree of evolutionary
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53 conservation of those codons affected by somatic/germline mutations, orthologous tumour
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55 suppressor cDNA and protein sequences from different vertebrate species were retrieved from
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57 NCBI’s Entrez Gene database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). The species
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3 used as a source of these cDNA and protein sequences are listed in Supp. Table 1 for each
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6 tumour suppressor gene/protein. ClustalX (<http://www.clustal.org/>) was used to align the protein
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9 sequences. A program was written to replace all amino acids in the protein alignments by cDNA-
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12 derived codons, thereby avoiding the introduction of gaps within codons.

13 The evolutionary constraints acting upon the 17 human tumour suppressor genes at the codon
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15 level were inferred by calculating the $\frac{K_a}{K_a + K_s}$ ratio for each codon where K_s and K_a are
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18 respectively the relative numbers of synonymous and nonsynonymous substitutions between
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21 codons in two aligned sequences [Walker et al., 1999]. If two aligned codons required more than
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23
24 one substitution to be transformed into each other, then the minimum number of substitutions
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26 was assumed, and the most parsimonious path was determined using a PAM100 matrix and the
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28 Nei & Gojobori [1986] pathway method. Gaps inserted into the non-human vertebrate
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30 orthologous cDNA sequences during alignment were treated as being equivalent to a non-
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33 synonymous substitution. Codons that were not present in the human cDNA sequence were not
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36 considered. A value representing the median level of evolutionary conservation across all codons
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38 was then derived for each mutational spectrum.

41 42 43 *Relative mutability rates*

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45 To assess the likelihood of observing a certain nucleotide change in a given position and in a
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48 specific context, two tabulated measures of the nearest neighbour-dependent mutation rate were
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50 employed. The first was derived from 20,200 single base-pair substitutions inferred from
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52 alignments of paired human gene/pseudogene sequences [Hess et al., 1994]. This was termed the
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54 *non-disease-associated mutability rate* and, since it approximates to the neutral mutation
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56 frequency, it should reflect the intrinsic mutability of the underlying DNA sequence. One would
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58 expect the non-disease-associated mutation rates associated with pathological mutations to be
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3 low implying that these specific substitutions are much less likely to occur as neutral
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5 substitutions.
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8 The nearest neighbour-dependent mutation rates derived from germline single base-pair
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10 substitutions [using data from the Human Gene Mutation Database (HGMD); Stenson et al.,
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12 2009] by Krawczak et al. [1998] were used as an approximation of the *disease-associated*
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14 *mutability rate*. This mutation rate is a function of selection for loss of biological function as
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16 well as the underlying intrinsic mutability of the DNA sequence.
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19 20 21 22 *Repetitive sequence elements*

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24 A variety of repetitive sequence elements have been reported in association with human gene
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26 mutations causing both inherited disease and cancer. Direct and inverted repeats and symmetric
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28 elements [see Chuzhanova et al. 2003 for definitions] of length ≥ 8 bp, and less than 21 bp apart,
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30 capable of forming non-B DNA structures, were therefore sought within the extended cDNA
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32 sequences (comprising exons and up to ± 85 bp of flanking sequence) using purposely designed
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34 software. In addition, DNA sequences were screened for the presence of mononucleotide runs of
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36 ≥ 4 bp.
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43 44 *Mutation descriptors*

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46 Each missense mutation was ascribed various descriptors indicating (a) the type of mutation [i.e.
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48 shared mutation (i.e. found to occur both somatically and in the germline); exclusively somatic;
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50 exclusively germline; shared recurrent mutation (i.e. found to occur not only in the germline but
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52 also somatically on more than one occasion; somatic recurrent mutations (recorded in the soma
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54 more than once, but not in the germline); potential mutation (as defined above)] and (b) its
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56 location [i.e. C \rightarrow T and G \rightarrow A within a CpG dinucleotide or within a CpHpG trinucleotide
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58 (where H=A, C or T) or in a repeat sequence (as described above)]. Mutations that have been
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3 reported as being exclusively somatic or exclusively germline will henceforth be referred to
4 simply as 'somatic' and 'germline', respectively. The shared mutations, comprising the overlap
5 between the somatic and germline mutations, may be visualized in the form of a Venn diagram
6 (Figure 1). All somatic missense (including shared) mutations were further described as being
7 either recurrent or non-recurrent (in the soma, see above; Figure 1). No such division was made
8 for the relatively small number of recurrent micro-deletions and micro-insertions available; both
9 recurrent and non-recurrent somatic mutations were therefore included in either the somatic or
10 the shared datasets and labelled accordingly (Figure 1).
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22 All micro-lesions (*viz.* missense mutations, micro-deletions and micro-insertions) in each gene
23 were also labelled with respect to their occurrence within a region spanning a repetitive element
24 or mononucleotide run including ± 5 bp of flanking sequence. If a missense mutation (or at least
25 one micro-deletion/micro-insertion breakpoint) was found to occur within this extended region,
26 the micro-lesion was labelled as being found in association with the corresponding type of
27 repeat.
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39 *Assessing the statistical significance of the results generated*

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41 To assess the similarity (or dissimilarity) of the germline and somatic mutational spectra with
42 respect to (i) the frequency with which the missense mutations were located within CpG/non-
43 CpG dinucleotides or CpHpG/non-CpHpG trinucleotides and (ii) the frequency with which the
44 micro-deletions/micro-insertions were found within/outwith repeats, the various non-overlapping
45 mutation datasets (bearing specific descriptors) were compared by means of the χ^2 test. Since the
46 normality assumption did not hold for the datasets studied, the Wilcoxon rank-sum test was used
47 to compare and contrast missense mutational spectra with respect to the Grantham score, degree
48 of evolutionary conservation, and both the non-disease- and disease-associated mutability rates.
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3 The permutation-based method [Olshen and Jain, 2002] was used to estimate the significance
4 of our findings and to allow for multiple testing wherever appropriate. For each comparison, the
5 null hypothesis [viz. no overall difference between two groups of mutations (e.g. somatic and
6 potential) with respect to the specific property in question (e.g. occurrence in CpG or non-CpG
7 nucleotides)], was tested for, either in the context of each gene or all genes combined. χ^2 or rank-
8 sum statistics were calculated for the observed germline and somatic mutations as well as for
9 10,000 control sets of mutations created from the original sets by random permutation of the
10 assigned mutational descriptors (e.g. randomly chosen mutations labelled as 'somatic' were re-
11 labelled as 'germline'; randomly chosen mutations labelled as 'shared' were re-labelled as
12 'somatic', etc.). The test statistic (χ^2 or rank-sum) for the original datasets that exceeded the 95th
13 percentile of χ^2 maxima for 10,000 control sets was deemed to be statistically significant; the
14 corresponding p-value was termed the 'gene-wise' p-value. To allow for multiple testing in those
15 cases where specific mutations in all genes were combined, a Bonferroni correction was applied;
16 the corresponding p-value was termed the 'experiment-wise' p-value.

38 *Naïve Bayes classifier*

39
40 A decision tree classifier known as a Naïve Bayes tree [NBTree; Kohavi, 1996], implemented in
41 the Weka machine learning package [Witten and Frank, 2005], was trained to discriminate
42 between somatic, germline, shared, recurrent somatic and recurrent shared missense mutations.
43 Each mutation was described by a total of six features including the degree of evolutionary
44 conservation, the non-disease-associated and disease-associated relative mutability rates,
45 Grantham score, and occurrence in CpG/CpHpG, non-CpG/non-CpHpG doublets/triplets or in
46 repeats/mononucleotide runs. Ten-fold cross-validation was used to assess the accuracy of
47 classification. The mutation datasets were balanced using random oversampling [Kotsiantis et
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3 al., 2006] by replicating random instances from the minority classes until all classes were
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5 represented by the same number of instances as the majority class.
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10 11 12 **Results and Discussion**

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15 The availability of both germline and somatic mutational spectra from tumour suppressor genes
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17 provides us with an ideal opportunity to study the nature of mutation of the same gene sequences
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19 in both the germline and the soma. The analysis reported here explores for the first time the
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21 similarities and differences exhibited by the germline, somatic (and shared) micro-lesion
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23 mutational spectra in 17 human tumour suppressor genes. The study presented here focussed
24
25 upon missense mutations and micro-deletions as well as micro-insertions. Nonsense mutations in
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27 tumour suppressor genes have already been addressed elsewhere in the context of a general
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29 meta-analysis of this type of lesion [Mort et al., 2008].
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37 *Characteristics of germline and somatic missense mutations with respect to mutation type*

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39 Taken together, the combined mutational spectra for all 17 tumour suppressor genes contained
40
41 twice as many somatic (61%) as germline (31%) mutations. For five genes (*APC*, *CDKN2A*,
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43 *NF2*, *PTEN* and *TP53*), a predominance of somatic over germline mutations was noted, with the
44
45 *TP53* gene having the highest proportion of somatic mutations (92%). For the majority of genes,
46
47 however (namely *ATM*, *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTCH1*, *RBI*, *STK11*, *TSC1*, *TSC2*, *VHL*
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49 and *WT1*), the analysed dataset included more germline than somatic mutations, with >97% of all
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51 mutations in the *BRCA1*, *NF1*, *TSC2* and *WT1* genes being germline in origin.
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56 Shared mutations are of particular interest because identical mutational mechanisms operating
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58 in the germline and the soma may be inferred for such lesions. The expected number of shared
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60 mutations for each gene was calculated as $p_{\text{somatic}} \times p_{\text{germline}} \times (\text{total number of mutations})$,

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3 where p denotes the relative frequencies of somatic and germline mutations. Although the
4
5 proportion of shared mutations varies markedly between genes (from 0% to 25% of the total),
6
7 only two genes (*TP53* and *VHL*) were found to have a higher than expected number of shared
8
9 mutations as calculated above.
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12 13 14 15 *Patterns of germline and somatic missense mutations by mutation type*

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17 Missense mutations were characterised by a predominance of transitions over transversions
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19 (Figure 2). The transition:transversion ratio was at its highest for shared recurrent mutations (3.5)
20
21 and shared non-recurrent mutations (2.7). By contrast, the transition:transversion ratio for the
22
23 control group (i.e. potential mutations) was 0.85. Significant differences in the
24
25 transition:transversion ratio were observed between all mutation types ($p < 0.05$) with the
26
27 exception of germline vs. shared mutations (Figure 2).
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32 Not surprisingly, a strong positive correlation was noted between somatic and shared
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34 mutational spectra (Pearson's correlation $r = 0.986$, $p = 2.91 \times 10^{-4}$) with respect to the frequencies
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36 of six mutational changes viz. A.T>C.G, A.T>G.C, A.T>T.A, C.G>A.T, C.G>G.C and
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38 C.G>T.A. Weaker negative correlations were found between somatic mutations and the control
39
40 dataset of mutations ($r = -0.887$, $p = 0.019$) and between shared and the control ($r = -0.837$,
41
42 $p = 0.038$) mutational spectra, indicative of the non-randomness of somatic mutation.
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47 C.G>T.A transitions constituted the most frequent type of mutation in shared (46%), germline
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49 (29%) and somatic (25%) mutational spectra, significantly higher proportions than noted in the
50
51 spectrum of mutations within our control dataset (13%, $p < 0.001$) (Figure 2). Intriguingly, the
52
53 number of A.T>G.C mutations was significantly higher (28%) in the germline as compared to
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55 the somatic (16%), shared (17%) and control (16%) mutational spectra (Figure 2). A.T>C.G
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57 mutations were significantly under-represented in the shared mutational spectrum (7%, $p < 0.001$)
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59 as compared to the other spectra whereas A.T>T.A mutations were under-represented (7%,
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4 p<0.001) in both the germline and shared mutational spectra compared to both somatic and
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6 potential mutations (Figure 2). Finally, C.G>A.T mutations were significantly underrepresented
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8 in the germline mutational spectrum (10%) as compared to the somatic (16%, $p=1.2\times 10^{-5}$) and
9
10 potential (15%, $p=2.6\times 10^{-5}$) spectra. Thus, the main similarity between the somatic and germline
11
12 missense mutational spectra was in relation to C.G>T.A transitions whereas the main differences
13
14 between these spectra involved the A.T>G.C, A.T>T.A and C.G>A.T mutations. It should be
15
16 noted that the patterns of somatic nucleotide substitution exhibited by the 17 tumour suppressor
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18 genes studied here were markedly different from the genome-wide patterns of somatic nucleotide
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20 substitution previously observed in various cancer genome sequencing studies [Sjöblom et al.,
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25 2006; Greenman et al., 2007; Kan et al., 2010].
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29 *CpG- and CpHpG-located missense mutations*

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32 The CpG dinucleotide is a well known mutational hotspot in the human genome as a
33
34 consequence of the spontaneous (and endogenous) deamination of 5-methylcytosine. In addition,
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36 Lister et al. [2009] reported abundant DNA methylation in CpHpG trinucleotides in the human
37
38 genome, where H is either A, C or T, raising the possibility that CpHpG might also be a
39
40 generalized mutation hotspot [Cooper et al., 2010].
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44 The proportion of missense mutations that were either C>T or G>A within CpG or CpHpG
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46 oligonucleotides in the 17 tumour suppressor genes was found to vary between 0% and 100%
47
48 (Table 2). This wide range in values may be attributed to the small size of some of the gene
49
50 mutation datasets under study. Importantly, the CpG and CpHpG oligonucleotides were found to
51
52 be disproportionately likely to harbour shared mutations; thus, 34% of shared recurrent
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54 mutations and 21% of shared non-recurrent mutations were C>T and G>A mutations in CpG
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56 dinucleotides with an additional 10% and 9% of mutations, respectively, occurring within
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58 CpHpG trinucleotides. Since driver mutations tend to occur disproportionately frequently within
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3 CpG dinucleotides [Talavera et al., 2010], we postulate that missense mutations identified as
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5
6 being shared are highly likely to be driver mutations.
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8 Significant differences were noted between the relative frequencies of CpG- and CpHpG-
9
10 located mutations for somatic, germline, shared, somatic recurrent and shared recurrent missense
11
12 mutations (Supp. Table 2).
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15 We have previously shown that 18.2% and 9.9% of all missense/nonsense mutations recorded
16
17 in the HGMD are C>T and G>A transitions in CpG and CpHpG oligonucleotides respectively
18
19 [Cooper et al., 2010]. In the present study, we observed that the mutational spectra of shared and
20
21 shared recurrent missense mutations in tumour suppressor genes were both found to be
22
23 significantly enriched in CpG-located mutations (χ^2 -test; p-values, 0.028 and 1.1×10^{-9}
24
25 respectively). This implies that the CpG dinucleotide is a generalized mutation hotspot in both
26
27 the soma and the germline as a consequence of the endogenous mutational mechanism of
28
29 methylation-mediated deamination of 5-methylcytosine. By contrast, the number of CpG-located
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31 mutations was significantly underrepresented (χ^2 -test; p-values $< 5 \times 10^{-14}$) in the other mutational
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33 spectra (i.e. non-recurrent somatic, somatic recurrent and germline mutations) by comparison
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35 with HGMD data. To perform these comparisons, missense mutations (Table 2) and nonsense
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37 mutations [previously reported in Mort et al., 2008; see Table 6 therein] in all 17 tumour
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39 suppressor genes were combined. The proportion of shared recurrent missense mutations in
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41 tumour suppressor genes that were CpHpG-located was found to be significantly higher
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43 ($p=0.023$) than for mutations recorded in the HGMD whereas CpHpG-located somatic and
44
45 recurrent somatic mutations were significantly under-represented ($p < 4 \times 10^{-10}$). Significant
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47 enrichment in CpHpG-located mutations was observed for germline mutations as compared to
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49 somatic mutations ($p < 3 \times 10^{-10}$) consistent with the reported decrease in CpHpG methylation in
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51 differentiated cells [Lister et al., 2009]. In summary, germline and shared missense mutations
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53 were found to be significantly enriched at CpG and CpHpG oligonucleotides.
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3 The numbers of somatic and shared C>T and G>A transitions recorded within CpG
4 dinucleotides for each gene (Table 2) did not correlate with the numbers of CpG dinucleotides
5 found in these genes ($r < -0.5$, $p > 0.127$) and hence do not simply reflect intragenic CpG
6 frequency. A weak positive correlation between CpG-located mutations and the number of genic
7 CpG dinucleotides was however noted for germline mutations ($r = 0.489$, $p = 0.046$) indicating that
8 CpG methylation is not entirely unrelated to the number of CpG dinucleotides, at least with
9 respect to the germline; the relationship is however clearly more complex in the soma, possibly
10 due to inter-tissue differences in gene methylation patterns [Tornaletti and Pfeifer, 1995] or
11 transcription-coupled repair [Rubin and Green, 2009].
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24 No correlation was found between the numbers of somatic, germline and shared mutations
25 recorded within CpHpG trinucleotides and the corresponding numbers of CpHpG trinucleotides
26 for these genes ($r = -0.316, 0.373, -0.414$; p -values 0.281, 0.216 and 0.098, respectively)
27 indicating that mutation within CpHpG trinucleotides is likely to be very much a gene-specific
28 phenomenon (presumably dependent on both the extent and the degree of spatial localization of
29 CpHpG methylation in the germline and/or soma).
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38 Finally, the number of CpG dinucleotides in the various tumour suppressor genes studied
39 (Table 2) was not found to correlate with gene length ($r = 0.3$, p -value=0.241). By contrast, we
40 found a significant correlation ($r = 0.885$, p -value= 2.35×10^{-6}) between tumour suppressor gene
41 length and the number of CpHpG trinucleotides (excluding those with mutations), indicating that
42 the tumour suppressor genes under study possess a similar density of CpHpG trinucleotides per
43 unit length. We surmise that the factors that govern the establishment of the methylation pattern
44 of CpHpG trinucleotides are likely to be quite complex.
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57 *Evolutionary conservation of tumour suppressor genes in relation to the sites of somatic and*
58 *germline missense mutations*
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3 For all 17 tumour suppressor genes, the degree of evolutionary conservation, as measured by
4
5 Ka/Ks , was less than unity, indicating that these genes (and proteins) have been highly
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7
8 conserved evolutionarily as a consequence of the action of purifying selection. Indeed, the
9
10 degree of evolutionary conservation displayed by most of the studied genes was markedly lower
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12 than the average (~0.18) noted in a comparison of 1880 human, rat and mouse gene orthologues
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14 [Makalowski and Boguski, 1998]. However, three genes (*CDKN2A*, *BRCA1* and *BRCA2*) were
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16 found to exhibit a higher rate of evolutionary conservation than the average between human and
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18 rodents.
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24 The evolutionary conservation of each mutated codon was inferred by calculating the $\frac{Ka}{Ka + Ks}$
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26 ratio; for each gene/spectrum, the mean value was then calculated across all mutations in the
27
28 corresponding gene/spectrum. Shared recurrent missense mutations were found to occur
29
30 disproportionately in highly conserved amino acid residues (mean degree of evolutionary
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32 conservation, 0.072) followed by shared non-recurrent mutations (0.138), somatic recurrent
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34 (0.169), germline (0.175), non-recurrent somatic (0.265), and control dataset mutations (0.255).
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36 The observed differences in the degree of evolutionary conservation for the different mutational
37
38 spectra are shown in Supp. Table 2. These quite specific findings are consistent with the
39
40 previously reported general tendency for cancer-associated mutations to occur frequently at
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42 evolutionarily conserved sites [Greenblatt et al., 2003; Tavgigian et al., 2009; Talavera et al.,
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44 2010].
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51 Somatic non-recurrent mutations were found to occur in codons characterized by the highest
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53 mean value of $\frac{Ka}{Ka + Ks}$ ratios as compared not only to the shared recurrent and shared non-
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55 recurrent mutations (see above) but also to the mutations within the control dataset. This is
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57 consistent with the interpretation that a high proportion of non-recurrent somatic mutations, and
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3 most notably those which are located in less evolutionarily conserved regions, are likely to be
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6 'passenger' mutations.
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10 *Missense mutations in relation to the disease- and non-disease-associated substitution rates*

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12 Employing alignments of paired human gene/pseudogene sequences, Hess et al. [1994] derived
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14 relative (non-disease-associated) nearest-neighbour-dependent mutability rates using the lowest
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16 frequency substitution type, C(T>G)A/T(A>C)G, as a baseline. These mutability rates were
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18 found to vary over a 52-fold range, with unity being assigned to the lowest frequency
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20 substitution type. This *non-disease-associated* mutability rate approximates to the neutral
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22 mutation frequency and hence reflects the intrinsic mutability of the underlying DNA sequence.
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24 Depending upon the observed nearest-neighbour context, we retrieved the corresponding non-
25
26 disease-associated mutability rate (from the data of Hess et al. 1994) for each mutation (either
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28 observed or from the control dataset) and calculated the median value for each mutational
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30 spectrum. These median values are indicative of the relative mutability of each tumour
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32 suppressor gene. The median values were found to vary between 4 (*NF2*) and 8.9 (*STK11*) for
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34 somatic mutations, 4.1 (*TP53*) and 10.1 (*WT1*) for germline mutations, and 7.2 (*RBI*) and 11
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36 (*PTEN*) for shared mutations (values given only for genes with more than three mutations in the
37
38 corresponding category; see Supp. Table 3, indicating that many of the median values are quite
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40 low and hence the corresponding mutations are unlikely to be neutral.
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49 When data from all 17 genes were combined, shared recurrent mutations were found to be
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51 characterised by intrinsically low non-disease-associated mutability (median=11), followed by
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53 even lower median mutability values for shared non-recurrent mutations (7.9), germline
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55 mutations (7.2), somatic recurrent and non-recurrent (4.7) and control dataset mutations (4.1).
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57 Such low median mutability values across all groups indicates that at least half of the mutations
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59 within observed triplets are unlikely to be neutral in the sense defined by Hess et al. [1994] and
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3 hence are not simply explicable in terms of intrinsic DNA mutability. The low median mutability
4 values for the control dataset of mutations within tumour suppressor genes reflect the high level
5 of evolutionary conservation manifested by tumour suppressor gene coding sequences across
6 different species, implying that any mutation within a triplet characterized by a low non-disease-
7 associated mutation rate is very likely to have pathological consequences and would thus be
8 subject to purifying selection.
9

10
11 In contrast to the non-disease-associated mutability rate (which is purely a reflection of the
12 intrinsic DNA mutability), the disease-associated mutability rate reflects (in addition to the
13 intrinsic DNA mutability) the increased likelihood of coming to clinical attention conferred by
14 the loss of biological function. The C(G>T)T mutation is one of the most frequent types of
15 mutation associated with the loss of biological function [disease-associated mutability rate
16 10.255; Krawczak et al., 1998] but occurs much less frequently among neutral mutations [non-
17 disease-associated mutability rate 4.4; Hess et al., 1994].
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21 For each tumour suppressor gene and each mutational spectrum, the disease-associated median
22 mutability values were calculated using mutability rates derived from Krawczak et al. [1998].
23 The disease-associated median value was found to be 0.85 for the germline mutations. The
24 highest and lowest disease-associated median values for the mutation rates were noted for
25 somatic mutations in the *STK11* gene (1.7; Supp. Table 3) and for germline mutations in the
26 *TP53* (0.42) gene (values given only for genes with more than three mutations in the
27 corresponding category). We found that shared recurrent and shared non-recurrent mutational
28 spectra were characterized by higher median values of the disease-associated mutability rates
29 (1.42 and 1.01 respectively) whereas somatic non-recurrent, somatic recurrent and control
30 dataset mutations exhibited lower median mutability rates (0.5, 0.5 and 0.4 respectively) as
31 compared to germline mutations (0.85). The finding that the shared mutations (which, by
32 definition, occur in both the germline and the soma) are characterized by higher disease-
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3 associated mutability rates is not surprising since mutations that occur with the highest
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5 probability are among those most likely to be shared.
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8 We postulated that those mutations which occur both in the germline and the soma, and which
9
10 are characterised by higher disease-associated mutability rates are disproportionately likely to be
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12 drivers of tumour development. Consistent with this postulate, somatic recurrent and non-
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14 recurrent mutational spectra are characterized by lower median disease-associated mutability
15
16 rates as compared to the germline spectrum. However, given that higher disease-associated
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18 mutability rates are a characteristic feature of driver mutations, a certain proportion of the
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20 somatic mutations, namely those characterised by higher disease-associated mutability rates, may
21
22 correspond to functionally significant driver mutations.
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27 In assessing the significance of our results, it was appropriate to consider the possibility that
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29 somatic mutations might display quite different nearest-neighbour-dependent disease-associated
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31 mutability rates from germline mutations. However, since a good correlation was observed
32
33 between the mutability rates derived from inherited disease data [Krawczak et al., 1998] and the
34
35 neighbour-dependent mutability rates calculated for the somatic mutations of the 17 tumour-
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37 suppressor genes studied here (Pearson's correlation $r=0.703$, $p=6.6\times 10^{-30}$), this *caveat* appears
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39 not to be an issue.
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46 *Distribution of Grantham scores with respect to tumour suppressor gene mutations*

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48 Shared recurrent mutations were found to exhibit the largest median chemical difference value
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50 (Grantham scores) between the wild-type and mutated amino acid residues (100) followed by
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52 shared non-recurrent mutations and germline mutations (both 93), somatic recurrent (85),
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54 somatic non-recurrent (80) and potential mutations (78). Since there was an obvious trend for
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56 shared recurrent and non-recurrent mutations to cause the most dramatic chemical changes of the
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58 affected codon, we may infer that these types of lesion are also more likely to be driver
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3 mutations. However, bearing in mind that the range of theoretically possible values varies
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5 between 5 (Leu ↔ Ile) and 215 (Cys ↔ Trp), less elevated median values may simply indicate
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7 that a proportion of the mutations in each mutational spectrum are likely to be chemically less
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9 dramatic (Grantham scores <100).
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12 13 14 15 *Missense mutations occurring within repeats and runs of identical nucleotides*

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17 A number of studies have noted that single base-pair substitutions associated with inherited
18
19 disease occur disproportionately either within, or in close proximity to, repetitive sequences
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21 [Jego et al., 1993; Greenblatt et al., 1996; Tappino et al., 2009; Thomas et al., 2010; Leclercq et
22
23 al., 2010]. Hence, we wished to assess whether either germline or somatic mutations occurred
24
25 disproportionately either within, or in the vicinity (see *Mutation descriptors*) of, direct, inverted
26
27 and symmetric repeats or mononucleotide runs in the 17 tumour suppressor genes under study
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29 (Table 3, Supplementary Tables 4-6).
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34 On average, direct repeats of length ≥ 8 bp were found to cover 5.6% of the cDNA lengths of
35
36 the 17 tumour suppressor genes, the coverage varying between 2.5% (*BRCA2*) and 17% (*PTEN*)
37
38 of the respective gene sequences. The corresponding proportion of the cDNA lengths for
39
40 inverted repeats ≥ 8 bp was 8.5%, with proportions varying between *PTCH1* (4.5%) and *RBI*
41
42 (15.7%) while symmetric elements ≥ 8 bp were found to encompass 25% of the cDNA lengths
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44 (varying between 15.5% for *APC* and 44% for *PTEN*).
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49 On average, mononucleotide runs ≥ 4 bp spanned 19.9% of the cDNA lengths, varying between
50
51 9.5% (*VHL*) and 29% (*TP53*). Approximately 24% of non-recurrent somatic and 20% of
52
53 germline missense mutations were found in mononucleotide runs; these proportions were
54
55 significantly higher than noted for shared non-recurrent missense mutations (4.9%, $p \leq 1.6 \times 10^{-4}$).
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57 A greater proportion of non-recurrent somatic missense mutations was found in direct repeats
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59 (7%) as compared to recurrent somatic missense mutations (2%, $p = 8.8 \times 10^{-7}$), germline missense
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3 (4%, $p=0.028$) and potential missense mutations (3.7%, $p=8.1\times 10^{-7}$). This result may reflect the
4
5 disproportionate number of CpG/CpHpG mutations among shared and recurrent somatic
6
7 missense mutations. Further, for all mutational spectra examined (with the exception of the
8
9 shared mutations), missense mutations were preferentially found in association with inverted and
10
11 symmetric repeats as compared to the control dataset of mutations ($p<0.05$). However, no
12
13 statistically significant differences were found between mutational spectra.
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17 No correlation was observed between the number of mutations located within repeats and the
18
19 fractional length of the cDNA covered by repeats, indicating that not every repeat sequence is
20
21 mutation-prone. However, a strong correlation between the fractional length of the cDNA
22
23 covered by repeats and cDNA length of genes ($r >0.87$ and $p<10^{-6}$) served to demonstrate that
24
25 repeat density per unit length was approximately the same for all tumour suppressor genes
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27 studied.
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34 *Towards a classification of somatic and germline missense mutations*

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36 All observed mutations within each mutational spectrum were re-categorized (Supp. Table 7)
37
38 with respect to the location of mutations within CpG/CpHpG oligonucleotides, within different
39
40 types of repeat/mononucleotide runs, within both CpG/CpHpG oligonucleotides and repeats. 4×2
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42 contingency tables were then used to measure the strength of the pairwise associations between
43
44 the various mutational distributions presented in Supp. Table 7, the significance of the
45
46 associations being assessed by means of a Chi-square test. Significant ($p<0.002$) pairwise
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48 differences were noted between somatic and germline, somatic and shared, and between
49
50 germline and shared mutational spectra ($p<0.002$) with respect to the features listed above and
51
52 each of four types of repeat, indicating that these features have great discriminant potential.
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57 All somatic, germline, shared non-recurrent, recurrent somatic and shared recurrent missense
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59 mutations (each described by a combination of different features (i.e. degree of evolutionary
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3 conservation, non-disease- and disease-associated mutability rates, Grantham score,
4 CpG/CpHpG location, occurrence within repeat/mononucleotide run) were then used to train a
5 Naïve Bayes Tree classifier. 63.1% of somatic, germline, shared, recurrent somatic and shared
6 recurrent mutations were correctly classified [the area under the Receiver Operating
7 Characteristic (ROC) curve being 0.869, indicating a reasonably good classification] implying
8 that the mutation groupings differ with respect to the different features in a consistent fashion.
9 The complete Naïve Bayes Tree classifier is depicted in Supp. Figure 1.

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20 An additional non-overlapping dataset of 568 missense somatic mutations, identified in the 17
21 tumour suppressor genes under study, were extracted from a collection of 2,488 mutations
22 identified as being probable driver mutations [Carter et al., 2009]. Features such as the degree of
23 evolutionary conservation, Grantham score, mutability rates, CpG/CpHpG location, occurrence
24 within repeats/mononucleotide runs were again determined for each of these mutations.
25 Employing our classifier, 7% and 10% respectively of these 568 mutations were found to possess
26 features consistent with their being shared recurrent and shared non-recurrent mutations. In
27 addition, 32% of these probable driver mutations were found to bear features characteristic of
28 recurrent somatic mutations (i.e. mutations documented in different tumours). A further 25% of
29 the probable (somatic) driver mutations were classified as possessing features characteristic of
30 germline mutations and hence could conceivably be treated as shared mutations missing from the
31 original training dataset. The remaining 25% of mutations were classified as non-recurrent
32 somatic mutations. Using this classifier, which is based on a very modest number (6) of
33 predictive features, to analyse an independent dataset of probable driver mutations, we were able
34 to predict that ~50% of these somatic missense mutations exhibited features specific to either
35 shared or recurrent mutations, indicating that a disproportionate number of such lesions are likely
36 to be drivers of tumorigenesis. This percentage is certainly lower (79%) than that obtained by
37 Carter et al., [2009] through the application of a Random Forest Classifier based on 500 trees and
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3 >50 predictive features (using an out-of-the-bag error estimate similar to the cross-validation
4 procedure) to the set of putative 2,488 driver mutations. However, based on the results of this
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6 study, we may conclude that, in general, the mutational spectrum of driver mutations is likely to
7
8 contain a disproportionate number of somatic mutations that have germline counterparts (~17%)
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10 whilst an additional 32% of the driver mutations are likely to occur recurrently in the soma.
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17 *Truncating vs non-truncating mutations in the germline and soma*

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19 Somatic mutational spectra from the *BRCA2*, *CDKN2A*, *STK11*, *TP53* and *TSC1* genes were
20 characterized by the predominance of non-truncating (i.e. missense) lesions over truncating
21 lesions (i.e. nonsense mutations, frameshift micro-deletions, micro-insertions and indels) when
22 nonsense mutations [reported in Mort et al. (2008)] and micro-indels (excluded from previous
23 analyses) were also considered (Supp. Table 8). A similar predominance of non-truncating over
24 truncating lesions was observed for the germline mutational spectra of the *CDKN2A*, *TP53*, *VHL*
25 and *WT1* genes. In general, the ratio of non-truncating to truncating lesions was found to be
26 significantly higher in the soma (0.85) than in the germline (0.30; p-value<2.20E-16). All other
27 mutational spectra were characterized by the predominance of truncating mutations.
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43 *Occurrence of micro-deletions and micro-insertions within repeats and runs of identical* 44 *nucleotides*

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47 The mutational spectrum of micro-deletions, combined for all 17 tumour suppressor genes,
48 comprised 55% germline, 43% somatic and 2% shared mutations. The mutational spectrum of
49 micro-insertions was similar to that of micro-deletions and comprised 60% germline, 38%
50 somatic and 2% shared mutations. Approximately 77% somatic, 87% germline and 91% shared
51 micro-deletions and micro-insertions were ≤ 4 bp in length. Strong ($r = \sim 1$) correlations were
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60 noted between the distributions of micro-deletions and micro-insertions with respect to the length

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3 of the deleted/inserted fragments, both gene-wise and for all genes combined ($r>0.9$, $p<10^{-8}$) for
4 all mutational spectra.
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8 Recent studies have revealed that simple repetitive DNA sequences are not only capable of
9 adopting non-B DNA conformations and are highly mutagenic [Bacolla et al., 2004; Bacolla and
10 Wells, 2004; Chuzhanova et al., 2009]. Indeed, both direct repeats and mononucleotide runs have
11 long been known to be mutation hotspots in the *TP53* gene [Jego et al., 1993; Greenblatt et al.,
12 1996]. The number of micro-lesions occurring in the vicinity (see *Mutation descriptors*) of
13 direct, symmetric and inverted repeats (capable respectively of slipped, triplex and cruciform
14 non-B structure formation), or within mononucleotide runs (which often mediate micro-
15 deletions/micro-insertions) were therefore determined. The number of mutations found in the
16 vicinity of all three types of repeat, and within mononucleotide runs, are given in Tables 3 and
17 Supp. Tables 4-6.
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31 The highest proportion of mutations in mononucleotide runs was found for the shared (39%),
32 germline (30%) and somatic (25%) mutational spectra. Significant differences were observed
33 between shared and germline ($p=0.0002$), somatic and shared ($p=0.045$), and between all
34 mutational spectra and potential mutations ($p<0.0001$) with respect to their occurrence within
35 mononucleotide runs, confirming that these simple repeats constitute an important hotspot for
36 micro-deletions and micro-insertions in both the soma and the germline. The preponderance of
37 such mutations in mononucleotide runs is unsurprising in the context of the shared mutations
38 since all mutations that occur with high frequency within mutation hotspots are more likely to be
39 shared between the germline and the soma (as previously noted for CpG and CpHpG mutations).
40 No other types of repeat were disproportionately associated (after correction for multiple testing)
41 with micro-deletions and micro-insertions.
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Hotspots in somatic and germline mutational spectra

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3 For the purposes of this analysis, a mutation hotspot was defined as a stretch of DNA of length
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5 ≤ 20 bp where four or more independent mutational events have been reported and a significant
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7 degree ($p \leq 0.05$) of clustering of these mutations was evident for a given stretch of DNA. In this
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9 definition of a hotspot, each recurrent mutation was considered only once. The order statistics, r-
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11 scans, as described by Karlin and Macken [1991] and applied in Bacolla et al. [2006], were used
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13 to detect significant clustering of mutations by comparison with a Poisson distribution of
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15 mutations along the gene sequence. Overlapping hotspot regions were considered as a single
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17 hotspot.
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22 The only mutational hotspot for somatic missense mutations was observed in the *PTEN* gene
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24 and comprised 18 mutations in the region between nucleotide positions 269 and 286. Several
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26 germline mutational hotspots were however detected for missense mutational spectra in the
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28 *ATM*, *BRCA1*, *BRCA2*, *NF1*, *PTEN*, *RBI*, *STK11*, *TP53* and *WT1* genes (Table 4). Several
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30 somatic mutational hotspots were found for micro-deletions/micro-insertions in the *APC* gene,
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32 the largest of which contained 33 mutations (positions 4303-4398) and forms part of a previously
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34 reported mutation cluster region [Miyoshi et al., 1992]. Hotspots identified in different
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36 mutational spectra were however unique to that spectrum. The only overlap noted between
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38 mutational hotspots identified in germline and somatic micro-deletion/micro-insertion mutational
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40 spectra was observed for the *APC* gene (the overlapping region comprising nucleotide positions
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42 3919-3933). This micro-deletion/micro-insertion hotspot also includes codon 1309 (cDNA
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44 positions 3925-3927) found to be frequently mutated in Greek and French patients with familial
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46 adenomatous polyposis [Fostira et al. 2010; Lagarde et al. 2010].
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53 Inspection of hotspot regions revealed that they are rich in repetitive elements, runs of identical
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55 nucleotides and CpG/CpHpG oligonucleotides, offering immediate explanations for the elevated
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57 mutability.
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Germline and somatic mutations located within specific hotspot motifs

The cDNA sequences of 17 tumour suppressor genes were screened for the presence of nine specific motifs (and their complements) previously reported as being hotspots for mutation. These motifs included the putative somatic (cancer) mutation hotspot, WKVNRRRRNVWK [the ‘THEMIS motif’; Makridakis et al., 2009], the RGYW motif that correlates with the DNA polymerase eta error spectrum [Rogozin et al., 2001] and several so-called ‘super hotspot’ motifs originally found in germline micro-insertions and micro-deletions [Ball et al., 2005] and indels [Chuzhanova et al., 2003]. For the purposes of this analysis, the shared mutations were added to both the germline and somatic mutational spectra. Both germline and somatic micro-deletions and micro-insertions were found to be significantly overrepresented ($p \leq 0.002$) in the ‘indel super hotspot’ motif GTAAGT and its complement. Somatic micro-deletions and micro-insertions were also significantly overrepresented ($p = 0.009$) with respect to the micro-deletion/micro-insertion super hotspot AAATCT and its complement. The number of germline (but not somatic) micro-deletions/micro-insertions in the THEMIS motif were significantly overrepresented ($p = 0.003$) as compared to the controls. No significant difference was however observed in the number of missense mutations occurring in any motifs analysed.

Conclusions

A number of important conclusions may be drawn from the results reported here. Firstly, it would appear that missense mutations that are found both in the soma and the germline (shared mutations) are disproportionately more likely to exert profound effects on tumour development and/or progression (i.e. more likely to be driver mutations) than exclusively somatic non-recurrent missense mutations (at least for the *TP53* and *CDKN2A* genes whose mutations contributed the bulk of the documented shared mutations in our tumour suppressor gene mutation dataset). Shared mutations also occur preferentially in CpG/CpHpG oligonucleotides

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3 and are characterised by higher mutability rates (both non-disease- and disease-associated).
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6 Further, we found that shared mutations tend to occur in those codons that have been more
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8 highly conserved evolutionarily, and are associated with more dramatic chemical differences
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10 between the substituted (wild-type) and substituting amino acids. Taken together, it would thus
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12 appear that shared mutations are influenced to a greater extent by the local nucleotide sequence
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14 context than either germline or somatic non-recurrent missense mutations. Since this implies that
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16 shared mutations (the mutation category most likely to harbour driver mutations) have a
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18 tendency to arise through the action of similar endogenous mutational mechanisms, we may infer
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20 that endogenous mechanisms of mutagenesis exert a disproportionate effect on tumorigenesis.
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25 In an analysis of an unrelated dataset, we demonstrated that 17% of somatic missense
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27 mutations previously identified as being probable drivers [Carter et al., 2009] were found to
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29 possess the same features as shared (both recurrent and non-recurrent) mutations. A further 32%
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31 of these probable driver mutations shared the features expected of recurrent somatic mutations.
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33 Thus, we may conclude that ~50% of these somatic missense mutations possess features
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35 consistent with their being either shared or recurrent, suggesting that a disproportionate number
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37 of such lesions are likely to be drivers of tumorigenesis.
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42 A sizeable proportion of shared (39%) and germline (30%) micro-lesions were found to be
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44 located in runs of identical nucleotides ≥ 4 bp, making mononucleotide runs a hotspot for micro-
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46 deletion and micro-insertions. The most likely underlying causative mechanism for these
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48 mutations is slipped mispairing at DNA replication mediating duplications and 'de-duplications'
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50 [Kondrashov & Rogozin, 2004]. With regard to missense mutations, CpG and CpHpG
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52 oligonucleotides were found to be hotspots for shared recurrent and shared non-recurrent
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54 missense mutations; 34% (10%) and 21% (9%) of respective mutations were found in CpG
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56 (CpHpG) oligonucleotides. Further, 12% of the 568 probable driver mutations [derived from
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58 Carter et al., 2009] were found to occur in CpG/CpHpG oligonucleotides. 41% of probable
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3 driver mutations were found in repeats that were capable of non-B DNA structure formation (cf.
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5 23% for potential mutations). Several hotspot regions were found in the mutational spectra of
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7 various genes; one of these, in the *APC* gene, was a hotspot for both somatic and germline
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9 micro-deletions/micro-insertions and corresponded to a previously recognized mutation hotspot
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11 [Miyoshi et al., 1992].
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15 Taken together, the results and analysis presented herein strongly suggest that algorithms that
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17 attempt to predict the relative impact of tumour-associated micro-lesions on (tumour suppressor)
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19 gene and protein function [Tavtigian et al., 2008; Couch et al., 2008; Thusberg and Vihinen,
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21 2009], should take into consideration the origin (i.e. somatic, germline or shared) of the
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23 mutations, their sequence context and repetitivity, as well as their frequency of occurrence.
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Table 1. Summary of mutational spectra in the 17 tumour suppressor genes studied

Gene symbol	Gene length (in bp)	Number of observed missense mutations						Number of observed micro-deletions and micro-insertions			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	8532	34	25	1	4	0	64	181	399	15	595
ATM	9171	10	81	0	1	0	92	5	157	0	162
BRCA1	5592	5	172	0	0	1	178	9	338	5	352
BRCA2	10257	19	91	2	2	0	114	9	332	3	344
CDH1	2649	14	19	1	0	0	34	15	20	0	35
CDKN2A	471	173	35	30	6	1	245	100	16	2	118
NF1	8457	2	85	0	0	0	87	16	323	3	342
NF2	1788	20	22	0	3	0	45	204	66	5	275
PTCH1	4344	13	25	1	0	0	39	20	74	0	94
PTEN	1212	154	23	11	49	12	249	192	41	10	243
RB1	2787	22	35	3	1	1	62	42	165	4	211
STK11	1302	16	28	4	3	0	51	4	69	2	75
TP53	1182	358	6	9	793	87	1253	738	11	12	761
TSC1	3495	2	7	0	0	0	9	1	78	0	79
TSC2	5424	0	93	1	0	1	95	5	156	0	161
VHL	642	41	98	39	5	9	192	209	86	14	309
WT1	1350	1	41	0	0	0	42	7	12	0	19
TOTAL	68655	884	886	102	867	112	2851	1757	2343	75	4175

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Table 2. Missense mutations found in CpG and CpHpG oligonucleotides for the 17 tumour suppressor genes under study.

Gene symbol	Number of possible missense mutations		Number of observed CpG-located mutations						Number of observed CpHpG-located mutations					
	in CpG	in CpHpG	somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	total	somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	total
<i>APC</i>	177	300	1	6	0	0	0	7	0	1	0	0	0	1
<i>ATM</i>	157	232	0	12	0	1	0	13	0	3	0	0	0	3
<i>BRCA1</i>	70	192	0	12	0	0	0	12	0	6	0	0	0	6
<i>BRCA2</i>	116	310	3	15	2	0	0	20	0	2	0	0	0	2
<i>CDHI</i>	135	116	1	5	0	0	0	6	0	0	0	0	0	0
<i>CDKN2A</i>	50	16	35	3	10	0	0	48	9	0	0	0	0	9
<i>NF1</i>	226	275	0	6	0	0	0	6	0	1	3	0	0	4
<i>NF2</i>	89	59	1	4	0	1	0	6	0	0	0	0	0	0
<i>PTCH1</i>	345	213	2	4	0	0	0	6	0	0	0	0	0	0
<i>PTEN</i>	14	33	1	1	0	5	4	11	2	0	0	0	0	2
<i>RB1</i>	80	81	4	3	2	0	0	9	1	1	1	1	0	3
<i>STK11</i>	137	60	4	3	2	2	0	11	0	0	0	0	0	0
<i>TP53</i>	15	22	8	0	0	35	28	71	10	0	0	23	8	41
<i>TSC1</i>	147	139	0	1	0	0	0	1	0	0	0	0	0	0
<i>TSC2</i>	454	238	0	19	1	0	1	21	0	7	1	0	1	8
<i>VHL</i>	78	24	7	2	4	0	5	18	0	2	4	0	2	6
<i>WT1</i>	143	70	0	9	0	0	0	9	0	4	0	0	0	4
TOTAL	2433	2380	67	105	21	44	38	275	22	27	9	24	11	63

Table 3. Summary of mutations occurring in runs of identical nucleotides ≥ 4 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by runs (%)	Number of missense mutations found in runs						Number of micro-deletions and micro-insertions found in runs			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	13	5	3	0	2	0	10	74	108	6	188
ATM	26	2	20	0	0	0	22	3	55	0	58
BRCA1	16	3	37	0	0	0	40	2	120	3	125
BRCA2	19	4	27	0	0	0	31	5	151	2	158
CDH1	18	5	7	0	0	0	12	3	11	0	14
CDKN2A	17	42	7	2	0	1	52	30	5	0	35
NF1	24	1	15	0	0	0	16	5	74	2	81
NF2	19	3	2	0	0	0	5	40	8	0	48
PTCH1	15	4	7	0	0	0	11	6	24	0	30
PTEN	32	41	8	1	15	1	66	56	12	2	70
RB1	37	5	9	1	0	0	15	14	54	3	71
STK11	24	1	7	0	2	0	10	2	23	2	27
TP53	29	89	2	1	166	13	271	177	3	7	187
TSC1	15	1	2	0	0	0	3	0	15	0	15
TSC2	17	0	10	0	0	0	10	0	36	0	36
VHL	10	2	3	0	0	0	5	15	6	2	23
WT1	20	0	12	0	0	0	12	2	4	0	6
TOTAL	20	208	178	5	185	15	591	434	709	29	1172

Table 4. Mutational hotspots found in 17 tumour suppressor genes. The number of mutations within the hotspots is shown in parentheses. Shared overlapping hotspot regions for somatic and germline micro-deletions/insertions is shown in bold. Positions are given with respect to the corresponding cDNA sequences.

Gene symbol	Missense mutations		Micro-deletions/insertions	
	somatic	germline	somatic	germline
<i>APC</i>			3856-3882 (9) 3897-3933 (15) 3977-3989 (5) 4117-4140 (7) 4178-4200 (9) 4231-4271 (17) 4303-4398 (33) 4450-4495 (27) 4662-4669 (5)	1484-1492 (4) 1857-1882 (11) 2306-2313 (4) 2789-2821 (13) 3919-3935 (7)
<i>ATM</i>		8479-8494 (6)		
<i>BRCA1</i>		181-191 (6) 5085-5098 (8) 5201-5222 (9) 5236-5258 (8)		
<i>BRCA2</i>		8165-8182 (4)		6196-6203 (4) 6443-6450 (8)
<i>NF1</i>		2329-2352 (6) 2530-2543 (5) 4255-4274 (6)		6788-6798 (5)
<i>PTEN</i>	269-287 (18)	367-371 (4)		
<i>RBI</i>		1960-1970 (5)		202-220 (7)
<i>STK11</i>		526-545 (5)		150-197 (11) 737-757 (6)
<i>TP53</i>		832-848 (11)		
<i>TSC1</i>				2101-2112 (5)
<i>TSC2</i>				2059-2074 (5) 4247-4268 (5)
<i>WT1</i>		1174-1201 (13)		

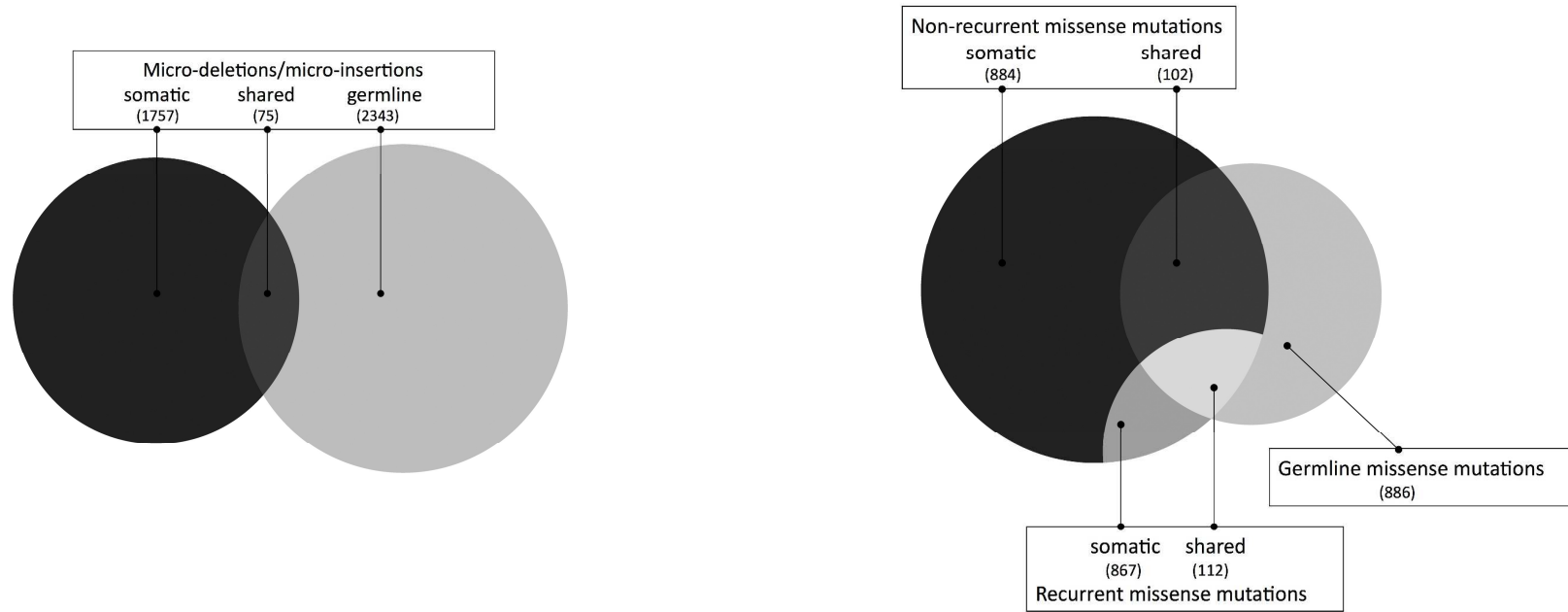


Figure 1. Diagrammatic representation of the number of various types of mutations analysed in the present study.

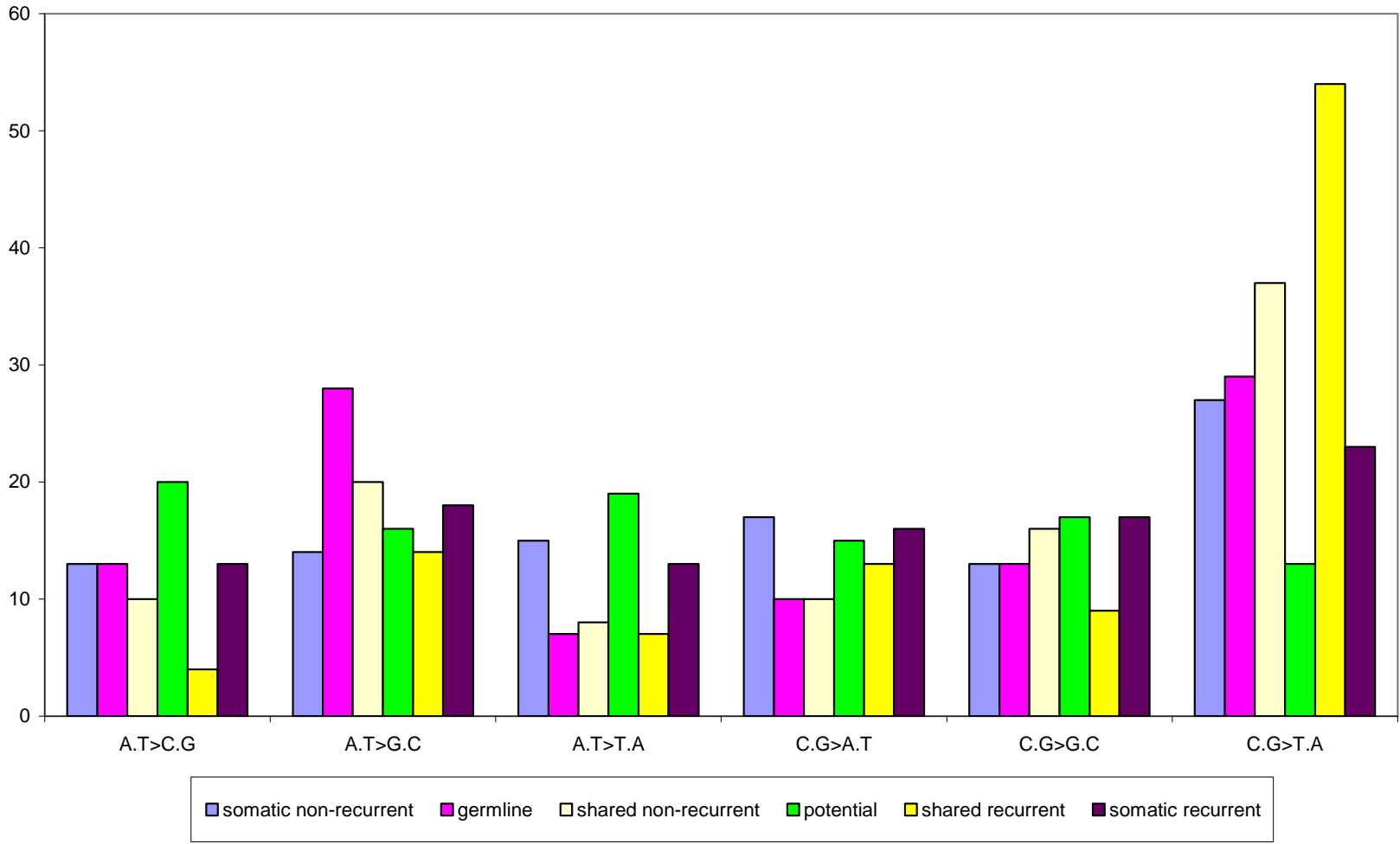


Figure 2. Nucleotide substitution patterns of missense mutations in 17 tumour suppressor genes.

Supplementary Table 1. Tumour suppressor gene orthologues used to estimate the degree of evolutionary conservation of the various gene coding sequences

Gene	Species	cDNA sequence identifier	Protein sequence identifier
APC	<i>Xenopus laevis</i>	U64442.1	AAB41671.1
	<i>Bos taurus</i>	XM_865627.1	XP_870720.1
	<i>Rattus norvegicus</i>	NM_012499.1	NP_036631.1
	<i>Mus musculus</i>	NM_007462.1	NP_031488.1
ATM	<i>Gallus gallus</i>	XM_417160.1	XP_417160.1
	<i>Xenopus laevis</i>	AY668954.1	AAT72929.1
	<i>Rattus norvegicus</i>	XM_236275.3	XP_236275.3
	<i>Sus scrofa</i>	AY587061	AAT01608.1
	<i>Canis familiaris</i>	XM_845871.1	XP_850964.1
	<i>Mus musculus</i>	NM_007499	NP_031525.1
BRCA1	<i>Gallus gallus</i>	NM_204169.1	NP_989500.1
	<i>Xenopus laevis</i>	AF416868.1	AAL13037.1
	<i>Bos taurus</i>	NM_178573.1	NP_848668.1
	<i>Rattus norvegicus</i>	NM_012514.1	NP_036646.1
	<i>Canis familiaris</i>	NM_001013416.1	NP_001013434.1
	<i>Mus musculus</i>	NM_009764.2	NP_033894.2
BRCA2	<i>Gallus gallus</i>	NM_204276.1	NP_989607.1
	<i>Danio rerio</i>	XM_690042.1	XP_695134.1
	<i>Bos taurus</i>	XM_583622.2	XP_583622.2
	<i>Rattus norvegicus</i>	NM_031542.1	NP_113730.1
	<i>Canis familiaris</i>	NM_001006653.4	NP_001006654.2
	<i>Mus musculus</i>	NM_009765.1	NP_033895.1
CDH1	<i>Xenopus laevis</i>	BC068940.1	AAH68940.1
	<i>Danio rerio</i>	NM_131820.1	NP_571895.1
	<i>Bos taurus</i>	NM_001002763.1	NP_001002763.1
	<i>Rattus norvegicus</i>	NM_031334.1	NP_112624.1
	<i>Canis familiaris</i>	XM_536807.2	XP_536807.2
	<i>Mus musculus</i>	NM_009864.1	NP_033994.1
CDKN2A	<i>Gallus gallus</i>	NM_204433.1	NP_989764.1
	<i>Takifugu rubripes</i>	AJ250231.1	CAC12808.1
	<i>Bos taurus</i>	XM_868375.1	XP_873468.1
	<i>Rattus norvegicus</i>	NM_031550.1	NP_113738.1
	<i>Canis familiaris</i>	XM_538685.2	XP_538685.2
	<i>Mus musculus</i>	AF044336.1	AAC08963.1
NF1	<i>Gallus gallus</i>	XM_415914.1	XP_415914.1
	<i>Takifugu rubripes</i>	AF064564.2	AAD15839.1
	<i>Rattus norvegicus</i>	NM_012609.1	NP_036741.1
	<i>Canis familiaris</i>	XM_537738.2	XP_537738.2
	<i>Mus musculus</i>	NM_010897.1	NP_035027.1
NF2	<i>Gallus gallus</i>	NM_204497.2	NP_989828.2
	<i>Danio rerio</i>	NM_212951.1	NP_998116.1
	<i>Bos taurus</i>	XM_611643.2	XP_611643.2
	<i>Rattus norvegicus</i>	XM_341248.2	XP_341249.2
	<i>Canis familiaris</i>	XM_534729.2	XP_534729.2
	<i>Mus musculus</i>	NM_010898.2	NP_035028.2
PTCH1	<i>Xenopus laevis</i>	AF302765.1	AAK15463.1
	<i>Gallus gallus</i>	NM_204960.1	NP_990291.1
	<i>Danio rerio</i>	NM_130988.1	NP_571063.1
	<i>Meriones unguiculatus</i>	AB188226.1	BAE78534.1
	<i>Rattus norvegicus</i>	NM_053566.1	NP_446018.1
	<i>Mus musculus</i>	NM_008957.1	NP_032983.1
PTEN	<i>Xenopus laevis</i>	AF144732.1	AAD46165.1

	<i>Gallus gallus</i>	XM_421555.1	XP_421555.1
	<i>Bos taurus</i>	XM_613125.2	XP_613125.2
	<i>Canis familiaris</i>	NM_001003192.1	NP_001003192.1
	<i>Rattus norvegicus</i>	NM_031606.1	NP_113794.1
	<i>Mus musculus</i>	NM_008960.2	NP_032986.1
	<i>Gallus gallus</i>	NM_204419.1	NP_989750.1
	<i>Rattus norvegicus</i>	XM_344434.2	XP_344435.2
	<i>Canis familiaris</i>	XM_534118.2	XP_534118.2
	<i>Mus musculus</i>	NM_009029.1	NP_033055.1
	<i>Oncorhynchus mykiss</i>	AF102861.1	AAD13390.1
	<i>Notophthalmus viridescens</i>	Y09226.1	CAA70428.1
	<i>Xenopus laevis</i>	U24435.1	AAC59904.1
	<i>Danio rerio</i>	NM_001017839.1	NP_001017839.1
	<i>Rattus norvegicus</i>	XM_234900.2	XP_234900.2
	<i>Raja erinacea</i>	AF486831.1	AAL92113.1
	<i>Canis familiaris</i>	XM_542206.2	XP_542206.2
	<i>Mus musculus</i>	NM_011492.1	NP_035622.1
	<i>Gallus gallus</i>	NM_205264.1	NP_990595.1
	<i>Danio rerio</i>	NM_131327.1	NP_571402.1
	<i>Bos taurus</i>	NM_174201.2	NP_776626.1
	<i>Rattus norvegicus</i>	NM_030989.1	NP_112251.1
	<i>Canis familiaris</i>	NM_001003210.1	NP_001003210.1
	<i>Mus musculus</i>	NM_011640.1	NP_035770.1
	<i>Gallus gallus</i>	XM_415449.1	XP_415449.1
	<i>Danio rerio</i>	XM_691747.1	XP_696839.1
	<i>Bos taurus</i>	XM_612846.2	XP_612846.2
	<i>Rattus norvegicus</i>	NM_021854.1	NP_068626.1
	<i>Canis familiaris</i>	XM_537808.2	XP_537808.2
	<i>Mus musculus</i>	NM_022887.2	NP_075025.2
	<i>Gallus gallus</i>	XM_414853.1	XP_414853.1
	<i>Takifugu rubripes</i>	AF013614	AAB86682.1
	<i>Bos taurus</i>	XM_581197.2	XP_581197.2
	<i>Rattus norvegicus</i>	NM_012680.2	NP_036812.2
	<i>Canis familiaris</i>	XM_537008.2	XP_537008.2
	<i>Mus musculus</i>	NM_011647.2	NP_035777.2
	<i>Gallus gallus</i>	XM_414447.1	XP_414447.1
	<i>Danio rerio</i>	XM_681176.1	XP_686268.1
	<i>Bos taurus</i>	XM_613870.2	XP_613870.2
	<i>Rattus norvegicus</i>	NM_052801.1	NP_434688.1
	<i>Canis familiaris</i>	NM_001008552.1	NP_001008552.1
	<i>Mus musculus</i>	NM_009507.2	NP_033533.1
	<i>Xenopus laevis</i>	U42011.1	AAB53152.1
	<i>Gallus gallus</i>	NM_205216.1	NP_990547.1
	<i>Rattus norvegicus</i>	NM_031534.1	NP_113722.1
	<i>Canis familiaris</i>	XM_846479.1	XP_851572.1
	<i>Sus scrofa</i>	NM_001001264.1	NP_001001264.1
	<i>Mus musculus</i>	NM_144783.1	NP_659032.1

SupplementaryTable 2. Differences in distribution of parameters for somatic, germline, shared, somatic recurrent and shared recurrent missense mutations. Observed median and/or mean values are shown in brackets. DAVID: I prefer 'with respect' In my view according means that Hess and KR did the study

Parameter	Observed trend (p<0.05)
Median non-disease associated mutability rate according to Hess et al. [1994]	shared recurrent >>shared non-recurrent >germline>>somatic~somatic recurrent* [10.7] [7.9] [7.3] [4.7] [4.7]
Median disease-associated mutability rate according to Krawczak et al. [1998]	shared recurrent>shared non-recurrent >germline>>somatic~somatic recurrent [1.42] [1.01] [0.85] [0.53] [0.53]
Mean/median degree of evolutionary conservation	shared recurrent < shared << somatic [0.072/0] [0.138/0] [0.265/0.24] somatic >> germline [0.265/0.24] [0.18/0]
Mean Grantham score	germline >somatic recurrent ~somatic non-recurrent [93] [85] [80] shared recurrent~shared non-recurrent >> somatic recurrent [100] [93] [85]
Proportion of CpG-located mutations	shared recurrent~shared >>germline>>somatic ~somatic recurrent [0.34] [0.21] [0.12] [0.08] [0.05]
Proportion of CpHpG-located mutations	shared recurrent~shared >> somatic recurrent [0.098] [0.082] [0.028]
Proportion of mutations located within or in the vicinity of direct repeats	somatic>>germline>>recurrent somatic [0.07] [0.04] [0.02]

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Proportion of mutations located within (or in the vicinity of) runs of identical nucleotides	somatic>>shared		somatic>>shared recurrent	
	[0.24]	[0.05]	[0.24]	[0.16]
	germline>>shared		somatic recurrent>>shared	
	[0.20]	[0.05]	[0.21]	[0.05]

*Inequality **shared>germline>somatic** implies that a significant difference ($p < 0.05$) in the corresponding parameter was observed between each pair of mutational spectra, i.e. shared vs germline, shared vs somatic and germline vs somatic. Symbol '~' denotes the absence of any significant difference between any two mutational spectra with respect to a given parameter. Symbols '>>' or '<<' indicate experiment-wise statistical significance of the observed inequality whereas symbols '<' or '>' indicate gene-wise statistical significance.

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Supplementary Table 3. Various parameters of gene-wise somatic and germline missense mutational spectra vs. potential mutational spectra exhibiting either gene-wise ($p < 0.05$) or experiment-wise differences ($p < 0.05$; shaded in light grey) with respect to the parameters measured.

	Non-disease associated mutation rate		Disease-associated mutation rate		Evolutionary conservation rate		Grantham score		CpG-located missense mutations		CpHpG-located missense mutations	
	Gene symbol	Median	Gene symbol	Median	Gene symbol	Median	Gene symbol	Median	Gene symbol	%	Gene symbol	%
Somatic mutations			<i>STK11</i>	1.66					<i>STK11</i>	25		
			<i>PTCH1</i>	1.06								
		8.4	<i>CDKN2A</i>	1.01	<i>CDKN2A</i>	0.38			<i>CDKN2A</i>	20	<i>CDKN2A</i>	5.2
		7.9	<i>APC</i>	0.83								
		5.6	<i>PTEN</i>	0.53								
		4.6	<i>TP53</i>	0.5	<i>TP53</i>	0.17			<i>RB1</i>	18	<i>TP53</i>	2.8
					<i>VHL</i>	0.14			<i>BRCA2</i>	16		
								<i>PTCH1</i>	15			
for all 17 genes combined	somatic	4.7	somatic	0.53	somatic	0	somatic	78	somatic	8	somatic	2.5
	control	4.1	control	0.4	control	0.2	control	74	control	2	control	2
	germline	7.2	germline	0.85	germline	0	germline	94	germline	12	germline	3
Germline mutations												
		7.2			<i>TSC2</i>	0			<i>BRCA1</i>	7	<i>BRCA1</i>	3.6
		7.3					<i>NF1</i>	98				
		7.6							<i>NF1</i>	7		
		7.9	<i>ATM</i>	0.79	<i>ATM</i>	0	<i>ATM</i>	98	<i>ATM</i>	15	<i>ATM</i>	3.8
		7.9	<i>BRCA1</i>	0.81	<i>VHL</i>	0	<i>VHL</i>	99	<i>BRCA1</i>	16		
		8.7	<i>BRCA2</i>	0.81					<i>NF1</i>	18		
			<i>PTEN</i>	0.92							<i>TSC2</i>	8.1
			<i>RB1</i>	0.99							<i>WT1</i>	10.8
		<i>NF1</i>	1.03									
		<i>TSC2</i>	1.03									

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	<i>WT1</i>	10.1	<i>WT1</i>	1.22	<i>WT1</i>	0			<i>TSC2</i>	21		
			<i>CDH1</i>	1.27	<i>BRCA1</i>	0.14			<i>APC</i>	24		
					<i>CDKN2A</i>	0.29			<i>CDH1</i>	26		

For Peer Review

Supplementary Table 4. Summary of mutations occurring in direct repeats of length ≥ 8 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by repeats (%)	Number of missense mutations found in repeats						Number of micro-deletions and micro-insertions found in repeats			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	4	3	0	0	0	0	3	17	21	1	17
ATM	7	2	0	0	0	0	2	0	11	0	0
BRCA1	5	0	9	0	0	0	9	1	8	0	1
BRCA2	2	0	0	0	0	0	0	1	12	0	1
CDH1	3	0	0	0	0	0	0	0	1	0	0
CDKN2A	17	25	8	3	0	0	36	28	2	0	28
NF1	7	0	2	0	0	0	2	0	15	0	0
NF2	3	0	0	0	0	0	0	1	1	0	1
PTCH1	3	0	0	0	0	0	0	0	0	0	0
PTEN	17	7	0	0	4	2	13	20	5	1	20
RB1	12	0	1	0	0	0	1	2	12	0	2
STK11	10	0	3	1	0	0	4	0	6	0	0
TP53	14	24	1	0	13	2	40	21	0	0	21
TSC1	5	0	1	0	0	0	1	0	4	0	0
TSC2	5	0	10	1	0	0	11	0	6	0	0
VHL	6	0	1	0	0	0	1	0	1	0	0
WT1	7	1	0	0	0	0	1	0	0	0	0
TOTAL	6	62	36	5	17	4	124	91	105	2	91

Supplementary Table 5. Summary of mutations occurring in inverted repeats of length ≥ 8 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by repeats (%)	Number of missense mutations found in repeats						Number of micro-deletions and micro-insertions found in repeats			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	6	5	4	1	1	0	5	21	27	2	50
ATM	13	1	14	0	0	0	1	1	16	0	17
BRCA1	6	0	15	0	0	0	0	0	22	1	23
BRCA2	7	3	1	0	0	0	3	1	27	0	28
CDH1	5	0	1	0	0	0	0	1	0	0	1
CDKN2A	8	30	5	6	2	1	30	13	2	1	16
NF1	11	0	3	0	0	0	0	1	24	0	25
NF2	10	1	3	0	0	0	1	11	6	0	17
PTCH1	5	1	0	0	0	0	1	0	2	0	2
PTEN	6	10	1	1	4	1	10	9	2	0	11
RB1	16	4	5	1	0	0	4	7	28	0	35
STK11	13	1	5	0	1	0	1	1	9	0	10
TP53	5	13	0	0	51	9	13	53	2	0	55
TSC1	5	0	1	0	0	0	0	0	7	0	7
TSC2	9	0	6	0	0	0	0	1	13	0	14
VHL	12	9	8	1	1	0	9	36	15	2	53
WT1	7	0	2	0	0	0	0	0	0	0	0
TOTAL	9	78	74	10	60	11	78	156	202	6	364

Supplementary Table 6. Summary of mutations occurring within symmetric repeats of length ≥ 8 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by repeats (%)	Number of missense mutations found in repeats						Number of micro-deletions and micro-insertions found in repeats			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	16	5	2	0	2	0	9	58	87	6	151
ATM	32	2	11	0	0	0	13	2	43	0	45
BRCA1	20	1	30	0	0	0	31	0	82	2	84
BRCA2	18	6	18	0	0	0	24	2	79	3	84
CDH1	24	4	0	0	0	0	4	5	8	0	13
CDKN2A	24	49	13	5	2	0	69	35	7	1	43
NF1	31	1	20	0	0	0	21	2	85	2	89
NF2	24	6	3	0	1	0	10	49	12	3	64
PTCH1	23	5	8	1	0	0	14	5	23	0	28
PTEN	44	27	3	1	9	0	40	42	13	1	56
RB1	48	3	10	1	0	0	14	4	41	1	46
STK11	33	3	6	0	2	0	11	1	20	1	22
TP53	30	60	2	1	132	23	218	147	1	0	148
TSC1	23	0	3	0	0	0	3	0	27	0	27
TSC2	23	0	13	0	0	0	13	1	29	0	30
VHL	17	3	9	2	0	2	16	25	7	2	34
WT1	26	0	6	0	0	0	6	3	4	0	7
TOTAL	25	175	157	11	148	25	516	381	568	22	971

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Supplementary Table 7. Occurrence of missense mutations in repeats/runs of identical nucleotides and/or CpG/CpHpG oligonucleotides

Type of Repeats	Type of mutational spectrum	Number of mutations			Remaining number of mutations
		exclusively in repeats/runs	exclusively in CpG/CpHpG	in both repeats/runs and CpG/CpHpG	
Runs	somatic non-recurrent	184	58	24	618
	germline	151	100	27	608
	somatic recurrent	167	46	18	636
	shared non-recurrent	5	28	0	69
	shared recurrent	10	38	5	59
	potential	32861	3902	765	111495
Direct	somatic non-recurrent	52	72	10	750
	germline	31	122	5	728
	somatic recurrent	14	61	3	789
	shared non-recurrent	3	26	2	71
	shared recurrent	2	41	2	67
	potential	5252	4431	236	139104

Inverted	somatic non-recurrent	65	69	13	737
	germline	64	117	10	695
	somatic recurrent	55	59	5	748
	shared non-recurrent	8	26	2	66
	shared recurrent	7	39	4	62
	potential	10790	4314	353	133566
Symmetric	somatic non-recurrent	155	62	20	647
	germline	140	110	17	619
	somatic recurrent	137	53	11	666
	shared non-recurrent	7	24	4	67
	shared recurrent	16	34	9	53
	potential	28646	3752	915	115710

Supplementary Table 8. Truncating vs. non-truncating lesions

Gene		Missense	Nonsense	Micro-deletions	Micro-insertions	Micro-indels	Non-truncating lesions	Truncating lesions	Ratio of non-truncating to truncating lesions	Ratio of truncating somatic to truncating germline lesions
<i>APC</i>	Somatic	39	79	152	44	3	39	278	0.14	0.46
	Germline	23	180	299	115	12	23	606	0.04	
<i>ATM</i>	Somatic	11	7	4	1	0	11	12	0.92	0.05
	Germline	76	75	122	35	14	76	246	0.31	
<i>BRCA1</i>	Somatic	6	9	9	5	0	6	23	0.26	0.05
	Germline	170	121	259	85	12	170	477	0.36	
<i>BRCA2</i>	Somatic	21	1	8	4	0	21	13	1.62	0.03
	Germline	86	76	247	90	11	86	424	0.20	
<i>CDH1</i>	Somatic	15	7	13	2	0	15	22	0.68	0.69
	Germline	19	11	12	8	1	19	32	0.59	
<i>CDKN2A</i>	Somatic	198	18	77	25	8	198	128	1.55	4.74
	Germline	62	7	11	7	2	62	27	2.30	
<i>NF1</i>	Somatic	2	11	16	3	0	2	30	0.07	0.07
	Germline	83	115	221	105	8	83	449	0.18	
<i>NF2</i>	Somatic	23	42	182	28	6	23	258	0.09	2.22
	Germline	20	43	55	16	2	20	116	0.17	
<i>PTCH1</i>	Somatic	14	9	14	6	1	14	30	0.47	0.28
	Germline	24	27	42	32	8	24	109	0.22	
<i>PTEN</i>	Somatic	226	56	152	51	4	226	263	0.86	3.21
	Germline	45	28	29	22	3	45	82	0.55	
<i>RBI</i>	Somatic	25	27	34	12	3	25	76	0.33	0.30

	Germline	37	76	117	53	11	37	257	0.14	
<i>STK11</i>	Somatic	20	10	5	1	1	20	17	1.18	0.17
	Germline	30	27	47	24	3	30	101	0.30	
<i>TP53</i>	Somatic	1229	96	512	238	0	1229	846	1.45	24.89
	Germline	94	10	16	5	3	94	34	2.76	
<i>TSC1</i>	Somatic	2	1	1	0	0	2	2	1.00	0.02
	Germline	7	37	53	25	4	7	119	0.06	
<i>TSC2</i>	Somatic	2	1	3	2	1	2	7	0.29	0.03
	Germline	89	74	110	46	3	89	233	0.38	
<i>VHL</i>	Somatic	88	15	180	44	1	88	240	0.37	1.82
	Germline	143	27	63	37	5	143	132	1.08	
<i>WT1</i>	Somatic	1	3	4	3	0	1	10	0.10	0.37
	Germline	40	14	8	4	1	40	27	1.48	
Total	Somatic	1922	392	1366	469	28	1922	2255	0.85	0.65
	Germline	1048	948	1711	709	103	1048	3471	0.30	

Supplementary Figure 1. Naive Bayes Tree Classifier. Number in parenthesis shows the probability of a mutations being somatic non-recurrent, germline, shared non-recurrent, somatic recurrent and shared recurrent respectively.

Attributes:

Mut_Type
Hess_value
Krawczak_value
Evol
Grantham_score
CpG/CHG
Repeats

Test mode: 10-fold cross-validation

NBTree

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Evol <= 0.205
| Repeats = 0
| | CpG/CHG = 0
| | | Krawczak_value <= 1.0465
| | | | Evol <= 0.155
| | | | | Evol <= 0.12
| | | | | | Krawczak_value <= 0.811
| | | | | | | Krawczak_value <= 0.099
| | | | | | | | Hess_value <= 3.1: (0.42) (0.08) (0.08) (0.33) (0.08)
| | | | | | | | Hess_value > 3.1: (0.23) (0.13) (0.03) (0.10) (0.52)
| | | | | | | | Krawczak_value > 0.099
| | | | | | | | | Hess_value <= 2.5
| | | | | | | | | | Grantham_score <= 146.5
| | | | | | | | | | | Hess_value <= 2.15: (0.27) (0.47) (0.02) (0.22) (0.02)
| | | | | | | | | | | Hess_value > 2.15: (0.14) (0.24) (0.05) (0.52) (0.05)
| | | | | | | | | | | Grantham_score > 146.5: (0.47) (0.07) (0.07) (0.33) (0.07)
| | | | | | | | | | | Hess_value > 2.5
| | | | | | | | | | | | Hess_value <= 5.45
| | | | | | | | | | | | | Grantham_score <= 30.5
| | | | | | | | | | | | | | Hess_value <= 5.2
| | | | | | | | | | | | | | | Hess_value <= 4.55
| | | | | | | | | | | | | | | | Hess_value <= 2.75: (0.27) (0.09) (0.09) (0.45) (0.09)
| | | | | | | | | | | | | | | | Hess_value > 2.75: (0.25) (0.43) (0.03) (0.28) (0.03)

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5 | | | | | | | | Hess_value > 4: (0.48) (0.28) (0.04) (0.16) (0.04)
6 | | | | | | | | Krawczak_value > 0.6155: (0.22) (0.5) (0.06) (0.17) (0.06)
7 | | | | | | | | Krawczak_value > 1.083: (0.17) (0.08) (0.08) (0.58) (0.08)
8 | | | | | | | | Grantham_score > 40.5
9 | | | | | | | | Hess_value <= 5.05
10 | | | | | | | | Grantham_score <= 194.5
11 | | | | | | | | Krawczak_value <= 0.365
12 | | | | | | | | Hess_value <= 3.95
13 | | | | | | | | Grantham_score <= 66.5
14 | | | | | | | | Hess_value <= 2.65: (0.21) (0.07) (0.38) (0.07) (0.28)
15 | | | | | | | | Hess_value > 2.65
16 | | | | | | | | Evol <= 0.275: (0.05) (0.05) (0.79) (0.05) (0.05)
17 | | | | | | | | Evol > 0.275: (0.32) (0.08) (0.36) (0.2) (0.04)
18 | | | | | | | | Grantham_score > 66.5
19 | | | | | | | | Grantham_score <= 159.5: (0.36) (0.37) (0.12) (0.15) (0.01)
20 | | | | | | | | Grantham_score > 159.5: (0.19) (0.04) (0.3) (0.07) (0.41)
21 | | | | | | | | Hess_value > 3.95
22 | | | | | | | | Krawczak_value <= 0.229: (0.26) (0.19) (0.04) (0.48) (0.04)
23 | | | | | | | | Krawczak_value > 0.229: (0.39) (0.07) (0.04) (0.04) (0.46)
24 | | | | | | | | Krawczak_value > 0.365
25 | | | | | | | | Hess_value <= 4.55
26 | | | | | | | | Hess_value <= 4.3
27 | | | | | | | | Grantham_score <= 105.5: (0.51) (0.14) (0.03) (0.29) (0.03)
28 | | | | | | | | Grantham_score > 105.5
29 | | | | | | | | Hess_value <= 3.3: (0.50) (0.33) (0.06) (0.06) (0.06)
30 | | | | | | | | Hess_value > 3.3: (0.36) (0.16) (0.04) (0.28) (0.16)
31 | | | | | | | | Hess_value > 4.3: (0.06) (0.24) (0.06) (0.29) (0.35)
32 | | | | | | | | Hess_value > 4.55: (0.39) (0.04) (0.04) (0.48) (0.04)
33 | | | | | | | | Grantham_score > 194.5: (0.09) (0.09) (0.73) (0.05) (0.05)
34 | | | | | | | | Hess_value > 5.05
35 | | | | | | | | Grantham_score <= 45.5: (0.04) (0.11) (0.54) (0.29) (0.04)
36 | | | | | | | | Grantham_score > 45.5
37 | | | | | | | | Evol <= 0.51
38 | | | | | | | | Hess_value <= 7.25
39 | | | | | | | | Evol <= 0.28: (0.07) (0.43) (0.07) (0.36) (0.07)
40 | | | | | | | | Evol > 0.28: (0.27) (0.27) (0.24) (0.18) (0.03)
41 | | | | | | | | Hess_value > 7.25
42 | | | | | | | | Hess_value <= 7.6: (0.09) (0.18) (0.09) (0.55) (0.09)
43 | | | | | | | | Hess_value > 7.6
44 | | | | | | | | Grantham_score <= 69: (0.57) (0.09) (0.04) (0.26) (0.04)
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												Evol <= 0.585:	(0.38)	(0.18)	(0.03)	(0.38)	(0.03)
												Evol > 0.585:	(0.70)	(0.14)	(0.03)	(0.11)	(0.03)
												Hess_value > 4.8:	(0.71)	(0.07)	(0.07)	(0.07)	(0.07)
												Krawczak_value > 0.417:	(0.08)	(0.31)	(0.08)	(0.46)	(0.08)
												Krawczak_value > 0.4675					
												Krawczak_value <= 0.5205:	(0.24)	(0.06)	(0.53)	(0.12)	(0.06)
												Krawczak_value > 0.5205:	(0.78)	(0.07)	(0.04)	(0.07)	(0.04)
												Hess_value > 6.75					
												Grantham_score <= 57:	(0.11)	(0.53)	(0.05)	(0.26)	(0.05)
												Grantham_score > 57:	(0.47)	(0.22)	(0.03)	(0.25)	(0.03)
												CpG/CHG = 1:	(0.40)	(0.10)	(0.10)	(0.30)	(0.10)
												Hess_value > 8.55					
												Evol <= 0.54:	(0.27)	(0.20)	(0.07)	(0.40)	(0.07)
												Evol > 0.54:	(0.03)	(0.03)	(0.84)	(0.06)	(0.03)
												Krawczak_value > 1.27					
												Grantham_score <= 86:	(0.52)	(0.04)	(0.04)	(0.37)	(0.04)
												Grantham_score > 86:	(0.40)	(0.40)	(0.03)	(0.13)	(0.03)
												Grantham_score > 123					
												Evol <= 0.445					
												Hess_value <= 3.45					
												Krawczak_value <= 0.4665:	(0.03)	(0.19)	(0.03)	(0.16)	(0.59)
												Krawczak_value > 0.4665:	(0.25)	(0.08)	(0.08)	(0.50)	(0.08)
												Hess_value > 3.45:	(0.43)	(0.05)	(0.05)	(0.43)	(0.05)
												Evol > 0.445:	(0.44)	(0.09)	(0.03)	(0.41)	(0.03)
												Hess_value > 9.65					
												Hess_value <= 42.75					
												Hess_value <= 12.1					
												Repeats = 0					
												Evol <= 0.325:	(0.32)	(0.39)	(0.21)	(0.04)	(0.04)
												Evol > 0.325					
												Hess_value <= 11.4					
												Evol <= 0.705:	(0.26)	(0.33)	(0.04)	(0.33)	(0.04)
												Evol > 0.705:	(0.06)	(0.75)	(0.06)	(0.06)	(0.06)
												Hess_value > 11.4:	(0.18)	(0.23)	(0.05)	(0.14)	(0.41)
												Repeats = 1					
												Grantham_score <= 91.5					
												Grantham_score <= 85					
												Hess_value <= 11.4:	(0.18)	(0.24)	(0.47)	(0.08)	(0.03)
												Hess_value > 11.4:	(0.05)	(0.32)	(0.05)	(0.14)	(0.45)
												Grantham_score > 85:	(0.20)	(0.45)	(0.05)	(0.25)	(0.05)

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5 | | | | | Grantham_score > 91.5:      (0.33) (0.17) (0.03) (0.03) (0.43)
6 | | | | | Hess_value > 12.1
7 | | | | |   Evol <= 0.51
8 | | | | |     Repeats = 0
9 | | | | |       Grantham_score <= 44.5: (0.20) (0.07) (0.07) (0.60) (0.07)
10 | | | | |         Grantham_score > 44.5
11 | | | | |           Grantham_score <= 51: (0.09) (0.27) (0.09) (0.09) (0.45)
12 | | | | |             Grantham_score > 51: (0.24) (0.53) (0.03) (0.18) (0.03)
13 | | | | |               Repeats = 1:      (0.32) (0.45) (0.05) (0.14) (0.05)
14 | | | | |                 Evol > 0.51
15 | | | | |                   Hess_value <= 13.35: (0.27) (0.15) (0.04) (0.5) (0.04)
16 | | | | |                     Hess_value > 13.35: (0.28) (0.44) (0.04) (0.2) (0.04)
17 | | | | | Hess_value > 42.75
18 | | | | |   Repeats = 0
19 | | | | |     Evol <= 0.59
20 | | | | |       Evol <= 0.255:      (0.08) (0.12) (0.73) (0.04) (0.04)
21 | | | | |         Evol > 0.255
22 | | | | |           Evol <= 0.375: (0.18) (0.03) (0.03) (0.28) (0.49)
23 | | | | |             Evol > 0.375: (0.40) (0.13) (0.07) (0.33) (0.07)
24 | | | | |               Evol > 0.59
25 | | | | |                 Grantham_score <= 139: (0.02) (0.20) (0.75) (0.02) (0.02)
26 | | | | |                   Grantham_score > 139: (0.36) (0.43) (0.07) (0.07) (0.07)
27 | | | | | Repeats = 1
28 | | | | |   Hess_value <= 59.5
29 | | | | |     Hess_value <= 50.35: (0.40) (0.15) (0.05) (0.35) (0.05)
30 | | | | |       Hess_value > 50.35: (0.67) (0.13) (0.04) (0.13) (0.04)
31 | | | | |         Hess_value > 59.5: (0.19) (0.63) (0.06) (0.06) (0.06)

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32 === Stratified cross-validation ===

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33 === Summary ===

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34 Correctly Classified Instances      2797          63.1377 %
35 Incorrectly Classified Instances   1633          36.8623 %
36 Kappa statistic                    0.5392
37 Mean absolute error                 0.1878
38 Root mean squared error            0.3177
39 Relative absolute error            58.6858 %

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Root relative squared error 79.4156 %
Total Number of Instances 4430

=== Detailed Accuracy By Class ===

	TP Rate	FP Rate	Precision	Recall	F-Measure	ROC Area	Class
	0.505	0.106	0.544	0.505	0.523	0.826	1
	0.426	0.082	0.566	0.426	0.486	0.778	2
	0.894	0.091	0.712	0.894	0.792	0.967	3
	0.475	0.109	0.52	0.475	0.497	0.809	4
	0.858	0.073	0.745	0.858	0.797	0.964	5
Weighted Avg.	0.631	0.092	0.617	0.631	0.619	0.869	

=== Confusion Matrix ===

a	b	c	d	e	<-- classified as
447	125	63	207	44	a = 1
170	377	89	153	97	b = 2
12	9	792	9	64	c = 3
181	144	85	421	55	d = 4
12	11	84	19	760	e = 5

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Comparative Analysis of Germline and Somatic Micro-lesion Mutational Spectra in
17 Human Tumour Suppressor Genes

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Abstract

Mutations associated with tumorigenesis may either arise somatically or can be inherited through the germline. We performed a comparison of somatic, germline, shared (found in both soma and germline) and somatic recurrent mutational spectra for 17 human tumour suppressor genes which included missense single base-pair substitutions and micro-deletions/micro-insertions. Somatic and germline mutational spectra were similar in relation to C.G>T.A transitions but differed with respect to the frequency of A.T>G.C, A.T>T.A and C.G>A.T substitutions. Shared missense mutations were characterised by higher mutability rates, greater physicochemical differences between wild-type and mutant residues, and a tendency to occur in evolutionarily conserved residues and within CpG/CpHpG oligonucleotides. Mononucleotide runs (≥ 4 bp) were identified as hotspots for shared micro-deletions/micro-insertions. Both germline and somatic micro-deletions/micro-insertions were found to be significantly overrepresented within the 'indel-hotspot' motif, GTAAGT. Using a naïve Bayes' classifier trained to discriminate between five missense mutation groups, 63% of mutations in our dataset were on average correctly recognized. Applying this classifier to an independent dataset of probable driver mutations, we concluded that ~50% of these somatic missense mutations possess features consistent with their being either shared or recurrent, suggesting that a disproportionate number of such lesions are likely to be drivers of tumorigenesis.

Key Words: germline and somatic mutational spectra; tumour suppressor genes; recurrent mutation; mutation hotspot; non-B DNA; driver mutations

Deleted: In this study

Introduction

A major distinction to be made between somatic and germline mutations is that the former occur during mitotic cell cycles whereas the latter are generally meiotic in origin. In addition, whilst somatic cancer-causing gene lesions come to clinical attention by conferring a growth advantage upon the affected cells or tissue, germ-line gene mutations causing inherited disease normally come to attention by conferring a disadvantage upon the individual, usually through haploinsufficiency. Finally, whereas inherited disease usually implies only one or two pathological mutations at a specific locus, cancer is often characterized by multiple somatic mutations distributed genome-wide. Those somatic mutations which confer a growth advantage on the cells in which they occur, which are positively selected for in the emerging tumour mass and which have therefore been causally implicated in tumorigenesis, are termed 'driver' mutations [Stratton *et al.*, 2009]. By contrast, those mutations which do not confer any growth advantage and have not been subject to selection during tumorigenesis, are termed 'passenger' mutations [Stratton *et al.*, 2009]. Such passenger mutations may arise at high frequency as a consequence either of increased genomic instability or simply due to the considerable number of cell divisions required to convert a single transformed cell into a clinically detectable tumour [Lengauer *et al.*, 1998; Boland and Ricciardiello, 1999; Simpson 2008; Parmigiani *et al.* 2009; Stratton *et al.*, 2009].

Despite these basic differences, the mutational spectra (and hence the underlying mutational mechanisms) associated with single base-pair substitutions [Krawczak *et al.*, 1995; Schmutte and Jones, 1998; Cole *et al.*, 2008; Lobo *et al.*, 2009], micro-deletions and micro-insertions [Jego *et al.*, 1993; Greenblatt *et al.* 1996] and gross gene rearrangements [Oldenburg *et al.*, 2000; Kolomietz *et al.*, 2002] in specific genes often appear to exhibit marked similarities between the germline and the soma. Further, certain triplet repeats associated with a number of inherited human conditions are known to be unstable in both the germline and somatic tissues, a finding

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2 which serves to explain not only the phenomenon of genetic anticipation characteristic of these
3 disorders but also their inherent inter-individual clinical variability [Giovannone et al., 1997;
4 Leeflang et al., 1999; Martorell et al., 2000; Sharma et al., 2002; Pollard et al., 2004]. However,
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9 by contrast, highly variable human minisatellites can display markedly different degrees of
10 instability between the soma and the germline [Buard et al., 2000; Stead and Jeffreys, 2000;
11 Shanks et al., 2008]. These studies notwithstanding, few attempts have so far been made to
12 compare the nature, location and relative frequency of germline and somatic mutations.
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16 Human cancer genes usually harbour either somatic or germline mutations [Goode et al., 2002;
17 Futreal et al., 2004; Vogelstein and Kinzler, 2004]. There is, however, one category of cancer
18 gene, broadly termed tumour suppressors, that by virtue of their being mutated in both the
19 germline and the soma, provides us with an ideal model system to compare somatic vs. germline
20 mutational spectra [Futreal et al., 2004]. Tumour suppressor genes, defined as “genes that sustain
21 loss-of-function mutations in the development of cancer” [Haber and Harlow, 1997], are
22 involved in the regulation of a diverse array of different cellular functions including cell cycle
23 checkpoint control, detection and repair of DNA damage, protein ubiquitination and degradation,
24 mitogenic signalling, cell specification, differentiation and migration, and tumour angiogenesis
25 [Sherr, 2004]. They encode proteins with a regulatory role in cell cycle progression (e.g. Rb),
26 DNA-binding transcription factors (e.g. p53) and inhibitors of cyclin-dependent kinases required
27 for cell cycle progression (e.g. p16). In inherited cancer syndromes, the mutational inactivation
28 of both tumour suppressor alleles is required to change the phenotype of the cell. This ‘two hit
29 hypothesis’ provides the basis for our mechanistic understanding of tumour suppressor gene
30 mutagenesis: a first (inherited) mutation in one tumour suppressor allele is followed by the
31 somatic loss of the remaining wild-type allele via a number of different mutational mechanisms
32 [Knudson, 2001]. Whereas the inherited lesion is usually fairly subtle, the second (somatic) hit
33 may also involve the deletional loss of the entire gene or even a substantial portion of the
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2 chromosome involved. Alternatively, both 'hits' may constitute somatic mutations: whatever the
3 actual mechanism, the end result is the same – the loss or inactivation of both gene copies. Some
4 interplay may however occur between the soma and the germline in that the location of the
5 germline mutation can in some instances influence the nature, frequency and location of the
6 subsequent somatic mutation [Lamlum et al., 1999; Groves et al., 2002; Latchford et al., 2007;
7 Dallosso et al., 2009; Dworkin et al., 2010].

8
9 Tumour suppressor genes are often somatically inactivated by mutational mechanisms that are
10 almost exclusively confined to the soma and which are found only infrequently in the germline
11 (e.g. gross mutations characterized by loss of heterozygosity, epi-mutations such as methylation-
12 mediated promoter inactivation, and micro-lesions within highly repetitive sequence elements
13 that are consequent to microsatellite instability). However, a typical spectrum of somatic
14 mutations associated with tumorigenesis may also include gross rearrangements, copy number
15 variation, and various types of micro-lesion (e.g. micro-deletions, micro-insertions and indels)
16 including single base-pair substitutions [Loeb and Harris, 2008; Stratton et al., 2009]. Although
17 the somatic micro-lesions are often quite similar to their germline counterparts, few studies of
18 tumour suppressor genes have so far attempted to compare and contrast germline and somatic
19 mutational spectra with respect to these relatively subtle types of mutation. However, several
20 such studies have indicated that germline and somatic micro-lesions can display remarkable
21 similarities in terms of mutation type, location and relative frequency of occurrence, and hence
22 by inference the putative underlying mechanisms of mutagenesis [Marshall et al., 1997; Ali et
23 al., 1999; Gallou et al., 1999; Richter et al., 2003; Upadhyaya et al., 2004; Glazko et al., 2004;
24 Tartaglia et al., 2006; Baser et al., 2006; Upadhyaya et al., 2008].

25
26 We attempt here a first formal comparison between germline and somatic micro-lesion
27 mutational spectra for a total of 17 different human tumour suppressor genes [*APC* (MIM#
28 611731), *ATM* (MIM# 607585), *BRCA1* (MIM# 113705), *BRCA2* (MIM# 600185), *CDHI*

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2 (MIM# 192090), *CDKN2A* (MIM# 600160), *NF1* (MIM# 162200), *NF2* (MIM# 607379),
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4 *PTCH1* (MIM# 601309), *PTEN* (MIM# 601728), *RBI* (MIM# 180200), *STK11* (MIM# 602216),
5
6 *TP53* (MIM# 191170), *TSC1* (MIM# 605284), *TSC2* (MIM# 191092), *VHL* (MIM# 608537) and
7
8 *WT1* (MIM# 607102)].
9

10 11 12 **Materials and Methods**

13 14 *Sources of germline and somatic mutation data*

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16 Data on germline and somatic micro-lesions (viz. missense mutations, micro-deletions and
17
18 micro-insertions involving ≤ 20 bp) were collated for 17 different human tumour suppressor
19
20 genes. Germline mutation data were obtained from the Human Gene Mutation Database
21
22 [HGMD; <http://www.hgmd.org>; Stenson et al., 2009]. [HGMD lists mutations for which there is](#)
23
24 [direct evidence for a pathological effect but includes only one example of every lesion. Apart](#)
25
26 [from this, no specific filters were applied to the available data.](#) Somatic mutation data were
27
28 compiled from a number of different sources including online somatic mutational databases viz.
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30 *Catalogue of Somatic Mutations in Cancer* (<http://www.sanger.ac.uk/genetics/CGP/cosmic>; *RBI*
31
32 and *PTEN*), the *Breast Cancer Information Core* (<http://research.nhgri.nih.gov/bic>; *BRCA1*), the
33
34 *RBI Gene Mutation Database* (<http://www.verandi.de/joomla>; *RBI*), the *International NF2*
35
36 *Mutation Database* (<http://www.hgmd.cf.ac.uk/nf2>; *NF2*), the *CDKN2A Database*
37
38 (<https://biodesktop.uvm.edu/perl/p16>; *CDKN2A*) and the *IARC TP53 Mutation Database*
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40 (<http://www-p53.iarc.fr>; *TP53*), the *VHL Mutations Database* (<http://www.umd.be/VHL/>), and
41
42 data privately communicated by Eamonn Maher (*VHL*) and Gareth Evans (*NF2*). Additional
43
44 somatic mutation data [for *APC*, *ATM*, *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTCH1*, *STK11*, *TSC1*,
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46 *TSC2* and *WT1*] were obtained by searching PubMed.

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48 To be regarded as *bona fide* somatic mutations, and therefore suitable for inclusion in this
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50 analysis, reported lesions had to have been shown not only to be present in a tumour tissue but
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2 also to be absent from a non-tumour tissue (usually blood) from the same patient. Hence,
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4 mutational data derived from 'sporadic' patients were not included unless a non-tumour tissue
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6 had also been examined in order to exclude the possibility that the lesions detected were
7
8 constitutional in origin. Depending upon the number of independent occurrences, f , of a given
9
10 somatic or shared mutation described in the literature, these mutation types were further
11
12 subdivided into two categories: *recurrent mutations* ($f > 1$) and *non-recurrent mutations* ($f = 1$). At
13
14 the time this study was initiated (October 2006), the number of available germline and somatic
15
16 missense mutations for each of the 17 studied tumour suppressor genes were as listed in Table 1.
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18 The analysis reported here focussed exclusively on missense mutations and micro-deletions/
19
20 micro-insertions. Nonsense mutations in tumour suppressor genes have already been addressed
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22 in the context of a general meta-analysis of this type of lesion [Mort et al., 2008]. Indels
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24 (~~complex lesions representing combined micro-deletion/micro-insertions~~) were excluded from
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26 the analysis owing to their paucity.

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30 *Control datasets of potential mutations*

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32 For every tumour suppressor gene examined, all possible single base-pair substitutions in the
33
34 gene coding sequence that (i) could potentially have given rise to a missense mutation and (ii)
35
36 were not already included in either of the corresponding observed somatic and/or germline
37
38 mutational spectra, were generated. These 'potential missense mutations' were used as a control
39
40 dataset.
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42 For each tumour suppressor gene, a matching control dataset of 'potential micro-deletions' was
43
44 also generated by randomly selecting a first breakpoint and then choosing the length of the
45
46 simulated micro-deletion (and hence the position of the second breakpoint) by reference to the
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48 probability distribution calculated for micro-deletions (from 1 bp to 20 bp) observed in the
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50 corresponding dataset of mutations. A matching dataset of micro-insertions was generated in
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2 similar fashion, with the sites of insertion being randomly selected. Since some of the micro-
3 deletion/micro-insertion breakpoints occurred within an intron, extended cDNA sequences
4 comprising exons and additional flanking intronic sequence were used to generate corresponding
5 control datasets.
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10 11 12 *Grantham scores*

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14 The 'Grantham score' or 'Grantham difference' [Grantham, 1974] measures the chemical
15 difference between wild-type and mutated amino acid residues in terms of their side chain
16 composition (i.e. the weight ratio of non-carbon components in end-groups or rings to carbons in
17 side chains), polarity (i.e. basic, acidic or nonpolar depending upon side chain charge) and
18 molecular volume.
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24 On average, the physicochemical differences manifested by orthologous amino acid
25 substitutions that have accumulated over evolutionary time will tend to be relatively small. By
26 contrast, disease-causing substitutions are expected to exhibit higher Grantham scores, indicative
27 of more dramatic physicochemical differences between the wild-type and mutated amino acid
28 residues [Krawczak et al., 1998]. The values tabulated by Grantham [1974] were used in this
29 study to calculate a median Grantham score for each set of missense mutations for each tumour
30 suppressor gene.
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41 *Degree of evolutionary conservation*

42 Amino acid residues that are highly conserved in orthologous proteins frequently represent sites
43 of structural or functional importance. Hence, such highly conserved amino acid residues/protein
44 regions often constitute hotspots for observed pathological mutations as a consequence of
45 phenotype selection (rather than intrinsic mutability). To assess the degree of evolutionary
46 conservation of those codons affected by somatic/germline mutations, orthologous tumour
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2 suppressor cDNA and protein sequences from different vertebrate species were retrieved from
3
4 NCBI's Entrez Gene database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). The species
5
6 used as a source of these cDNA and protein sequences are listed in Supp. Table 1 for each
7
8 tumour suppressor gene/protein. ClustalX (<http://www.clustal.org/>) was used to align the protein
9
10 sequences. A program was written to replace all amino acids in the protein alignments by cDNA-
11
12 derived codons, thereby avoiding the introduction of gaps within codons.
13

14 The evolutionary constraints acting upon the 17 human tumour suppressor genes at the codon
15
16 level were inferred by calculating the $\frac{K_a}{K_a + K_s}$ ratio for each codon where K_s and K_a are
17
18 respectively the relative numbers of synonymous and nonsynonymous substitutions between
19
20 codons in two aligned sequences [Walker et al., 1999]. If two aligned codons required more than
21
22 one substitution to be transformed into each other, then the minimum number of substitutions
23
24 was assumed, and the most parsimonious path was determined using a PAM100 matrix and the
25
26 Nei & Gojobori [1986] pathway method. Gaps inserted into the non-human vertebrate
27
28 orthologous cDNA sequences during alignment were treated as being equivalent to a non-
29
30 synonymous substitution. Codons that were not present in the human cDNA sequence were not
31
32 considered. A value representing the median level of evolutionary conservation across all codons
33
34 was then derived for each mutational spectrum; the higher values correspond to less conserved
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36 genes whereas the lower values refer to more highly conserved ones.
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41 *Relative mutability rates*

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43 To assess the likelihood of observing a certain nucleotide change in a given position and in a
44
45 specific context, two tabulated measures of the nearest neighbour-dependent mutation rate were
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47 employed. The first was derived from 20,200 single base-pair substitutions inferred from
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49 alignments of paired human gene/pseudogene sequences [Hess et al., 1994]. This was termed the
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51 *non-disease-associated mutability rate* and, since it approximates to the neutral mutation
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2 frequency, it should reflect the intrinsic mutability of the underlying DNA sequence. One would
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4 expect the non-disease-associated mutation rates associated with pathological mutations to be
5
6 low implying that these specific substitutions are much less likely to occur as neutral
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8 substitutions.
9

10 The nearest neighbour-dependent mutation rates derived from germline single base-pair
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12 substitutions [using data from the Human Gene Mutation Database (HGMD); Stenson et al.,
13
14 2009] by Krawczak et al. [1998] were used as an approximation of the *disease-associated*
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16 *mutability rate*. This mutation rate is a function of selection for loss of biological function as
17
18 well as the underlying intrinsic mutability of the DNA sequence. This mutability rate varies
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20 between 0.032 for the C(A>T)G mutation and 13.023 for the C(G>A)G mutation [Krawczak et
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22 al., 1998].
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25 26 27 *Repetitive sequence elements*

28 A variety of repetitive sequence elements have been reported in association with human gene
29
30 mutations causing both inherited disease and cancer. Direct and inverted repeats and symmetric
31
32 elements [see Chuzhanova et al. 2003 for definitions] of length ≥ 8 bp, and less than 21 bp apart,
33
34 capable of forming non-B DNA structures, were therefore sought within the extended cDNA
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36 sequences (comprising exons and up to ± 85 bp of flanking sequence) using purposely designed
37
38 software. In addition, DNA sequences were screened for the presence of mononucleotide runs of
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40 ≥ 4 bp.
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43 44 45 *Mutation descriptors*

46 Each missense mutation was ascribed various descriptors indicating (a) the type of mutation [i.e.
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48 shared mutation (i.e. found to occur both somatically and in the germline); exclusively somatic;
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50 exclusively germline; shared recurrent mutation (i.e. found to occur not only in the germline but
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2 also somatically on more than one occasion; somatic recurrent mutation (recorded in the soma
3 more than once, but not in the germline); potential mutation (as defined above)] and (b) its
4 location [i.e. C→T and G→A within a CpG dinucleotide or within a CpHpG trinucleotide
5 (where H=A, C or T) or in a repeat sequence (as described above)]. Mutations that have been
6 reported as being exclusively somatic or exclusively germline will henceforth be referred to
7 simply as 'somatic' and 'germline', respectively. The shared mutations, comprising the overlap
8 between the somatic and germline mutations, may be visualized in the form of a Venn diagram
9 (Figure 1). All somatic missense (including shared) mutations were further described as being
10 either recurrent or non-recurrent (in the soma, see above; Figure 1). No such division was made
11 for the relatively small number of recurrent micro-deletions and micro-insertions available; both
12 recurrent and non-recurrent somatic mutations were therefore included in either the somatic or
13 the shared datasets and labelled accordingly (Figure 1).

14
15 All micro-lesions (*viz.* missense mutations, micro-deletions and micro-insertions) in each gene
16 were also labelled with respect to their occurrence within a region spanning a repetitive element
17 or mononucleotide run including ± 5 bp of flanking sequence. If a missense mutation (or at least
18 one micro-deletion/micro-insertion breakpoint) was found to occur within this extended region,
19 the micro-lesion was labelled as being found in association with the corresponding type of
20 repeat.

21 *Assessing the statistical significance of the results generated*

22 To assess the similarity (or dissimilarity) of the germline and somatic mutational spectra with
23 respect to (i) the frequency with which the missense mutations were located within CpG/non-
24 CpG dinucleotides or CpHpG/non-CpHpG trinucleotides and (ii) the frequency with which the
25 micro-deletions/micro-insertions were found within/outwith repeats, the various non-overlapping
26 mutation datasets (bearing specific descriptors) were compared by means of the χ^2 test. Since the
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2 normality assumption did not hold for the datasets studied, the Wilcoxon rank-sum test was used
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4 to compare and contrast missense mutational spectra with respect to the Grantham score, degree
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6 of evolutionary conservation, and both the non-disease- and disease-associated mutability rates.
7

8
9 The permutation-based method [Olshen and Jain, 2002] was used to estimate the significance
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11 of our findings and to allow for multiple testing wherever appropriate. For each comparison, the
12
13 null hypothesis [viz. no overall difference between two groups of mutations (e.g. somatic and
14
15 potential) with respect to the specific property in question (e.g. occurrence in CpG or non-CpG
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17 nucleotides)], was tested for, either in the context of each gene or all genes combined. χ^2 or rank-
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19 sum statistics were calculated for the observed germline and somatic mutations as well as for
20
21 10,000 control sets of mutations created from the original sets by random permutation of the
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23 assigned mutational descriptors (e.g. randomly chosen mutations labelled as 'somatic' were re-
24
25 labelled as 'germline'; randomly chosen mutations labelled as 'shared' were re-labelled as
26
27 'somatic', etc.). The test statistic (χ^2 or rank-sum) for the original datasets that exceeded the 95th
28
29 percentile of χ^2 maxima for 10,000 control sets was deemed to be statistically significant; the
30
31 corresponding p-value was termed the 'gene-wise' p-value. To allow for multiple testing in those
32
33 cases where specific mutations in all genes were combined, a Bonferroni correction was applied;
34
35 the corresponding p-value was termed the 'experiment-wise' p-value.

36
37 Power calculations for the χ^2 tests were performed using the Pwr.Chisq.test package, part of
38
39 the R Statistical Language (<http://cran.r-project.org/>). A data based simulation method [Walters
40
41 2004] was used to perform power calculations for the Wilcoxon rank-sum tests. Only results
42
43 showing $\geq 80\%$ power to detect experiment- or gene-wise significance were reported.
44

45 46 47 *Naïve Bayes classifier*

48
49 A decision tree classifier known as a Naïve Bayes tree [NBTree; Kohavi, 1996], implemented in
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51 the Weka machine learning package [Witten and Frank, 2005], was trained to discriminate
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2 between somatic, germline, shared, recurrent somatic and recurrent shared missense mutations.
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4 Each mutation was described by a total of six features including the degree of evolutionary
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6 conservation, the non-disease-associated and disease-associated relative mutability rates,
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8 Grantham score, and occurrence in CpG/CpHpG, non-CpG/non-CpHpG doublets/triplets or in
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10 repeats/mononucleotide runs. Ten-fold cross-validation was used to assess the accuracy of
11
12 classification. The mutation datasets were balanced using random oversampling [Kotsiantis et
13
14 al., 2006] by replicating random instances from the minority classes until all classes were
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16 represented by the same number of instances as the majority class.
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23 Results and Discussion

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25 The availability of both germline and somatic mutational spectra from tumour suppressor genes
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27 provides us with an ideal opportunity to study the nature of mutation of the same gene sequences
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29 in both the germline and the soma. The analysis reported here explores for the first time the
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31 similarities and differences exhibited by the germline, somatic (and shared) micro-lesion
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33 mutational spectra in 17 human tumour suppressor genes. The study presented here focussed
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35 upon missense mutations and micro-deletions as well as micro-insertions. Nonsense mutations in
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37 tumour suppressor genes have already been addressed elsewhere in the context of a general
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39 meta-analysis of this type of lesion [Mort et al., 2008].
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43 *Characteristics of germline and somatic missense mutations with respect to mutation type*

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45 Taken together, the combined mutational spectra for all 17 tumour suppressor genes contained
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47 twice as many somatic (61%) as germline (31%) mutations. [Further details are provided in the](#)
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49 [Supplementary Text online.](#)
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Deleted: For five genes (*APC*, *CDKN2A*, *NF2*, *PTEN* and *TP53*), a predominance of somatic over germline mutations was noted, with the *TP53* gene having the highest proportion of somatic mutations (92%). For the majority of genes, however (namely *ATM*, *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTCH1*, *RBI*, *STK11*, *TSC1*, *TSC2*, *VHL* and *WT1*), the analysed dataset included more germline than somatic mutations, with >97% of all mutations in the *BRCA1*, *NF1*, *TSC2* and *WT1* genes being germline in origin.

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2 Shared mutations are of particular interest because identical mutational mechanisms operating
3 in the germline and the soma may be inferred for such lesions. The expected number of shared
4 mutations for each gene was calculated as $p_{\text{somatic}} \times p_{\text{germline}} \times (\text{total number of mutations})$,
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6 where p denotes the relative frequencies of somatic and germline mutations. Although the
7
8 proportion of shared mutations varies markedly between genes (from 0% to 25% of the total),
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10 only two genes (*TP53* and *VHL*) were found to have a higher than expected number of shared
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12 mutations as calculated above.
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19 *Patterns of germline and somatic missense mutations by mutation type*

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21 Missense mutations were characterised by a predominance of transitions over transversions
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23 (Figure 2). The transition:transversion ratio was at its highest for shared recurrent mutations (3.5)
24
25 and shared non-recurrent mutations (2.7). By contrast, the transition:transversion ratio for the
26
27 control group (i.e. potential mutations) was 0.85. Significant differences in the
28
29 transition:transversion ratio were observed between all mutation types ($p < 0.05$) with the
30
31 exception of germline vs. shared mutations (Figure 2).
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34 Not surprisingly, a strong positive correlation was noted between somatic and shared
35
36 mutational spectra (Pearson's correlation $r = 0.986$, $p = 2.91 \times 10^{-4}$) with respect to the frequencies
37
38 of six mutational changes viz. A.T>C.G, A.T>G.C, A.T>T.A, C.G>A.T, C.G>G.C and
39
40 C.G>T.A. Weaker negative correlations were found between somatic mutations and the control
41
42 dataset of mutations ($r = -0.887$, $p = 0.019$) and between the shared and control ($r = -0.837$,
43
44 $p = 0.038$) mutational spectra, indicative of the non-randomness of somatic mutation.

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46 C.G>T.A transitions constituted the most frequent type of mutation in shared (46%), germline
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48 (29%) and somatic (25%) mutational spectra, significantly higher proportions than noted in the
49
50 spectrum of mutations within our control dataset (13%, $p < 0.001$) (Figure 2). Intriguingly, the
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52 number of A.T>G.C mutations was significantly higher (28%) in the germline as compared to
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2
3 the somatic (16%), shared (17%) and control (16%) mutational spectra (Figure 2). A.T>C.G
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5 mutations were significantly under-represented in the shared mutational spectrum (7%, $p<0.001$)
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7 as compared to the other spectra whereas A.T>T.A mutations were under-represented (7%,
8
9 $p<0.001$) in both the germline and shared mutational spectra compared to both somatic and
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11 potential mutations (Figure 2). Finally, C.G>A.T mutations were significantly underrepresented
12
13 in the germline mutational spectrum (10%) as compared to the somatic (16%, $p=1.2\times 10^{-5}$) and
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15 potential (15%, $p=2.6\times 10^{-5}$) spectra. Thus, the main similarity between the somatic and germline
16
17 missense mutational spectra was in relation to C.G>T.A transitions whereas the main differences
18
19 between these spectra involved the A.T>G.C, A.T>T.A and C.G>A.T mutations. In passing, it
20
21 should be noted that the patterns of somatic nucleotide substitution exhibited by the 17 tumour
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23 suppressor genes studied here were markedly different from the genome-wide patterns of
24
25 somatic nucleotide substitution Deleted: previously observed in various cancer genome sequencing studies [Sjöblom
26
27 et al., 2006; Greenman et al., 2007; Kan et al., 2010]; [these mutation datasets are likely to differ](#)
28
29 [quite dramatically with respect to their relative proportions of 'passenger' mutations.](#)
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32 *CpG- and CpHpG-located missense mutations*

34 The CpG dinucleotide is a well known mutational hotspot in the human genome as a
35
36 consequence of the spontaneous (and endogenous) deamination of 5-methylcytosine. In addition,
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38 Lister et al. [2009] reported abundant DNA methylation in CpHpG trinucleotides in the human
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40 genome, where H is either A, C or T, raising the possibility that CpHpG might also be a
41
42 generalized mutation hotspot [Cooper et al., 2010].
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44 The proportion of missense mutations that were either C>T or G>A within CpG or CpHpG
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46 oligonucleotides in the 17 tumour suppressor genes was found to vary between 0% and 100%
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48 (Table 2). This wide range in values may be attributed to the small size of some of the gene
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50 mutation datasets under study. Importantly, the CpG and CpHpG oligonucleotides were found to
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1
2 be disproportionately likely to harbour shared mutations; thus, 34% of shared recurrent
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4 mutations and 21% of shared non-recurrent mutations were C>T and G>A mutations in CpG
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6 dinucleotides with an additional 10% and 9% of mutations, respectively, occurring within
7
8 CpHpG trinucleotides. Since driver mutations tend to occur disproportionately frequently within
9
10 CpG dinucleotides [Talavera et al., 2010], we postulate that missense mutations identified as
11
12 being shared are highly likely to be driver mutations.
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15 Significant differences were noted between the relative frequencies of CpG- and CpHpG-
16
17 located mutations for somatic, germline, shared, somatic recurrent and shared recurrent missense
18
19 mutations (Supp. Table 2).

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21 We have previously shown that 18.2% and 9.9% of all missense/nonsense mutations recorded
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23 in the HGMD are C>T and G>A transitions in CpG and CpHpG oligonucleotides respectively
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25 [Cooper et al., 2010]. In the present study, we observed that the mutational spectra of shared and
26
27 shared recurrent missense mutations in tumour suppressor genes were both found to be
28
29 significantly enriched in CpG-located mutations (χ^2 -test; p-values, 0.028 and 1.1×10^{-9}
30
31 respectively). This implies that the CpG dinucleotide is a generalized mutation hotspot in both
32
33 the soma and the germline as a consequence of the endogenous mutational mechanism of
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35 methylation-mediated deamination of 5-methylcytosine. By contrast, the number of CpG-located
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37 mutations was significantly underrepresented (χ^2 -test; p-values $< 5 \times 10^{-14}$) in the other mutational
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39 spectra (i.e. non-recurrent somatic, somatic recurrent and germline mutations) by comparison
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41 with HGMD data. To perform these comparisons, missense mutations (Table 2) and nonsense
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43 mutations [previously reported in Mort et al., 2008; see Table 6 therein] in all 17 tumour
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45 suppressor genes were combined. The proportion of shared recurrent missense mutations in
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47 tumour suppressor genes that were CpHpG-located was found to be significantly higher
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49 ($p=0.023$) than for mutations recorded in the HGMD whereas CpHpG-located somatic and
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51 recurrent somatic mutations were significantly under-represented ($p < 4 \times 10^{-10}$). Significant
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2 enrichment in CpHpG-located mutations was observed for germline mutations as compared to
3 somatic mutations ($p < 3 \times 10^{-10}$) consistent with the reported decrease in CpHpG methylation in
4 differentiated cells [Lister et al., 2009]. In summary, germline and shared missense mutations
5 were found to be significantly enriched at CpG and CpHpG oligonucleotides.
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10 The numbers of somatic and shared C>T and G>A transitions recorded within CpG
11 dinucleotides for each gene (Table 2) did not correlate with the numbers of CpG dinucleotides
12 found in these genes ($r < -0.5$, $p > 0.127$) and hence do not simply reflect intragenic CpG
13 frequency. A weak positive correlation between CpG-located mutations and the number of genic
14 CpG dinucleotides was however noted for germline mutations ($r = 0.489$, $p = 0.046$) indicating that
15 CpG methylation is not entirely unrelated to the number of CpG dinucleotides, at least with
16 respect to the germline; the relationship is however clearly more complex in the soma, possibly
17 due to inter-tissue differences in gene methylation patterns [Tornaletti and Pfeifer, 1995] or
18 transcription-coupled repair [Rubin and Green, 2009].
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29 No correlation was found between the numbers of somatic, germline and shared mutations
30 recorded within CpHpG trinucleotides and the corresponding numbers of CpHpG trinucleotides
31 for these genes ($r = -0.316, 0.373, -0.414$; p -values 0.281, 0.216 and 0.098, respectively)
32 indicating that mutation within CpHpG trinucleotides is likely to be very much a gene-specific
33 phenomenon (presumably dependent on both the extent and the degree of spatial localization of
34 CpHpG methylation in the germline and/or soma).
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41 Finally, the number of CpG dinucleotides in the various tumour suppressor genes studied
42 (Table 2) was not found to correlate with gene length ($r = 0.3$, p -value=0.241). By contrast, we
43 found a significant correlation ($r = 0.885$, p -value= 2.35×10^{-6}) between tumour suppressor gene
44 length and the number of CpHpG trinucleotides (excluding those with mutations), indicating that
45 the tumour suppressor genes under study possess a similar density of CpHpG trinucleotides per
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2 unit length. We surmise that the factors that govern the establishment of the methylation pattern
3
4 of CpHpG trinucleotides are likely to be quite complex.
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9 *Evolutionary conservation of tumour suppressor genes in relation to the sites of somatic and*
10 *germline missense mutations*

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12 For all 17 tumour suppressor genes, the degree of evolutionary conservation, as measured by
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14 Ka/Ks , was less than unity, indicating that these genes (and proteins) have been highly
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16 conserved evolutionarily as a consequence of the action of purifying selection. Indeed, the
17
18 degree of evolutionary conservation displayed by most of the studied genes was markedly lower
19
20 than the average (~0.18) noted in a comparison of 1880 human, rat and mouse gene orthologues
21
22 [Makalowski and Boguski, 1998]. However, three genes (*CDKN2A*, *BRCA1* and *BRCA2*) were
23
24 found to exhibit a higher rate of evolutionary conservation than the average between human and
25
26 rodents.
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30 The evolutionary conservation of each mutated codon was inferred by calculating the $\frac{Ka}{Ka + Ks}$
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32 ratio; for each gene/spectrum, the mean value was then calculated across all mutations in the
33
34 corresponding gene/spectrum. Shared recurrent missense mutations were found to occur
35
36 disproportionately in highly conserved amino acid residues (mean degree of evolutionary
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38 conservation, 0.072) followed by shared non-recurrent mutations (0.138), somatic recurrent
39
40 (0.169), germline (0.175), non-recurrent somatic (0.265), and control dataset mutations (0.255).
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42 The observed differences in the degree of evolutionary conservation for the different mutational
43
44 spectra are shown in Supp. Table 2. These quite specific findings are consistent with the
45
46 previously reported general tendency for cancer-associated mutations to occur frequently at
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48 evolutionarily conserved sites [Greenblatt et al., 2003; Tavtigian et al., 2009; Talavera et al.,
49
50 2010].
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2 Somatic non-recurrent mutations were found to occur in codons characterized by the highest
3
4 mean value of $\frac{K_a}{K_a + K_s}$ ratios as compared not only to the shared recurrent and shared non-
5
6 recurrent mutations (see above) but also to the mutations within the control dataset. This is
7
8 consistent with the interpretation that a high proportion of non-recurrent somatic mutations, and
9
10 most notably those which are located in less evolutionarily conserved regions ([characterised by](#)
11
12 [higher values of the degree of evolutionary conservation](#)), are likely to be ‘passenger’ mutations.
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16 17 *Missense mutations in relation to the disease- and non-disease-associated substitution rates*

18 Employing alignments of paired human gene/pseudogene sequences, Hess et al. [1994] derived
19
20 relative (non-disease-associated) nearest-neighbour-dependent mutability rates using the lowest
21
22 frequency substitution type, C(T>G)A/T(A>C)G, as a baseline. These mutability rates were
23
24 found to vary over a 52-fold range, with unity being assigned to the lowest frequency
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26 substitution type. This *non-disease-associated* mutability rate approximates to the neutral
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28 mutation frequency and hence reflects the intrinsic mutability of the underlying DNA sequence.
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30 Depending upon the observed nearest-neighbour context, we retrieved the corresponding non-
31
32 disease-associated mutability rate (from the data of Hess et al. 1994) for each mutation (either
33
34 observed or from the control dataset) and calculated the median value for each mutational
35
36 spectrum. These median values are indicative of the relative mutability of each tumour
37
38 suppressor gene. [Further details are provided in the Supplementary Text online.](#)
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41
42 When data from all 17 genes were combined, shared recurrent mutations were found to be
43
44 characterised by intrinsically low non-disease-associated mutability (median=11), followed by
45
46 even lower median mutability values for shared non-recurrent mutations (7.9), germline
47
48 mutations (7.2), somatic recurrent and non-recurrent (4.7) and control dataset mutations (4.1).
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50 Such low median mutability values across all groups indicates that at least half of the mutations
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52 within observed triplets are unlikely to be neutral in the sense defined by Hess et al. [1994] and
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Deleted: The median values were found to vary between 4 (*NF2*) and 8.9 (*STK11*) for somatic mutations, 4.1 (*TP53*) and 10.1 (*WT1*) for germline mutations, and 7.2 (*RBI*) and 11 (*P TEN*) for shared mutations (values given only for genes with more than three mutations in the corresponding category; see Supp. Table 3, indicating that many of the median values are quite low and hence the corresponding mutations are unlikely to be neutral.¶)

1
2 hence are not simply explicable in terms of intrinsic DNA mutability. The low median mutability
3
4 values for the control dataset of mutations within tumour suppressor genes reflect the high level
5
6 of evolutionary conservation manifested by tumour suppressor gene coding sequences across
7
8 different species, implying that any mutation within a triplet characterized by a low non-disease-
9
10 associated mutation rate is very likely to have pathological consequences and would thus be
11
12 subject to purifying selection.

13
14 In contrast to the non-disease-associated mutability rate (which is purely a reflection of the
15
16 intrinsic DNA mutability), the disease-associated mutability rate reflects (in addition to the
17
18 intrinsic DNA mutability) the increased likelihood of coming to clinical attention conferred by
19
20 the loss of biological function. The C(G>T)T mutation is one of the most frequent types of
21
22 mutation associated with the loss of biological function [disease-associated mutability rate
23
24 10.255; Krawczak et al., 1998] but occurs much less frequently among neutral mutations [non-
25
26 disease-associated mutability rate 4.4; Hess et al., 1994].

27
28 For each tumour suppressor gene and each mutational spectrum, the disease-associated median
29
30 mutability values were calculated using mutability rates derived from Krawczak et al. [1998].

31
32 The disease-associated median value was found to be 0.85 for the germline mutations. [Further](#)
33
34 [details are provided in the Supplementary Text online.](#) We found that shared recurrent and

35
36 shared non-recurrent mutational spectra were characterized by higher median values of the
37
38 disease-associated mutability rates (1.42 and 1.01 respectively) whereas somatic non-recurrent,
39
40 somatic recurrent and control dataset mutations exhibited lower median mutability rates (0.5, 0.5
41
42 and 0.4 respectively) as compared to germline mutations (0.85). The finding that the shared
43
44 mutations (which, by definition, occur in both the germline and the soma) are characterized by
45
46 higher disease-associated mutability rates is not surprising since mutations that occur with the
47
48 highest probability are among those most likely to be shared.

Deleted: The highest and lowest disease-associated median values for the mutation rates were noted for somatic mutations in the *STK11* gene (1.7; Supp. Table 3) and for germline mutations in the *TP53* (0.42) gene (values given only for genes with more than three mutations in the corresponding category).

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3 We postulated that those mutations which occur both in the germline and the soma, and which
4 are characterised by higher disease-associated mutability rates are disproportionately likely to be
5 drivers of tumour development. Consistent with this postulate, somatic recurrent and non-
6 recurrent mutational spectra are characterized by lower median disease-associated mutability
7 rates as compared to the germline spectrum. However, given that higher disease-associated
8 mutability rates are a characteristic feature of driver mutations, a certain proportion of the
9 somatic mutations, namely those characterised by higher disease-associated mutability rates, may
10 correspond to functionally significant driver mutations.
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18 In assessing the significance of our results, it was appropriate to consider the possibility that
19 somatic mutations might display quite different nearest-neighbour-dependent disease-associated
20 mutability rates from germline mutations. However, since a good correlation was observed
21 between the mutability rates derived from inherited disease data [Krawczak et al., 1998] and the
22 neighbour-dependent mutability rates calculated for the somatic mutations of the 17 tumour-
23 suppressor genes studied here (Pearson's correlation $r=0.703$, $p=6.6\times 10^{-30}$), this *caveat* appears
24 not to be an issue.
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34 *Distribution of Grantham scores with respect to tumour suppressor gene mutations*

35 Shared recurrent mutations were found to exhibit the largest median chemical difference value
36 (Grantham scores) between the wild-type and mutated amino acid residues (100) followed by
37 shared non-recurrent mutations and germline mutations (both 93), somatic recurrent (85),
38 somatic non-recurrent (80) and potential mutations (78). Since there was an obvious trend for
39 shared recurrent and non-recurrent mutations to cause the most dramatic chemical changes of the
40 affected codon, we may infer that these types of lesion are also more likely to be driver
41 mutations. However, bearing in mind that the range of theoretically possible values varies
42 between 5 (Leu ↔ Ile) and 215 (Cys ↔ Trp), less elevated median values may simply indicate
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3 that a proportion of the mutations in each mutational spectrum are likely to be chemically less
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5 dramatic (Grantham scores <100).
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9 *Missense mutations occurring within repeats and runs of identical nucleotides*

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11 A number of studies have noted that single base-pair substitutions associated with inherited
12
13 disease occur disproportionately either within, or in close proximity to, repetitive sequences
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15 [Jego et al., 1993; Greenblatt et al., 1996; Tappino et al., 2009; Thomas et al., 2010; Leclercq et
16
17 al., 2010]. Hence, we wished to assess whether either germline or somatic mutations occurred
18
19 disproportionately either within, or in the vicinity (see *Mutation descriptors*) of, direct, inverted
20
21 and symmetric repeats or mononucleotide runs in the 17 tumour suppressor genes under study
22
23 (Table 3, Supplementary Tables 4-6).
24

25 On average, direct repeats of length ≥ 8 bp were found to cover 5.6% of the cDNA lengths of
26
27 the 17 tumour suppressor genes. [Further details are provided in the Supplementary Text online](#)

28
29 On average, mononucleotide runs ≥ 4 bp spanned 19.9% of the cDNA lengths. Approximately
30
31 24% of non-recurrent somatic and 20% of germline missense mutations were found in
32
33 mononucleotide runs; these proportions were significantly higher than noted for shared non-
34
35 recurrent missense mutations (4.9%, $p \leq 1.6 \times 10^{-4}$). A greater proportion of non-recurrent somatic
36
37 missense mutations was found in direct repeats (7%) as compared to recurrent somatic missense
38
39 mutations (2%, $p = 8.8 \times 10^{-7}$), germline missense (4%, $p = 0.028$) and potential missense mutations
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41 (3.7%, $p = 8.1 \times 10^{-7}$). This result may reflect the disproportionate number of CpG/CpHpG
42
43 mutations among shared and recurrent somatic missense mutations. Further, for all mutational
44
45 spectra examined (with the exception of the shared mutations), missense mutations were
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47 preferentially found in association with inverted and symmetric repeats as compared to the
48
49 control dataset of mutations ($p < 0.05$). However, no statistically significant differences were
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51 found between mutational spectra. [Further details are provided in the Supplementary Text online.](#)
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Deleted: , the coverage varying between 2.5% (*BRCA2*) and 17% (*PTEN*) of the respective gene sequences. The corresponding proportion of the cDNA lengths for inverted repeats ≥ 8 bp was 8.5%, with proportions varying between *PTCH1* (4.5%) and *RBI* (15.7%) while symmetric elements ≥ 8 bp were found to encompass 25% of the cDNA lengths (varying between 15.5% for *APC* and 44% for *PTEN*). ¶

Deleted: , varying between 9.5% (*VHL*) and 29% (*TP53*)

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Towards a classification of somatic and germline missense mutations

All observed mutations within each mutational spectrum were re-categorized (Supp. Table 7) with respect to the location of mutations within CpG/CpHpG oligonucleotides, within different types of repeat/mononucleotide runs, within both CpG/CpHpG oligonucleotides and repeats. 4x2 contingency tables were then used to measure the strength of the pairwise associations between the various mutational distributions presented in Supp. Table 7, the significance of the associations being assessed by means of a Chi-square test. Significant ($p < 0.002$) pairwise differences were noted between somatic and germline, somatic and shared, and between germline and shared mutational spectra ($p < 0.002$) with respect to the features listed above and each of four types of repeat, indicating that these features have great discriminant potential.

All somatic, germline, shared non-recurrent, recurrent somatic and shared recurrent missense mutations (each described by a combination of different features (i.e. degree of evolutionary conservation, non-disease- and disease-associated mutability rates, Grantham score, CpG/CpHpG location, occurrence within repeat/mononucleotide run) were then used to train a Naïve Bayes Tree classifier. On average, 63.1% of somatic, germline, shared, recurrent somatic and shared recurrent mutations were correctly classified [the area under the Receiver Operating Characteristic (ROC) curve being 0.869, indicating a reasonably good classification] with 71% and 75% respectively of shared and shared recurrent mutations being correctly recognized implying that the mutation groupings differ with respect to the different features in a consistent fashion. One would expect 20% of mutations to be assigned to each of the five groups by chance alone. Indeed, the average percentage did not exceed 20% when randomly selected datasets matching the number of somatic, germline, shared, recurrent somatic and shared mutations were drawn from the set of potential mutations; the average was taken over 10 matching datasets. The complete Naïve Bayes Tree classifier is depicted in Supp. Figure 1.

Deleted: No correlation was observed between the number of mutations located within repeats and the fractional length of the cDNA covered by repeats, indicating that not every repeat sequence is mutation-prone. However, a strong correlation between the fractional length of the cDNA covered by repeats and cDNA length of genes ($r > 0.87$ and $p < 10^{-6}$) served to demonstrate that repeat density per unit length was approximately the same for all tumour suppressor genes studied. ¶

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2 An additional non-overlapping dataset of 568 missense somatic mutations, identified in the 17
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4 tumour suppressor genes under study, were extracted from a collection of 2,488 mutations
5
6 identified as being probable driver mutations [Carter et al., 2009]. Features such as the degree of
7
8 evolutionary conservation, Grantham score, mutability rates, CpG/CpHpG location, occurrence
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10 within repeats/mononucleotide runs were again determined for each of these mutations.
11
12 Employing our classifier, 7% and 10% respectively of these 568 mutations were found to possess
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14 features consistent with their being shared recurrent and shared non-recurrent mutations. In
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16 addition, 32% of these probable driver mutations were found to bear features characteristic of
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18 recurrent somatic mutations (i.e. mutations documented in different tumours). A further 25% of
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20 the probable (somatic) driver mutations were classified as possessing features characteristic of
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22 germline mutations and hence could conceivably be treated as shared mutations missing from the
23
24 original training dataset. The remaining 25% of mutations were classified as non-recurrent
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26 somatic mutations. Using this classifier, which is based on a very modest number (6) of
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28 predictive features, to analyse an independent dataset of probable driver mutations, we were able
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30 to predict that ~50% of these somatic missense mutations exhibited features specific to either
31
32 shared or recurrent mutations, indicating that a disproportionate number of such lesions are likely
33
34 to be drivers of tumorigenesis. This percentage is certainly lower (79%) than that obtained by
35
36 Carter et al., [2009] through the application of a Random Forest Classifier based on 500 trees and
37
38 >50 predictive features (using an 'out-of-the-bag' error estimate similar to the cross-validation
39
40 procedure) to the set of putative 2,488 driver mutations. However, based on the results of this
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42 study, we may conclude that, in general, the mutational spectrum of driver mutations is likely to
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44 contain a disproportionate number of somatic mutations that have germline counterparts (~17%)
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46 whilst an additional 32% of the driver mutations are likely to occur recurrently in the soma.
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Occurrence of micro-deletions and micro-insertions within repeats and runs of identical nucleotides

The mutational spectrum of micro-deletions, combined for all 17 tumour suppressor genes, comprised 55% germline, 43% somatic and 2% shared mutations. The mutational spectrum of micro-insertions was similar to that of micro-deletions and comprised 60% germline, 38% somatic and 2% shared mutations. Approximately 77% somatic, 87% germline and 91% shared micro-deletions and micro-insertions were ≤ 4 bp in length. Strong ($r = \sim 1$) correlations were noted between the distributions of micro-deletions and micro-insertions with respect to the length of the deleted/inserted fragments, both gene-wise and for all genes combined ($r > 0.9$, $p < 10^{-8}$) for all mutational spectra.

Recent studies have revealed that simple repetitive DNA sequences are not only capable of adopting non-B DNA conformations and are highly mutagenic [Bacolla et al., 2004; Bacolla and Wells, 2004; Chuzhanova et al., 2009]. Indeed, both direct repeats and mononucleotide runs have long been known to be mutation hotspots in the *TP53* gene [Jego et al., 1993; Greenblatt et al., 1996]. The number of micro-lesions occurring in the vicinity (see *Mutation descriptors*) of direct, symmetric and inverted repeats (capable respectively of slipped, triplex and cruciform non-B structure formation), or within mononucleotide runs (which often mediate micro-deletions/micro-insertions) were therefore determined. The number of mutations found in the vicinity of all three types of repeat, and within mononucleotide runs, are given in Tables 3 and Supp. Tables 4-6.

The highest proportion of mutations in mononucleotide runs was found for the shared (39%), germline (30%) and somatic (25%) mutational spectra. Significant differences were observed between shared and germline ($p = 0.0002$), somatic and shared ($p = 0.045$), and between all mutational spectra and potential mutations ($p < 0.0001$) with respect to their occurrence within mononucleotide runs, confirming that these simple repeats constitute an important hotspot for

Deleted: Truncating vs non-truncating mutations in the germline and soma
Somatic mutational spectra from the *BRCA2*, *CDKN2A*, *STK11*, *TP53* and *TSC1* genes were characterized by the predominance of non-truncating (i.e. missense) lesions over truncating lesions (i.e. nonsense mutations, frameshift micro-deletions, micro-insertions and indels) when nonsense mutations [reported in Mort et al. (2008)] and micro-indels (excluded from previous analyses) were also considered (Supp. Table 8). A similar predominance of non-truncating over truncating lesions was observed for the germline mutational spectra of the *CDKN2A*, *TP53*, *VHL* and *WT1* genes. In general, the ratio of non-truncating to truncating lesions was found to be significantly higher in the soma (0.85) than in the germline (0.30; p -value $< 2.20 \times 10^{-16}$). All other mutational spectra were characterized by the predominance of truncating mutations. ¶

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2 micro-deletions and micro-insertions in both the soma and the germline. The preponderance of
3 such mutations in mononucleotide runs is unsurprising in the context of the shared mutations
4 since all mutations that occur with high frequency within mutation hotspots are more likely to be
5 shared between the germline and the soma (as previously noted for CpG and CpHpG mutations).
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7 No other types of repeat were disproportionately associated (after correction for multiple testing)
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9 with micro-deletions and micro-insertions.
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16 *Regional hotspots in somatic and germline mutational spectra*

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18 For the purposes of the following analysis, a regional mutation hotspot was defined as a stretch
19 of DNA of length ≤ 20 bp where four or more independent mutational events have been reported
20 and a significant degree ($p \leq 0.05$) of clustering of these mutations was evident for a given stretch
21 of DNA. In this definition of a regional hotspot, each recurrent mutation was considered only
22 once. The order statistics, r-scans, as described by Karlin and Macken [1991] and applied in
23 Bacolla et al. [2006], were used to detect significant clustering of mutations by comparison with
24 a Poisson distribution of mutations along the gene sequence. Overlapping hotspot regions were
25 considered as a single regional hotspot.
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34 The only regional mutational hotspot for somatic missense mutations was observed in the
35 *PTEN* gene and comprised 18 mutations in the region between nucleotide positions 269 and 286.
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37 Several germline regional mutational hotspots were however detected for missense mutational
38 spectra in the *ATM*, *BRCA1*, *BRCA2*, *NF1*, *PTEN*, *RBI*, *STK11*, *TP53* and *WT1* genes (Table 4).
39
40 Several somatic regional mutational hotspots were found for micro-deletions/micro-insertions in
41 the *APC* gene, the largest of which contained 33 mutations (positions 4303-4398) and forms part
42 of a previously reported mutation cluster region [Miyoshi et al., 1992]. Regional hotspots
43 identified in different mutational spectra were however unique to that spectrum. The only
44 overlap noted between regional mutational hotspots identified in germline and somatic micro-
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2 deletion/micro-insertion mutational spectra was observed for the *APC* gene (the overlapping
3 region comprising nucleotide positions 3919-3933). This micro-deletion/micro-insertion hotspot
4 also includes codon 1309 (cDNA positions 3925-3927) found to be frequently mutated in Greek
5 and French patients with familial adenomatous polyposis [Fostira et al. 2010; Lagarde et al.
6 2010].
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12 Inspection of regional hotspot sequences revealed that they are rich in repetitive elements, runs
13 of identical nucleotides and CpG/CpHpG oligonucleotides, offering immediate explanations for
14 the elevated mutability.
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19 *Germline and somatic mutations located within specific hotspot motifs*

20 The cDNA sequences of 17 tumour suppressor genes were screened for the presence of nine
21 specific motifs (and their complements) previously reported as being hotspots for mutation.
22 These motifs included the putative somatic (cancer) mutation hotspot, WKVNRRRNVWK [the
23 ‘THEMIS motif’; Makridakis et al., 2009], the RGYW motif that correlates with the DNA
24 polymerase eta error spectrum [Rogozin et al., 2001] and several so-called ‘super hotspot’ motifs
25 originally found in germline micro-insertions and micro-deletions [Ball et al., 2005] and indels
26 [Chuzhanova et al., 2003]. For the purposes of this analysis, the shared mutations were added to
27 both the germline and somatic mutational spectra. Both germline and somatic micro-deletions
28 and micro-insertions were found to be significantly overrepresented ($p \leq 0.002$) in the ‘indel super
29 hotspot’ motif GTAAGT and its complement. Somatic micro-deletions and micro-insertions
30 were also significantly overrepresented ($p = 0.009$) with respect to the micro-deletion/micro-
31 insertion super hotspot AAATCT and its complement. The number of germline (but not somatic)
32 micro-deletions/micro-insertions in the THEMIS motif were significantly overrepresented
33 ($p = 0.003$) as compared to the controls. No significant difference was however observed in the
34 number of missense mutations occurring in any motifs analysed.
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Conclusions

Several conclusions may be drawn from the results reported here. Firstly, it would appear that those missense mutations that are found both in the soma and the germline ('shared mutations') are disproportionately more likely to exert an effect on tumour development and/or progression (i.e. more likely to be driver mutations) than exclusively somatic non-recurrent missense mutations (at least for the *TP53* and *CDKN2A* genes whose mutations contributed the bulk of the documented shared mutations in our tumour suppressor gene mutation dataset). Shared mutations also occur preferentially in CpG/CpHpG oligonucleotides and are characterised by higher mutability rates (both non-disease- and disease-associated). Further, we found that shared mutations tend to occur in those codons that have been more highly conserved evolutionarily, and are associated with more dramatic chemical differences between the substituted (wild-type) and substituting amino acids. Taken together, it would thus appear that shared mutations are influenced to a greater extent by the local nucleotide sequence context than either germline or somatic non-recurrent missense mutations. Since this implies that shared mutations (the mutation category most likely to harbour driver mutations) have a tendency to arise through the action of similar endogenous mutational mechanisms, we may infer that endogenous mechanisms of mutagenesis exert a disproportionate effect on tumorigenesis.

In an analysis of an unrelated dataset, we demonstrated that 17% of somatic missense mutations previously identified as being probable drivers [Carter et al., 2009] were found to possess the same features as shared (both recurrent and non-recurrent) mutations. A further 32% of these probable driver mutations shared the features expected of recurrent somatic mutations. Thus, we may conclude that ~50% of these somatic missense mutations possess features consistent with their being either shared or recurrent, suggesting that a disproportionate number of such lesions are likely to be drivers of tumorigenesis.

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A sizeable proportion of shared (39%) and germline (30%) micro-lesions were found to be located in runs of identical nucleotides ≥ 4 bp, making mononucleotide runs a hotspot for micro-deletion and micro-insertions. The most likely underlying causative mechanism for these mutations is slipped mispairing at DNA replication mediating duplications and ‘de-duplications’ [Kondrashov & Rogozin, 2004]. With regard to missense mutations, CpG and CpHpG oligonucleotides were found to be hotspots for shared recurrent and shared non-recurrent missense mutations; 34% (10%) and 21% (9%) of respective mutations were found in CpG (CpHpG) oligonucleotides. Further, 12% of the 568 probable driver mutations [derived from Carter et al., 2009] were found to occur in CpG/CpHpG oligonucleotides. 41% of probable driver mutations were found in repeats that were capable of non-B DNA structure formation (cf. 23% for potential mutations). Several regional mutation hotspots were found in the mutational spectra of various genes; one of these, in the *APC* gene, was a regional hotspot for both somatic and germline micro-deletions/micro-insertions and corresponded to a previously recognized mutation hotspot [Miyoshi et al., 1992].

Taken together, the results and analysis presented herein strongly suggest that algorithms that attempt to predict the relative impact of tumour-associated micro-lesions on (tumour suppressor) gene and protein function [Tavtigian et al., 2008; Couch et al., 2008; Thusberg and Vihinen, 2009], should take into consideration the origin (i.e. somatic, germline or shared) of the mutations, their sequence context and repetitivity, as well as their frequency of occurrence.

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For Peer Review

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For Peer Review

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3 **Figure Legends**

4 **Figure 1.** Diagrammatic representation of the number of various types of mutations analysed in
5 the present study.
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8 **Figure 2.** Nucleotide substitution patterns of missense mutations in 17 tumour
9 suppressor genes.
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For Peer Review

1
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3 Comparative Analysis of Germline and Somatic Micro-lesion Mutational Spectra in
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6 17 Human Tumour Suppressor Genes
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9 (Supplementary Text)
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6 *Gene-wise characteristics of germline and somatic missense mutations with respect to mutation*
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8 *type*
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10 Taken together, the combined mutational spectra for all 17 tumour suppressor genes contained
11 twice as many somatic (61%) as germline (31%) mutations. For five genes (*APC*, *CDKN2A*,
12 *NF2*, *PTEN* and *TP53*), a predominance of somatic over germline mutations was noted, with the
13 *TP53* gene having the highest proportion of somatic mutations (92%). For the majority of genes,
14 however (namely *ATM*, *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTCH1*, *RBI*, *STK11*, *TSC1*, *TSC2*, *VHL*
15 and *WT1*), the analysed dataset included more germline than somatic mutations, with >97% of all
16 mutations in the *BRCA1*, *NF1*, *TSC2* and *WT1* genes being germline in origin.
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30 *Gene-wise characteristics of missense mutations in relation to the disease- and non-disease-*
31 *associated substitution rates*
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34 The median values were found to vary between 4 (*NF2*) and 8.9 (*STK11*) for somatic mutations,
35 4.1 (*TP53*) and 10.1 (*WT1*) for germline mutations, and 7.2 (*RBI*) and 11 (*PTEN*) for shared
36 mutations (values given only for genes with more than three mutations in the corresponding
37 category; see Supp. Table 3, indicating that many of the median values are quite low and hence
38 the corresponding mutations are unlikely to be neutral.
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46 The highest and lowest disease-associated median values for the mutation rates were noted for
47 somatic mutations in the *STK11* gene (1.7; Supp. Table 3) and for germline mutations in the
48 *TP53* (0.42) gene (values given only for genes with more than three mutations in the
49 corresponding category).
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58 *Gene-wise occurrence of missense mutations within repeats and runs of identical nucleotides*
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3 On average, the coverage of the respective gene sequences by direct repeats of length ≥ 8 bp
4 was found to vary between 2.5% (*BRCA2*) and 17% (*PTEN*). The corresponding proportion of
5 the cDNA lengths for inverted repeats ≥ 8 bp was found to vary between 4.5% (*PTCH1*) and *RBI*
6 15.7% (*RBI*) while symmetric elements ≥ 8 bp were found to vary between 15.5% for *APC* and
7 44% for *PTEN* genes.
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11 On average, mononucleotide runs ≥ 4 bp spanned 19.9% of the cDNA lengths, varying between
12 9.5% (*VHL*) and 29% (*TP53*).
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16 No correlation was observed between the number of mutations located within repeats and the
17 fractional length of the cDNA covered by repeats, indicating that not every repeat sequence is
18 mutation-prone. However, a strong correlation between the fractional length of the cDNA
19 covered by repeats and cDNA length of genes ($r > 0.87$ and $p < 10^{-6}$) served to demonstrate that
20 repeat density per unit length was approximately the same for all tumour suppressor genes
21 studied.
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34 *Truncating vs non-truncating mutations in the germline and soma*

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36 Somatic mutational spectra from the *BRCA2*, *CDKN2A*, *STK11*, *TP53* and *TSC1* genes were
37 characterized by the predominance of non-truncating (i.e. missense) lesions over truncating
38 lesions (i.e. nonsense mutations, frameshift micro-deletions, micro-insertions and indels) when
39 nonsense mutations [reported in Mort et al. (2008)] and micro-indels (excluded from previous
40 analyses) were also considered (Supp. Table 8). A similar predominance of non-truncating over
41 truncating lesions was observed for the germline mutational spectra of the *CDKN2A*, *TP53*, *VHL*
42 and *WT1* genes. In general, the ratio of non-truncating to truncating lesions was found to be
43 significantly higher in the soma (0.85) than in the germline (0.30; p -value $< 2.20 \times 10^{-16}$). All other
44 mutational spectra were characterized by the predominance of truncating mutations.
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Supplementary Figure 1. Naive Bayes Tree Classifier. Number in parenthesis shows the probability of a mutations being somatic non-recurrent, germline, shared non-recurrent, somatic recurrent and shared recurrent respectively.

Attributes:

Mut_Type
Hess_value
Krawczak_value
Evol
Grantham_score
CpG/CHG
Repeats

Test mode: 10-fold cross-validation

NBTree

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-----
Evol <= 0.205
| Repeats = 0
| | CpG/CHG = 0
| | | Krawczak_value <= 1.0465
| | | | Evol <= 0.155
| | | | | Evol <= 0.12
| | | | | | Krawczak_value <= 0.811
| | | | | | | Krawczak_value <= 0.099
| | | | | | | | Hess_value <= 3.1: (0.42) (0.08) (0.08) (0.33) (0.08)
| | | | | | | | Hess_value > 3.1: (0.23) (0.13) (0.03) (0.10) (0.52)
| | | | | | | | Krawczak_value > 0.099
| | | | | | | | | Hess_value <= 2.5
| | | | | | | | | | Grantham_score <= 146.5
| | | | | | | | | | | Hess_value <= 2.15: (0.27) (0.47) (0.02) (0.22) (0.02)
| | | | | | | | | | | Hess_value > 2.15: (0.14) (0.24) (0.05) (0.52) (0.05)
| | | | | | | | | | | Grantham_score > 146.5: (0.47) (0.07) (0.07) (0.33) (0.07)
| | | | | | | | | | | Hess_value > 2.5
| | | | | | | | | | | | Hess_value <= 5.45
| | | | | | | | | | | | | Grantham_score <= 30.5
| | | | | | | | | | | | | | Hess_value <= 5.2
| | | | | | | | | | | | | | | Hess_value <= 4.55
| | | | | | | | | | | | | | | | Hess_value <= 2.75: (0.27) (0.09) (0.09) (0.45) (0.09)
| | | | | | | | | | | | | | | | Hess_value > 2.75: (0.25) (0.43) (0.03) (0.28) (0.03)

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5 | | | | | Grantham_score > 91.5:      (0.33) (0.17) (0.03) (0.03) (0.43)
6 | | | | | Hess_value > 12.1
7 | | | | |   Evol <= 0.51
8 | | | | |     Repeats = 0
9 | | | | |       Grantham_score <= 44.5: (0.20) (0.07) (0.07) (0.60) (0.07)
10 | | | | |         Grantham_score > 44.5
11 | | | | |           Grantham_score <= 51: (0.09) (0.27) (0.09) (0.09) (0.45)
12 | | | | |             Grantham_score > 51: (0.24) (0.53) (0.03) (0.18) (0.03)
13 | | | | |               Repeats = 1:      (0.32) (0.45) (0.05) (0.14) (0.05)
14 | | | | |                 Evol > 0.51
15 | | | | |                   Hess_value <= 13.35: (0.27) (0.15) (0.04) (0.5) (0.04)
16 | | | | |                     Hess_value > 13.35: (0.28) (0.44) (0.04) (0.2) (0.04)
17 | | | | | Hess_value > 42.75
18 | | | | |   Repeats = 0
19 | | | | |     Evol <= 0.59
20 | | | | |       Evol <= 0.255:      (0.08) (0.12) (0.73) (0.04) (0.04)
21 | | | | |         Evol > 0.255
22 | | | | |           Evol <= 0.375: (0.18) (0.03) (0.03) (0.28) (0.49)
23 | | | | |             Evol > 0.375: (0.40) (0.13) (0.07) (0.33) (0.07)
24 | | | | |               Evol > 0.59
25 | | | | |                 Grantham_score <= 139: (0.02) (0.20) (0.75) (0.02) (0.02)
26 | | | | |                   Grantham_score > 139: (0.36) (0.43) (0.07) (0.07) (0.07)
27 | | | | | Repeats = 1
28 | | | | |   Hess_value <= 59.5
29 | | | | |     Hess_value <= 50.35: (0.40) (0.15) (0.05) (0.35) (0.05)
30 | | | | |       Hess_value > 50.35: (0.67) (0.13) (0.04) (0.13) (0.04)
31 | | | | |         Hess_value > 59.5: (0.19) (0.63) (0.06) (0.06) (0.06)

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32 === Stratified cross-validation ===

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33 === Summary ===

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34 Correctly Classified Instances      2797          63.1377 %
35 Incorrectly Classified Instances    1633          36.8623 %
36 Kappa statistic                     0.5392
37 Mean absolute error                  0.1878
38 Root mean squared error              0.3177
39 Relative absolute error              58.6858 %

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5 Root relative squared error 79.4156 %
6 Total Number of Instances 4430

7
8 === Detailed Accuracy By Class ===

	TP Rate	FP Rate	Precision	Recall	F-Measure	ROC Area	Class
	0.505	0.106	0.544	0.505	0.523	0.826	1
	0.426	0.082	0.566	0.426	0.486	0.778	2
	0.894	0.091	0.712	0.894	0.792	0.967	3
	0.475	0.109	0.52	0.475	0.497	0.809	4
	0.858	0.073	0.745	0.858	0.797	0.964	5
Weighted Avg.	0.631	0.092	0.617	0.631	0.619	0.869	

15
16 === Confusion Matrix ===

17
18 a b c d e <-- classified as
19 447 125 63 207 44 | a = 1
20 170 377 89 153 97 | b = 2
21 12 9 792 9 64 | c = 3
22 181 144 85 421 55 | d = 4
23 12 11 84 19 760 | e = 5

Supplementary Table 1. Tumour suppressor gene orthologues used to estimate the degree of evolutionary conservation of the various gene coding sequences

Gene	Species	cDNA sequence identifier	Protein sequence identifier
APC	<i>Xenopus laevis</i>	U64442.1	AAB41671.1
	<i>Bos taurus</i>	XM_865627.1	XP_870720.1
	<i>Rattus norvegicus</i>	NM_012499.1	NP_036631.1
	<i>Mus musculus</i>	NM_007462.1	NP_031488.1
ATM	<i>Gallus gallus</i>	XM_417160.1	XP_417160.1
	<i>Xenopus laevis</i>	AY668954.1	AAT72929.1
	<i>Rattus norvegicus</i>	XM_236275.3	XP_236275.3
	<i>Sus scrofa</i>	AY587061	AAT01608.1
	<i>Canis familiaris</i>	XM_845871.1	XP_850964.1
	<i>Mus musculus</i>	NM_007499	NP_031525.1
BRCA1	<i>Gallus gallus</i>	NM_204169.1	NP_989500.1
	<i>Xenopus laevis</i>	AF416868.1	AAL13037.1
	<i>Bos taurus</i>	NM_178573.1	NP_848668.1
	<i>Rattus norvegicus</i>	NM_012514.1	NP_036646.1
	<i>Canis familiaris</i>	NM_001013416.1	NP_001013434.1
	<i>Mus musculus</i>	NM_009764.2	NP_033894.2
BRCA2	<i>Gallus gallus</i>	NM_204276.1	NP_989607.1
	<i>Danio rerio</i>	XM_690042.1	XP_695134.1
	<i>Bos taurus</i>	XM_583622.2	XP_583622.2
	<i>Rattus norvegicus</i>	NM_031542.1	NP_113730.1
	<i>Canis familiaris</i>	NM_001006653.4	NP_001006654.2
	<i>Mus musculus</i>	NM_009765.1	NP_033895.1
CDH1	<i>Xenopus laevis</i>	BC068940.1	AAH68940.1
	<i>Danio rerio</i>	NM_131820.1	NP_571895.1
	<i>Bos taurus</i>	NM_001002763.1	NP_001002763.1
	<i>Rattus norvegicus</i>	NM_031334.1	NP_112624.1
	<i>Canis familiaris</i>	XM_536807.2	XP_536807.2
	<i>Mus musculus</i>	NM_009864.1	NP_033994.1
CDKN2A	<i>Gallus gallus</i>	NM_204433.1	NP_989764.1
	<i>Takifugu rubripes</i>	AJ250231.1	CAC12808.1
	<i>Bos taurus</i>	XM_868375.1	XP_873468.1
	<i>Rattus norvegicus</i>	NM_031550.1	NP_113738.1
	<i>Canis familiaris</i>	XM_538685.2	XP_538685.2
	<i>Mus musculus</i>	AF044336.1	AAC08963.1
NF1	<i>Gallus gallus</i>	XM_415914.1	XP_415914.1
	<i>Takifugu rubripes</i>	AF064564.2	AAD15839.1
	<i>Rattus norvegicus</i>	NM_012609.1	NP_036741.1
	<i>Canis familiaris</i>	XM_537738.2	XP_537738.2
	<i>Mus musculus</i>	NM_010897.1	NP_035027.1
NF2	<i>Gallus gallus</i>	NM_204497.2	NP_989828.2
	<i>Danio rerio</i>	NM_212951.1	NP_998116.1
	<i>Bos taurus</i>	XM_611643.2	XP_611643.2
	<i>Rattus norvegicus</i>	XM_341248.2	XP_341249.2
	<i>Canis familiaris</i>	XM_534729.2	XP_534729.2
	<i>Mus musculus</i>	NM_010898.2	NP_035028.2
PTCH1	<i>Xenopus laevis</i>	AF302765.1	AAK15463.1
	<i>Gallus gallus</i>	NM_204960.1	NP_990291.1
	<i>Danio rerio</i>	NM_130988.1	NP_571063.1
	<i>Meriones unguiculatus</i>	AB188226.1	BAE78534.1
	<i>Rattus norvegicus</i>	NM_053566.1	NP_446018.1
	<i>Mus musculus</i>	NM_008957.1	NP_032983.1
PTEN	<i>Xenopus laevis</i>	AF144732.1	AAD46165.1

	<i>Gallus gallus</i>	XM_421555.1	XP_421555.1
	<i>Bos taurus</i>	XM_613125.2	XP_613125.2
	<i>Canis familiaris</i>	NM_001003192.1	NP_001003192.1
	<i>Rattus norvegicus</i>	NM_031606.1	NP_113794.1
	<i>Mus musculus</i>	NM_008960.2	NP_032986.1
<i>RBI</i>	<i>Gallus gallus</i>	NM_204419.1	NP_989750.1
	<i>Rattus norvegicus</i>	XM_344434.2	XP_344435.2
	<i>Canis familiaris</i>	XM_534118.2	XP_534118.2
	<i>Mus musculus</i>	NM_009029.1	NP_033055.1
	<i>Oncorhynchus mykiss</i>	AF102861.1	AAD13390.1
	<i>Notophthalmus viridescens</i>	Y09226.1	CAA70428.1
<i>STK11</i>	<i>Xenopus laevis</i>	U24435.1	AAC59904.1
	<i>Danio rerio</i>	NM_001017839.1	NP_001017839.1
	<i>Rattus norvegicus</i>	XM_234900.2	XP_234900.2
	<i>Raja erinacea</i>	AF486831.1	AAL92113.1
	<i>Canis familiaris</i>	XM_542206.2	XP_542206.2
	<i>Mus musculus</i>	NM_011492.1	NP_035622.1
<i>TP53</i>	<i>Gallus gallus</i>	NM_205264.1	NP_990595.1
	<i>Danio rerio</i>	NM_131327.1	NP_571402.1
	<i>Bos taurus</i>	NM_174201.2	NP_776626.1
	<i>Rattus norvegicus</i>	NM_030989.1	NP_112251.1
	<i>Canis familiaris</i>	NM_001003210.1	NP_001003210.1
	<i>Mus musculus</i>	NM_011640.1	NP_035770.1
<i>TSC1</i>	<i>Gallus gallus</i>	XM_415449.1	XP_415449.1
	<i>Danio rerio</i>	XM_691747.1	XP_696839.1
	<i>Bos taurus</i>	XM_612846.2	XP_612846.2
	<i>Rattus norvegicus</i>	NM_021854.1	NP_068626.1
	<i>Canis familiaris</i>	XM_537808.2	XP_537808.2
	<i>Mus musculus</i>	NM_022887.2	NP_075025.2
<i>TSC2</i>	<i>Gallus gallus</i>	XM_414853.1	XP_414853.1
	<i>Takifugu rubripes</i>	AF013614	AAB86682.1
	<i>Bos taurus</i>	XM_581197.2	XP_581197.2
	<i>Rattus norvegicus</i>	NM_012680.2	NP_036812.2
	<i>Canis familiaris</i>	XM_537008.2	XP_537008.2
	<i>Mus musculus</i>	NM_011647.2	NP_035777.2
<i>VHL</i>	<i>Gallus gallus</i>	XM_414447.1	XP_414447.1
	<i>Danio rerio</i>	XM_681176.1	XP_686268.1
	<i>Bos taurus</i>	XM_613870.2	XP_613870.2
	<i>Rattus norvegicus</i>	NM_052801.1	NP_434688.1
	<i>Canis familiaris</i>	NM_001008552.1	NP_001008552.1
	<i>Mus musculus</i>	NM_009507.2	NP_033533.1
<i>WT1</i>	<i>Xenopus laevis</i>	U42011.1	AAB53152.1
	<i>Gallus gallus</i>	NM_205216.1	NP_990547.1
	<i>Rattus norvegicus</i>	NM_031534.1	NP_113722.1
	<i>Canis familiaris</i>	XM_846479.1	XP_851572.1
	<i>Sus scrofa</i>	NM_001001264.1	NP_001001264.1
	<i>Mus musculus</i>	NM_144783.1	NP_659032.1

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SupplementaryTable 2. Differences in distribution of parameters for somatic, germline, shared, somatic recurrent and shared recurrent missense mutations. Observed median and/or mean values are shown in brackets. (Note that the higher values correspond to less conserved genes whereas the low values refer to highly conserved ones).

Parameter	Observed trend (p<0.05)
Median non-disease associated mutability rate according to Hess et al. [1994]	shared recurrent >>shared non-recurrent >germline>>somatic~somatic recurrent* [10.7] [7.9] [7.3] [4.7] [4.7]
Median disease-associated mutability rate according to Krawczak et al. [1998]	shared recurrent>shared non-recurrent >germline>>somatic~somatic recurrent [1.42] [1.01] [0.85] [0.53] [0.53]
Mean/median degree of evolutionary conservation	shared recurrent < shared non-recurrent << somatic non-recurrent [0.072/0] [0.138/0] [0.265/0.24] somatic non-recurrent >> germline [0.265/0.24] [0.18/0]
Mean Grantham score	germline >somatic recurrent ~somatic non-recurrent [93] [85] [80] shared recurrent~shared non-recurrent >> somatic recurrent [100] [93] [85]
Proportion of CpG-located mutations	shared recurrent~shared >>germline>>somatic ~somatic recurrent [0.34] [0.21] [0.12] [0.08] [0.05]
Proportion of CpHpG-located mutations	shared recurrent~shared >> somatic recurrent [0.098] [0.082] [0.028]
Proportion of mutations located within or in the vicinity of direct repeats	somatic>>germline>>recurrent somatic [0.07] [0.04] [0.02]

Proportion of mutations located within (or in the vicinity of) runs of identical nucleotides	somatic>>shared	somatic>>shared recurrent
	[0.24] [0.05]	[0.24] [0.16]
	germline>>shared	somatic recurrent>>shared
	[0.20] [0.05]	[0.21] [0.05]

*Inequality **shared>germline>somatic** implies that a significant difference ($p < 0.05$) in the corresponding parameter was observed between each pair of mutational spectra, i.e. shared vs germline, shared vs somatic and germline vs somatic. Symbol '~' denotes the absence of any significant difference between any two mutational spectra with respect to a given parameter. Symbols '>>' or '<<' indicate experiment-wise statistical significance of the observed inequality whereas symbols '<' or '>' indicate gene-wise statistical significance.

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Supplementary Table 3. Various parameters of gene-wise somatic and germline missense mutational spectra vs. potential mutational spectra exhibiting either gene-wise (p<0.05) or experiment-wise differences (p<0.05; shaded in light grey) with respect to the parameters measured.

	Non-disease associated mutation rate		Disease-associated mutation rate		Evolutionary conservation rate		Grantham score		CpG-located missense mutations		CpHpG-located missense mutations	
	Gene symbol	Median	Gene symbol	Median	Gene symbol	Median	Gene symbol	Median	Gene symbol	%	Gene symbol	%
Somatic mutations			<i>STK11</i>	1.66					<i>STK11</i>	25		
			<i>PTCH1</i>	1.06								
		8.4	<i>CDKN2A</i>	1.01	<i>CDKN2A</i>	0.38			<i>CDKN2A</i>	20	<i>CDKN2A</i>	5.2
		7.9	<i>APC</i>	0.83								
		5.6	<i>PTEN</i>	0.53								
		4.6	<i>TP53</i>	0.5	<i>TP53</i>	0.17			<i>RB1</i>	18	<i>TP53</i>	2.8
					<i>VHL</i>	0.14			<i>BRCA2</i>	16		
								<i>PTCH1</i>	15			
for all 17 genes combined	somatic	4.7	somatic	0.53	somatic	0	somatic	78	somatic	8	somatic	2.5
	control	4.1	control	0.4	control	0.2	control	74	control	2	control	2
	germline	7.2	germline	0.85	germline	0	germline	94	germline	12	germline	3
Germline mutations												
		7.2			<i>TSC2</i>	0			<i>BRCA1</i>	7	<i>BRCA1</i>	3.6
		7.3					<i>NF1</i>	98				
		7.6							<i>NF1</i>	7		
		7.9	<i>ATM</i>	0.79	<i>ATM</i>	0	<i>ATM</i>	98	<i>ATM</i>	15	<i>ATM</i>	3.8
		7.9	<i>BRCA1</i>	0.81	<i>VHL</i>	0	<i>VHL</i>	99	<i>BRCA1</i>	16		
		8.7	<i>BRCA2</i>	0.81					<i>NF1</i>	18		
			<i>PTEN</i>	0.92							<i>TSC2</i>	8.1
			<i>RB1</i>	0.99							<i>WT1</i>	10.8
			<i>NF1</i>	1.03								
		<i>TSC2</i>	1.03									

	<i>WT1</i>	10.1	<i>WT1</i>	1.22	<i>WT1</i>	0			<i>TSC2</i>	21		
			<i>CDH1</i>	1.27	<i>BRCA1</i>	0.14			<i>APC</i>	24		
					<i>CDKN2A</i>	0.29			<i>CDH1</i>	26		

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Supplementary Table 4. Summary of mutations occurring in direct repeats of length ≥ 8 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by repeats (%)	Number of missense mutations found in repeats						Number of micro-deletions and micro-insertions found in repeats			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	4	3	0	0	0	0	3	17	21	1	17
ATM	7	2	0	0	0	0	2	0	11	0	0
BRCA1	5	0	9	0	0	0	9	1	8	0	1
BRCA2	2	0	0	0	0	0	0	1	12	0	1
CDH1	3	0	0	0	0	0	0	0	1	0	0
CDKN2A	17	25	8	3	0	0	36	28	2	0	28
NF1	7	0	2	0	0	0	2	0	15	0	0
NF2	3	0	0	0	0	0	0	1	1	0	1
PTCH1	3	0	0	0	0	0	0	0	0	0	0
PTEN	17	7	0	0	4	2	13	20	5	1	20
RB1	12	0	1	0	0	0	1	2	12	0	2
STK11	10	0	3	1	0	0	4	0	6	0	0
TP53	14	24	1	0	13	2	40	21	0	0	21
TSC1	5	0	1	0	0	0	1	0	4	0	0
TSC2	5	0	10	1	0	0	11	0	6	0	0
VHL	6	0	1	0	0	0	1	0	1	0	0
WT1	7	1	0	0	0	0	1	0	0	0	0
TOTAL	6	62	36	5	17	4	124	91	105	2	91

Supplementary Table 5. Summary of mutations occurring in inverted repeats of length ≥ 8 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by repeats (%)	Number of missense mutations found in repeats						Number of micro-deletions and micro-insertions found in repeats			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	6	5	4	1	1	0	5	21	27	2	50
ATM	13	1	14	0	0	0	1	1	16	0	17
BRCA1	6	0	15	0	0	0	0	0	22	1	23
BRCA2	7	3	1	0	0	0	3	1	27	0	28
CDH1	5	0	1	0	0	0	0	1	0	0	1
CDKN2A	8	30	5	6	2	1	30	13	2	1	16
NF1	11	0	3	0	0	0	0	1	24	0	25
NF2	10	1	3	0	0	0	1	11	6	0	17
PTCH1	5	1	0	0	0	0	1	0	2	0	2
PTEN	6	10	1	1	4	1	10	9	2	0	11
RB1	16	4	5	1	0	0	4	7	28	0	35
STK11	13	1	5	0	1	0	1	1	9	0	10
TP53	5	13	0	0	51	9	13	53	2	0	55
TSC1	5	0	1	0	0	0	0	0	7	0	7
TSC2	9	0	6	0	0	0	0	1	13	0	14
VHL	12	9	8	1	1	0	9	36	15	2	53
WT1	7	0	2	0	0	0	0	0	0	0	0
TOTAL	9	78	74	10	60	11	78	156	202	6	364

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Supplementary Table 6. Summary of mutations occurring within symmetric repeats of length ≥ 8 bp in the 17 tumour suppressor genes.

Gene symbol	Proportion of gene length covered by repeats (%)	Number of missense mutations found in repeats						Number of micro-deletions and micro-insertions found in repeats			
		somatic non-recurrent	germline	shared non-recurrent	somatic recurrent	shared recurrent	Total	somatic non-recurrent	germline	shared	Total
APC	16	5	2	0	2	0	9	58	87	6	151
ATM	32	2	11	0	0	0	13	2	43	0	45
BRCA1	20	1	30	0	0	0	31	0	82	2	84
BRCA2	18	6	18	0	0	0	24	2	79	3	84
CDH1	24	4	0	0	0	0	4	5	8	0	13
CDKN2A	24	49	13	5	2	0	69	35	7	1	43
NF1	31	1	20	0	0	0	21	2	85	2	89
NF2	24	6	3	0	1	0	10	49	12	3	64
PTCH1	23	5	8	1	0	0	14	5	23	0	28
PTEN	44	27	3	1	9	0	40	42	13	1	56
RB1	48	3	10	1	0	0	14	4	41	1	46
STK11	33	3	6	0	2	0	11	1	20	1	22
TP53	30	60	2	1	132	23	218	147	1	0	148
TSC1	23	0	3	0	0	0	3	0	27	0	27
TSC2	23	0	13	0	0	0	13	1	29	0	30
VHL	17	3	9	2	0	2	16	25	7	2	34
WT1	26	0	6	0	0	0	6	3	4	0	7
TOTAL	25	175	157	11	148	25	516	381	568	22	971

Supplementary Table 7. Occurrence of missense mutations in repeats/runs of identical nucleotides and/or CpG/CpHpG oligonucleotides

Type of Repeats	Type of mutational spectrum	Number of mutations			Remaining number of mutations
		exclusively in repeats/runs	exclusively in CpG/CpHpG	in both repeats/runs and CpG/CpHpG	
Runs	somatic non-recurrent	184	58	24	618
	germline	151	100	27	608
	somatic recurrent	167	46	18	636
	shared non-recurrent	5	28	0	69
	shared recurrent	10	38	5	59
	potential	32861	3902	765	111495
Direct	somatic non-recurrent	52	72	10	750
	germline	31	122	5	728
	somatic recurrent	14	61	3	789
	shared non-recurrent	3	26	2	71
	shared recurrent	2	41	2	67
	potential	5252	4431	236	139104

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Inverted	somatic non-recurrent	65	69	13	737
	germline	64	117	10	695
	somatic recurrent	55	59	5	748
	shared non-recurrent	8	26	2	66
	shared recurrent	7	39	4	62
	potential	10790	4314	353	133566
Symmetric	somatic non-recurrent	155	62	20	647
	germline	140	110	17	619
	somatic recurrent	137	53	11	666
	shared non-recurrent	7	24	4	67
	shared recurrent	16	34	9	53
	potential	28646	3752	915	115710

Supplementary Table 8. Truncating vs. non-truncating lesions

Gene		Missense	Nonsense	Micro-deletions	Micro-insertions	Micro-indels	Non-truncating lesions	Truncating lesions	Ratio of non-truncating to truncating lesions	Ratio of truncating somatic to truncating germline lesions
<i>APC</i>	Somatic	39	79	152	44	3	39	278	0.14	0.46
	Germline	23	180	299	115	12	23	606	0.04	
<i>ATM</i>	Somatic	11	7	4	1	0	11	12	0.92	0.05
	Germline	76	75	122	35	14	76	246	0.31	
<i>BRCA1</i>	Somatic	6	9	9	5	0	6	23	0.26	0.05
	Germline	170	121	259	85	12	170	477	0.36	
<i>BRCA2</i>	Somatic	21	1	8	4	0	21	13	1.62	0.03
	Germline	86	76	247	90	11	86	424	0.20	
<i>CDH1</i>	Somatic	15	7	13	2	0	15	22	0.68	0.69
	Germline	19	11	12	8	1	19	32	0.59	
<i>CDKN2A</i>	Somatic	198	18	77	25	8	198	128	1.55	4.74
	Germline	62	7	11	7	2	62	27	2.30	
<i>NF1</i>	Somatic	2	11	16	3	0	2	30	0.07	0.07
	Germline	83	115	221	105	8	83	449	0.18	
<i>NF2</i>	Somatic	23	42	182	28	6	23	258	0.09	2.22
	Germline	20	43	55	16	2	20	116	0.17	
<i>PTCH1</i>	Somatic	14	9	14	6	1	14	30	0.47	0.28
	Germline	24	27	42	32	8	24	109	0.22	
<i>PTEN</i>	Somatic	226	56	152	51	4	226	263	0.86	3.21
	Germline	45	28	29	22	3	45	82	0.55	
<i>RBI</i>	Somatic	25	27	34	12	3	25	76	0.33	0.30

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STK11	Germline	37	76	117	53	11	37	257	0.14	0.17
	Somatic	20	10	5	1	1	20	17	1.18	
	Germline	30	27	47	24	3	30	101	0.30	
TP53	Somatic	1229	96	512	238	0	1229	846	1.45	24.89
	Germline	94	10	16	5	3	94	34	2.76	
TSC1	Somatic	2	1	1	0	0	2	2	1.00	0.02
	Germline	7	37	53	25	4	7	119	0.06	
TSC2	Somatic	2	1	3	2	1	2	7	0.29	0.03
	Germline	89	74	110	46	3	89	233	0.38	
VHL	Somatic	88	15	180	44	1	88	240	0.37	1.82
	Germline	143	27	63	37	5	143	132	1.08	
WT1	Somatic	1	3	4	3	0	1	10	0.10	0.37
	Germline	40	14	8	4	1	40	27	1.48	
Total	Somatic	1922	392	1366	469	28	1922	2255	0.85	0.65
	Germline	1048	948	1711	709	103	1048	3471	0.30	