HYPOTHESIS

Age related shift in the mutation spectra of germline and somatic *NF2* mutations: hypothetical role of DNA repair mechanisms

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It has been suggested that somatic mutations that accumulate due to an age related decline in the efficiency of DNA repair mechanisms might contribute to the increased incidence of cancer in older people. However, there is little direct evidence for this phenomenon. The spectra of germline and somatic mutations can be compared in cancer genes that cause inherited tumour syndromes and sporadic tumours, respectively. In addition, mosaic patients reflect the nature of mutations that occur in early development. Hence, we hypothesised that the "temporal mutation record" of a human cancer gene might provide insight into mechanisms of mutagenesis in the germline, in early development, and in adulthood. We compared the ratio of frameshift to nonsense mutations in three diseases that are related to the *NF2* tumour suppressor gene: classic neurofibromatosis 2 (NF2), caused by germline *NF2* mutations; mosaic NF2; and unilateral sporadic vestibular schwannoma (USVS), caused by somatic *NF2* inactivation. Nonsense mutations predominated in both classic and mosaic NF2, but the ratio of nonsense to frameshift mutations was reversed in USVS. Moreover, in USVS patients, the ratio of somatic frameshift to nonsense mutations increased significantly with increasing age at diagnosis. This pattern is consistent with an age related decline in the efficiency of DNA repair mechanisms. Similar studies for other familial cancer genes may provide further evidence for this hypothesis.

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Mutations in the human genome occur during spermatogenesis and oogenesis (meiosis) or during somatic growth and replication (mitosis). Inherited cancer syndromes are usually caused by mutations that occur during meiosis, while sporadic tumours are typically caused by somatic mutations and epigenetic events. Some cancer susceptibility genes cause both familial and sporadic cancers, and in these genes, comparisons of the germline and somatic mutational spectra have provided important insights into mechanisms of mutagenesis and tumorigenesis.

Germline mutations in the *VHL* gene cause von Hippel-Lindau disease, and somatic *VHL* mutations occur in most sporadic renal cell carcinomas. *VHL* mutations that are predicted to produce a truncated protein account for 78% of somatic mutations, but for only 37% of germline mutations.[1] VHL families with germline truncating mutations have a higher frequency of renal cell carcinoma than families with missense mutations,[1] suggesting that the preponderance of somatic truncating mutations in sporadic renal cell carcinoma reflects selection during tumorigenesis.

The *TP53* gene is unusual for the high frequency of missense mutations. There are common mutation hotspots in the spectra of germline and somatic *TP53* mutations, and there are also somatic mutation hotspots in specific tumour types.[2] In contrast, the germline and somatic mutation spectra of the *NF1* tumour suppressor gene are similar in types and frequencies of mutations.[3]

There are non-random associations between the type of first and second hits in germline and somatic *APC* tumour suppressor gene mutations in people with familial adenomatous polyposis and sporadic colorectal cancer, respectively.[4][5] *APC* mutations in the "mutation cluster region", around codon 1300, are associated with loss of the second *APC* allele, whereas tumours with mutations outside this region tend to have a second truncating mutation. The causes of this phenomenon have been hypothesised to be selection for retained N terminal and lost C terminal APC functions, effects on beta-catenin levels, and APC protein stability.[5]

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder caused by inactivating mutations in the *NF2* tumour suppressor gene. NF2 is characterised by nervous system tumours such as schwannomas and meningiomas. Consistent with the Knudsen two hit hypothesis, the germline *NF2* allele is inactivated and tumours occur when the wildtype *NF2* allele is inactivated by mutation, silencing, or allelic loss. Unilateral sporadic vestibular schwannoma (USVS) also has somatic biallelic *NF2* inactivation.

There is a high prevalence of mosaicism in NF2 (up to 30% of people with new mutations).[6] This is not the case in other familial cancer syndromes such as familial adenomatous polyposis (FAP) and VHL,[7] although it is possible that some patients with colorectal cancer or renal cell carcinoma could be mosaics who do not fulfil

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Abbreviations: FAP, familial adenomatous polyposis; NF2, neurofibromatosis 2; USVS, unilateral sporadic vestibular schwannoma

clinical diagnostic criteria for FAP or VHL. Mosaicism occurs in many genetic diseases, but there is little information on the likely mechanism of mutagenesis. Hence, NF2 provides an opportunity to determine if there is evidence for age related changes in mutation mechanisms by comparing *NF2* mutation spectra in classic (non-mosaic) NF2, in mosaic NF2, and in USVS. Classic NF2 refers to people who meet the Manchester clinical diagnostic criteria for NF2.[8]

In this study, we used an international database of germline and somatic *NF2* mutations to compare the distributions of germline and somatic truncating *NF2* mutations (nonsense and frameshift mutations).[9] In classic NF2, germline non-truncating *NF2* mutations cause milder disease than truncating mutations.[10] This would cause selection for truncating mutations in USVS if partial protein function caused by a missense mutation decreases Schwann cell proliferation and lessens the chance of inducing a second ''hit'' in the *NF2* gene. Indeed, *NF2* missense mutations were more common in the germline (34 of 562 family mutations; 6.0%) than in USVS (4 of 226 tumours; 1.8%) ($p = 0.009$). This is consistent with the mild phenotypic effect of missense mutations in NF2 and the lower likelihood of these mutations being expressed in somatic form, as occurs in *VHL*. We did not include *NF2* large deletions in this study because it is not possible to determine the true prevalence of *NF2* large deletions as opposed to loss of the entire chromosome 22 in most reports of *NF2* related sporadic tumours, and because identifying large *NF2* deletions is more difficult in mosaics than in the germline.[6]

The distributions of nonsense and frameshift *NF2* mutations for classic NF3 and mosaic NF2 and USVS are shown in table 1. Nonsense mutations were more common than frameshift mutations in classic NF2 and in mosaics, but the converse was true in USVS (Fisher's exact test, $p<0.001$). Nonsense and frameshift mutations are each predicted to produce truncated proteins, so it is likely that these differences reflect different mechanisms of mutagenesis rather than selection during sporadic tumorigenesis. The predominance of nonsense mutations in classic NF3 and in mosaic NF2 suggests that there are similar mechanisms of mutagenesis during meiosis and early embryonic development. The precise timing of mutations that occur in mosaic NF2 cannot be assessed, but at least half of the mosaic *NF2* mutations reported to date have not been identified in blood and therefore are probably present in <10% of cells. We did not find differences in mutation spectra between patients with a detectable mutation in blood and neural crest cells (that is, mutations that occurred early in development) and patients in whom the mutation was present in tumours but not in blood (that is, mutations that could have occurred after differentiation from toti potential cells in the neural crest).

Nonsense *NF2* mutations occur primarily at CpG dinucleotides. The *NF2* gene has six CpG sites in exons 1–15 in which nonsense mutations predominate. There is another CpG site in exon 16, but pathogenic *NF2* mutations have not been reported in exons 16 or 17. In the international *NF2* mutation database,[9] 102 (53%) of 193 constitutional nonsense *NF2* mutations occur in the six CpG sites. This proportion is similar for USVS: 19 (45%) of 42 somatic nonsense *NF2* mutations occur in the six CpG sites. This indicates that CpG methylation is a risk factor for *NF2* nonsense mutations during gametogenesis and post-zygotically. CpG dinucleotides are hypermutable,[11] particularly when there is a flanking pyrimidine residue.[12] Five of the six *NF2* CpG sites are flanked by a pyrimidine residue, which may explain the high ratio of nonsense mutations in *NF2* compared to other tumour prone disorders such as adenomatous polyposis coli.

Table 1 Mutation spectra in *NF2* related conditions

Condition	Nonsense mutation		Frameshift mutation	
	No.	% of total	No.	% of total
Classic NF2	191	57	145	43
Mosaic NF2	39	62	24	38
All USVS*	42	24	130	76
USVS diagnosed at ages <40 years	13	52	12	48
USVS diagnosed at ages ≥40 years	18	23	61	77

USVS, unilateral sporadic vestibular schwannoma. *Of the 172 USVS patients, 104 had information on age at diagnosis. The age of 40 years was chosen to define USVS age categories because USVS typically occur after this age.[23] Difference in proportion of frameshift in classic NF2 or mosaic NF2 compared to USVS, Fisher's exact test, $p<0.001$; difference in proportion of frameshift mutations between age categories in USVS, Fisher's exact test, $p=0.011$.

The mutability of CpG sites has been attributed to frequent methylation of cytosine, which results in deamination.

Methylation is required in the early embryo and occurs at CpG sites.[13] Methylation at CpG sites increases the risk of mutation, and inactivation of *Dnmt1* in embryonic stem cells reduces overall mutation rates, although this has not been demonstrated in CpG sites.[14] The relative paucity of somatic nonsense mutations in USVS could be due to a lower absolute rate of mutagenesis at CpG dinucleotides, as might be predicted if demethylation occurred, or to a higher absolute frequency of frameshift mutations with increasing age. The age related methylation status of intragenic *NF2* CpG sites has not been defined, but CpG promoter region methylation occurs in a subset of USVS,[15] suggesting that there is not a general tendency for *NF2* genomic demethylation with increasing age. Indeed, hypermethylation of the promoter region as a cause of tumours appears to increase with increasing age.[16 17]

To further investigate age related changes in *NF2* mutations, we evaluated the *NF2* mutation spectra in USVS patients with increasing age at diagnosis. The proportion of frameshift to nonsense mutations was significantly greater in older than in younger USVS patients (table 1). Using logistic regression, the ratio of frameshift to nonsense mutations increased 1.03 fold per year of age ($p = 0.040$).

The higher proportion of frameshift *NF2* mutations in older USVS patients might result from an increased rate of mutagenesis or from reduced mutation repair efficiency. To date, specific environmental risk factors for USVS have not been identified. In particular, chemical mutagens that cause frameshift mutations (for example, DNA intercalating agents such as ethidium bromide) have not been implicated in the pathogenesis of USVS. Hence, the most likely explanation for the results of this study may be an age related reduction in DNA repair efficiency.[18] In colorectal cancer, somatic inactivation of the *MLH1* mismatch repair gene by promoter methylation is associated with advanced age.[19] *Mlh1* inactivation results in susceptibility to somatic *apc* truncating mutations, most of which are frameshift mutations.[20] However, USVS is not a feature of hereditary non-polyposis colon cancer syndrome, and microsatellite instability is infrequent in USVS, so there is no direct evidence to implicate deficient mismatch repair gene activity in the age related preponderance of frameshift mutations in USVS.

Vestibular schwannoma is not a feature of other known inherited DNA repair defect syndromes. However, there is experimental evidence for an age related decline in other DNA repair mechanisms. An age associated decline in base excision repair mechanisms has been reported in rodents and

in human skin,[21–24] and a reporter transgene has a higher mutation rate in older animals.[25] Sedelnikova *et al*[26] suggested that aging cells may accumulate irreparable DNA double strand breaks, and it has been suggested that older animals preferentially accumulate large genomic rearrangements.[27] The observation that DNA end joining is less efficient and more error prone in senescent cells suggests that an age related impairment of non-homologous end joining might facilitate genomic rearrangements and deletions, and lead to an increased incidence of cancer in the elderly.[28]

The incidence of USVS increases exponentially with increasing age in adults.[29] Biallelic *NF2* inactivation in USVS arises through a two step process, and large deletions of chromosome 22 are the most common second event. Hence, the age related increase in the incidence of USVS could result from an increased susceptibility to frameshift mutations and genomic rearrangements in senescent cells. Repair mechanisms for frameshift mutations may become less efficient with increasing age than repair mechanisms for nonsense mutations, or age related mutagenesis may be more likely to generate frameshift mutations than nonsense mutations.

An age related accumulation of somatic mutations has been frequently implicated as the cause of the increased incidence of cancer in the elderly, but to our knowledge, *NF2* provides the first example of an age related shift in mutation pattern in humans. Similar findings for other human cancer genes would strengthen the case for a role of age related changes in genomic stability and the efficiency of DNA repair mechanisms in the pathogenesis of increased cancer risk in old age, and could uncover evidence for specific environmental mutagens in some groups of patients.[30]

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