

ORIGINAL ARTICLE

Rates of loss of heterozygosity and mitotic recombination in NF2 schwannomas, sporadic vestibular schwannomas and schwannomatosis schwannomas

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Biallelic inactivation of the *NF2* gene occurs in the majority of schwannomas. This usually involves a combination of a point mutation or multiexon deletion, in conjunction with either a second point mutation or loss of heterozygosity (LOH). We have performed DNA sequence and dosage analysis of the *NF2* gene in a panel of 239 schwannoma tumours: 97 neurofibromatosis type 2 (NF2)-related schwannomas, 104 sporadic vestibular schwannomas (VS) and 38 schwannomatosis-related schwannomas. In total, we identified germline *NF2* mutations in 86 out of 97 (89%) NF2 patients and a second mutational event in 77 out of 97 (79%). LOH was by far the most common form of second hit. A combination of microsatellite analysis with either conventional comparative genomic hybridization (CGH) or multiplex ligation-dependent probe amplification (MLPA) identified mitotic recombination (MR) as the cause of LOH in 14 out of 72 (19%) total evaluable tumours. Among sporadic VS, at least one *NF2* mutation was identified by sequence analysis or MLPA in 65 out of 98 (66%) tumours. LOH occurred in 54 out of 96 (56%) evaluable tumours, but MR only accounted for 5 out of 77 (6%) tested. LOH was present in 28 out of 34 (82%) schwannomatosis-related schwannomas. In all eight patients who had previously tested positive for a germline *SMARCB1* mutation, this involved loss of the whole, or part of the long arm, of chromosome 22. In contrast, 5 out of 22 (23%) tumours from patients with no germline *SMARCB1* mutation exhibited MR. High-resolution Affymetrix SNP6 genotyping and copy number (CN) analysis (Affymetrix, Santa Clara, CA, USA) were used to determine the chromosomal breakpoint locations in tumours with MR. A range of unique recombination sites, spanning approximately 11.4 Mb, were identified. This study shows that MR is a mechanism of LOH in NF2 and *SMARCB1*-negative schwannomatosis-related schwannomas, occurring less frequently in sporadic VS. We found no evidence of MR in *SMARCB1*-positive schwannoma-

tosis, suggesting that susceptibility to MR varies according to the disease context.

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Introduction

Schwannomas are benign peripheral nerve sheath tumours that occur within the context of the tumour suppressor syndromes, neurofibromatosis type 2 (NF2) and schwannomatosis. They also occur sporadically within the general population. In most schwannoma tumours, the *NF2* gene, located on chromosome 22q12.2, is biallelically inactivated. In NF2 patients, the first mutation is frequently a constitutional point mutation or a small deletion. This is followed by a second somatic mutation in the tumour, which is either a second point mutation, or, more commonly, by loss of the wild-type allele. Mutation within the *NF2* gene promoter or hypermethylation of key CpG dinucleotides within this region may be an additional mechanism of inactivation (Kino *et al.*, 2001). This region is not routinely tested and may account for a proportion of patients with unfound mutations. In schwannomas occurring outside NF2 disease, *NF2* mutations are only found somatically, occurring in the tumour, but not in the germline (MacCollin *et al.*, 2003), suggesting that other genes are involved in these cases.

The chromatin remodelling gene, *SMARCB1*, located approximately 6 Mb upstream of *NF2* at 22q11.23, was recently found to be mutated in germline DNA in approximately 50% of familial schwannomatosis patients, and, less commonly, in sporadic schwannomatosis (5–10%) (Hulsebos *et al.*, 2007; Boyd *et al.*, 2008; Hadfield *et al.*, 2008; Sestini *et al.*, 2008). Our previous study has shown that these germline *SMARCB1* mutations are frequently associated with a somatic *NF2* mutation in tumours (Boyd *et al.*, 2008; Hadfield *et al.*, 2008), and a multihit hypothesis has been suggested, involving both of these loci (Boyd *et al.*, 2008; Hadfield *et al.*, 2008; Sestini *et al.*, 2008).

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The region 22q11 contains several low copy repeat domains, and is known to be a particularly unstable region of the genome (Dunham *et al.*, 1999; Edelmann *et al.*, 1999). Recombination events in this region are known to be involved in several diseases, occurring both constitutionally (for example, cat eye syndrome, velocardiofacial syndrome, DiGeorge syndrome) and somatically (for example, chronic myelogenous leukaemia Burkitt's lymphoma, Ewing's sarcoma) (Kaplan *et al.*, 1987).

Mitotic recombination (MR) is known to be an important mutational mechanism in neurofibromatosis type 1 and other tumour syndromes. Loss of heterozygosity (LOH) of 17q is a common somatic hit in neurofibromas lacking a germline microdeletion mutation, and is frequently the result of MR (Serra *et al.*, 2001; De Raedt *et al.*, 2006). As LOH is also a common event in tumours of patients with NF2, as well as with sporadic vestibular schwannomas (VS) and schwannomatosis, and chromosome 22 is prone to a variety of recombinational events, we analysed a panel of tumours from each of these groups to determine rates of LOH and MR with respect to the diagnosis and to the initial genetic event. The Genome-Wide Human SNP Array 6.0 (Affymetrix) platform was used to confirm the results and refine identified breakpoints, as the combination of high density of small-nucleotide polymorphism (SNP) genotyping and high density copy number (CN) probes provides a more accurate and sensitive way to determine both CN and homozygosity than do conventional methods.

Results

Mutation spectrum of NF2, sporadic VS and schwannomatosis tumours

We have performed DNA sequence and dosage analysis of the *NF2* gene in a panel of 239 schwannoma tumours: 97 tumours from 97 patients with NF2, 104 sporadic VS and 38 tumours from 34 patients with schwannomatosis (Table 1). We identified germline mutations in *NF2* in 89% (86 out of 97) of NF2 patients. Out of these, 70 were found by direct sequence analysis and consisted predominantly of nonsense mutations (36%) and frameshift mutations (21%). The remainder consisted of splice mutations (13%), a single missense mutation and an in-frame deletion of a single amino acid. In all, 16 additional tumours harboured single or multiexon deletions found by multiplex ligation-dependent probe amplification (MLPA). A second mutational event was found in 77 (79%). This was a second point mutation in 13, MLPA deletion of a part of *NF2* in 2 and LOH in 62. MR was the cause of LOH in 14 out of 41 evaluable tumours. Excluding tumours with LOH, but not tested for MR, this leads to an overall rate of 14 out of 72 (19%). Analysis was not possible in some tumours, because of insufficient DNA or because of poor quality of some DNA samples extracted from paraffin blocks. In total, 7 of 15 NF2 patients with large multiexon

deletions had LOH as the mechanism of second hit (including 3 with >6 exons deleted), although this did not occur in the two patients with whole-gene deletions. MR was not found to be the cause of LOH in any of these tumours.

Of the 41 NF2 patients tested for MR, 27 were mosaic, 13 were non-mosaic and 1 was uncertain. MR was the cause of LOH in seven of the non-mosaic patients (age range: 7–40 years; average: 23 years). These seven patients developed a total of 191 spinal and peripheral nerve schwannomas, with an average of 27 tumours per person. Sixteen non-mosaic NF2 patients with no evidence of MR, including those with no LOH (age range: 9–57 years; average: 32 years), had a total of 113 schwannomas, with an average of seven tumours per person. This may suggest that the MR mechanism functions as a modifier for NF2, leading to a more severe schwannoma phenotype, although the difference in tumour burden did not reach statistical significance by the two-tailed *t*-test ($P=0.129$). Furthermore, 5 out of 7 MR patients had an underlying truncating (nonsense/frameshift) mutation, compared with only 5 out of 16 non-mosaic patients. Out of the 27 mosaic patients, seven (six out of seven not identifiable in blood) showed MR and, strikingly, all seven developed spinal and/or peripheral nerve schwannomas (three out of seven aged <20 years); one as young as 10 years of age, despite the mutation being undetectable in the lymphocyte DNA. Of 21 mosaic patients without evidence of MR (16 out of 21 not identifiable in blood), only three developed a spinal or peripheral nerve schwannoma <20 years and five had no evidence of either spinal or cutaneous schwannomas. There was no evidence that these effects were primarily due to the underlying mutation: 6 out of 7 mosaic MR patients and 16 out of 21 mosaic non-MR patients had the more severe truncating mutations.

Among sporadic VS, we identified at least one point mutation in the *NF2* gene in 65 out of 98 (66%) tumours, with a higher incidence of frameshift (34%) than nonsense mutations (17%) in comparison with NF2-related tumours. Splice mutations (10%) and missense mutations (1%) occurred in similar proportions to NF2 tumours. MLPA identified a single exon-11 deletion. LOH occurred in 54 out of 96 (56%), but MR only accounted for 5 out of 35 evaluable tumours, with an overall rate of 5 out of 7 (6%). The difference between MR rates in evaluable tumours between NF2 and sporadic VS is significant by χ^2 -test (P -value=0.0161) if we extrapolate our results to calculate the predicted overall number of tumours in each group by first extrapolating the predicted proportion of LOH hits in the total number of tumours and then extrapolating the proportion of LOH hits due to MR. This suggests that 22 out of 97 NF2 tumours and 8 out of 104 sporadic VS have been caused by MR in our cohort. This proportion is also significant by χ^2 -test, adjusting for the lower rate of LOH, in addition to MR in sporadic VS ($P=0.010$).

As expected, no germline *NF2* mutations were found in any schwannomatosis patients. However, germline

Table 1 Mutational analysis results for the *NF2* gene in NF2-related schwannomas, sporadic vestibular schwannomas and schwannomatosis-related schwannomas

	<i>NF2</i>	<i>Sporadic VS</i>	<i>SMARCB1</i> mutation +ve schwannomatosis	<i>SMARCB1</i> mutation -ve schwannomatosis
Total tumours analyzed	97	104	8	30
Tumours evaluated for <i>NF2</i> by sequence analysis	97	98	6	30
Tumours with at least one mutation identified by sequence analysis	86	65	6	18
Tumours with two mutations identified by sequence analysis	13	10	0	1
Tumours with intragenic deletions	14 ^a	1	0	0
Tumours evaluated for LOH	93	96	8	26
Tumours with LOH	62	54	8	20
% of tumours with LOH	62/93 (67%)	54/96 (56%)	8/8 (100%)	20/26 (77%)
Tumours with LOH evaluated for MR	41	35	8	16
Tumours with MR	14	5	0	5
MR as % of total evaluated tumours	14/72 (19%)	5/77 (6%)	0/8 (0%)	5/22 (23%)
Extrapolated MR as a proportion of all tumours ^b	22/97	8/104	NA	7/30

Abbreviations: LOH, loss of heterozygosity; NA, not applicable; NF2, neurofibromatosis type 2; MR, mitotic recombination; VS, vestibular schwannomas; %, percentage.

^aTwelve of these deletions were constitutional and two were somatic.

^bLOH was first extrapolated as a proportion of the total number of tumours. MR was then extrapolated as a total of the extrapolated LOH and, hence, of the total number of tumours.

Table 2 Chromosomal positions of recombination breakpoints determined by Affymetrix SNP6 array analysis

Patient	Diagnosis	Genomic position of breakpoint (Chr 22)	SNP ID at breakpoint location
1	NF2	26 560 066	rs16985600
2	NF2	23 061 070	rs5760352
3	NF2	19 448 306	rs4822497
4	NF2	28 018 632	rs3761426
5i	NF2	16 615 305	rs181408
5ii	NF2	16 965 064	rs467814
6	Sporadic VS	16 754 150	rs2075453
7	Schw	23 283 227	rs2298386
8	Schw	18 335 375	rs5993889

Abbreviations: Chr, chromosome; NF2, neurofibromatosis type 2; Schw, schwannoma; SNP, single nucleotide polymorphism; VS, vestibular schwannomas.

SMARCB1 mutations were found in eight patients (six from three different families and two sporadic patients) and somatic *NF2* point mutations were found in 24 tumours (Hadfield *et al.*, 2008). The proportions of *NF2* frameshift mutations (37%) and splice mutations (11%) were similar to those found in sporadic VS, but with fewer nonsense mutations (5%) and a greater number of missense (8%) mutations.

LOH was present in 28 out of 38 (74%) schwannomatosis-related schwannomas. In all eight tumours from schwannomatosis patients who tested positive for a germline *SMARCB1* mutation, this involved loss of the whole chromosome 22 or part of the long arm. In contrast, 5 out of 22 (23%) evaluable tumours from 19 patients who had tested negative for *SMARCB1* mutations exhibited MR.

Determination of mitotic recombination breakpoints

To investigate the potential mechanisms leading to MR, we sought to define a critical region of recombination

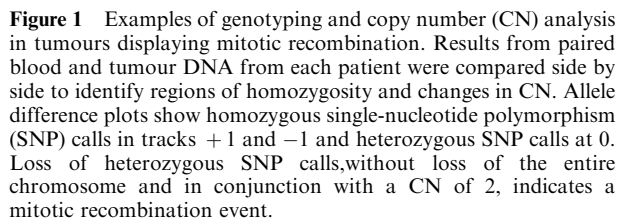
on chromosome 22q, relevant to the pathogenesis of schwannomas. We used Affymetrix SNP6.0 arrays to identify recombination breakpoints in a subset of tumour samples that were previously found to have undergone MR by a combination of dosage and heterozygosity analysis, consisting of either conventional CGH and microsatellite analysis or MLPA and microsatellite analysis. Genomic DNA was available for analysis from seven NF2-related schwannomas, one sporadic VS and two *SMARCB1* mutation-negative schwannomatosis-related schwannomas, along with their respective matched blood DNAs.

The data confirmed that MR was unique to chromosome 22 in each sample, as all other chromosomes showed normal CN and comparable heterozygosity in blood and tumour. We determined the breakpoint locations on chromosome 22q in six tumours obtained from five patients with NF2, one sporadic VS and two *SMARCB1* mutation-negative schwannomatosis-related schwannomas (Table 2). Examples of the SNP array results are shown in Figure 1.

NF2 tumours showed a range of unique breakpoints from 16 615 305 (SNP ID: rs181408) to 28 018 632 (SNP ID: rs3761426) on chromosome 22 (Figure 2a). Two tumours from the same patient (5i and 5ii) showed relatively close, but non-identical breakpoints, approximately 350 000 bases apart. For one further NF2-related sample, the combined genotyping and CN data analysis determined that the LOH previously identified as MR by a combination of microsatellite analysis and conventional comparative genomic hybridization (CGH) was in fact due to loss of genetic material.

A breakpoint was determined in a single sporadic VS after 16 754 150 (SNP ID: rs2075453).

Recombination breakpoints were determined for both *SMARCB1* mutation-negative sporadic schwannomatosis tumours tested. Interestingly, the breakpoint in one of these tumours occurred at 23 283 227 (SNP ID: rs2298386), which is downstream of the *SMARCB1*



We used high-density SNP analysis to refine the breakpoints and identify any common regions of recombination in 10 tumours. This method provides much more information than the combination of microsatellite analysis and conventional CGH or MLPA used previously. In this study, one NF2 tumour, originally identified with somatic LOH caused by MR, was shown to be due to loss of one chromosome 22. One reason for a false-positive result in the original analysis is the mixed population of normal and tumour cell DNA in some tumour samples, which is more difficult to

Aberrant methylation of three CpG islands in the *NF2* gene promoter has been shown to downregulate its

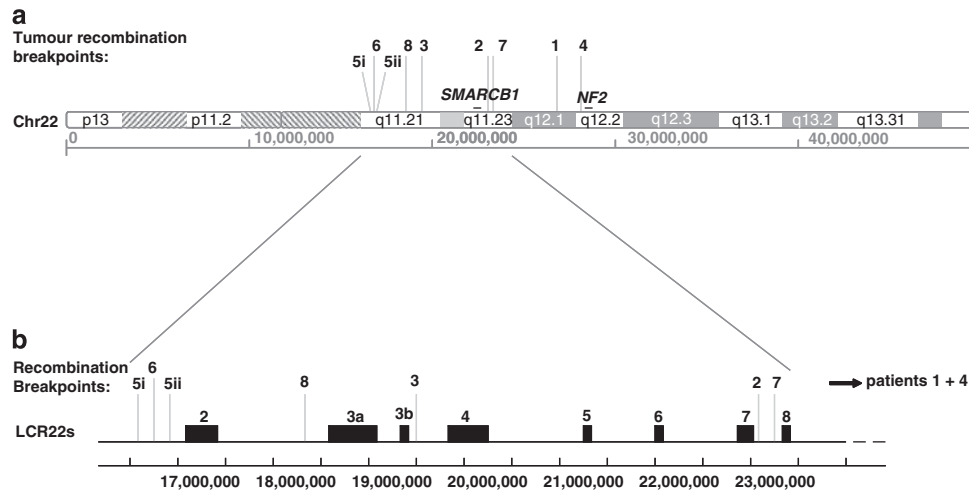


Figure 2 Schematic representation of the chromosomal locations of recombination breakpoints found in tumours, relative to *SMARCB1* and *NF2* genes (**a**), and a higher resolution schematic of the 22q11.2 region, showing breakpoint locations relative to low copy repeat (LCR) domains (**b**).

expression (Kino *et al.*, 2001) and has been suggested as a mechanism for progression to growth of VS (Gonzalez-Gomez *et al.*, 2003). It is possible that increased rates of aberrant methylation in sporadic VS may account for the reduced detection rate of *NF2* point mutations in sporadic VS in comparison with *NF2* tumours. As a reduction in the levels of methylation has also been proposed as a mechanism of increased occurrence of genomic recombination events (Carbone *et al.*, 2009), it is also possible that an increased incidence of methylation in certain tumours would reduce the likelihood of MR involvement. Further work is necessary to test this hypothesis.

A number of patients have shown MR in more than one tumour and there seems to be a trend towards an increased schwannoma formation in those patients who develop schwannomas by this mechanism. We were unable to demonstrate a formal statistical significance for this, but it remains possible that some individuals with non-mosaic *NF2* or *SMARCB1*-negative schwannomatosis and an increased susceptibility to MR have a higher risk of increased tumour burden.

In summary, our study demonstrates a role for mitotic recombination events in the development of a proportion of schwannoma tumours and shows that the rates of MR vary, depending on the disease context. The lack of *SMARCB1* mutations in schwannomatosis-related schwannomas with MR suggests that there are additional genetic causes of schwannomatosis, one of which may involve an increased susceptibility to mitotic recombination. Further studies will be required to determine the specific combination of genetic events that initiates tumour growth

Materials and methods

Patient tumours

Tumour DNA from a total of 239 patients was included in the study. In total, 97 patients fulfilled the clinical criteria for *NF2*

disease (Evans *et al.*, 1992). Another 104 patients had suffered only a unilateral VS and had tested negative for germline *NF2* mutation.

There were 38 tumours from 34 schwannomatosis patients; eight patients were from families with multiple affected members who were negative for VS and germline *NF2* mutations. The remaining 26 were sporadic patients with at least three schwannomas in more than one body segment and who were negative for VS and germline *NF2* mutations. Approval for the study was provided by the Central Manchester local ethics committee. Informed consent was obtained from patients and their families for publication of their details in this report.

Mutational analysis

DNA sequence analysis of *NF2* and *SMARCB1* genes, CN analysis of *SMARCB1* exon deletions or amplifications by quantitative RT-PCR (TaqMan) assay and LOH analysis by microsatellite markers *D22S303*, *D22S310*, *D22S446*, *D22S449*, *D22S1174*, *D22S275*, *NF2CA3* and *D22S268* were carried out as described by Hadfield *et al.*, 2008. The microsatellite markers spanned a total of approximately 12.7 Mb of chromosome 22, covering both the *NF2* and *SMARCB1* loci. Analysis of the centromeric microsatellite marker *D22S420*, which is found 6.3 Mb upstream of *SMARCB1*, was used to confirm that any observed LOH was not due to deletion, followed by reduplication of the homologous chromosome 22.

Hybridization, ligation and amplification of MLPA probes for exon CN analysis were carried out according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). The method for CGH was previously described in (James and Varley, 1996; James *et al.*, 1999; Warren *et al.*, 2003).

High-resolution genotyping and CN analysis

Matched blood and tumour samples for each patient were genotyped using the Genome-Wide Human SNP Array 6.0 (Affymetrix). Genomic DNA was purified by ethanol precipitation and 500 ng was hybridized to an SNP6 chip according to the manufacturer's instructions. This array contains a total of ~1.8 million probes, comprised of 906 600 SNP probes and 946 000 CN probes spaced throughout

the genome. Staining of the array was carried out on a Fluidics Station 450 (Affymetrix) and the array was scanned using the GeneChip scanner 3000 7G system (Affymetrix).

Data analysis

The CN data were generated using the BRLMM-P-Plus algorithm from Affymetrix using reference model files generated from a pool of 56 unaffected genomic DNA control samples. Genotype and CN analysis were carried out using the Genotyping Console 3.0.2 (Affymetrix). The genotyping no-call threshold was set to 0.01 and the CN/LOH detection threshold was set to 0.02. Mitotic recombination was demonstrated by tumour-specific, incomplete loss of heterozygosity, with no change in CN throughout the homozygous region. In tumour samples containing a heterogeneous population of normal and tumour cell DNA, regions of MR appeared as dual tracks of SNP calls on allele difference plots and were not called as LOH by the Genotyping Console. However, the copy number remained at 2, whereas heterozygous base calls were

lost in comparison with blood. For these samples, an approximate recombination breakpoint was determined from the allele difference plots.

Chromosomal numbering is taken from the NCBI Build 36 = hg18.

Conflict of interest

The authors declare no conflict of interest.

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