ORIGINAL ARTICLE

Molecular study of frequency of mosaicism in neurofibromatosis 2 patients with bilateral vestibular schwannomas

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Neurofibromatosis 2 (NF2) is a severe autosomal dominant disorder that predisposes to multiple tumours of the nervous system. About half of all patients are founders with clinically unaffected parents. The purpose of the present study was to examine the extent to which mosaicism is present in NF2 founders. A total of 233 NF2 founders with bilateral vestibular schwannomas (BVS) were screened by exon scanning. *NF2* mutations were detected in the blood samples of 122 patients (52%). In 10 of the 122 cases, the ratio of mutant to normal alleles was obviously less than 1, suggesting mosaicism. Tumour specimens were available from 35 of the 111 subjects in whom no mutation could be detected in blood specimens. Mutational analysis by exon scanning detected typical *NF2* mutations in 21 of the 35 tumours. In nine subjects, the alterations found in tumours could be confirmed to be the constitutional mutation based on finding of identical mutations in pathologically and/or anatomically distinct second tumours. In six other subjects with only a single tumour available, allelic loss of the *NF2* gene was found in addition to the mutation in each tumour, suggesting that either the mutation or the deletion of the *NF2* gene is probably the constitutional genetic alteration. Our results suggest that failure to find constitutional mutations in blood specimen from these 15 patients was not because of the limitation of the applied screening technique, but the lack of the mutations in their leucocytes, best explained by mosaicism. Extrapolating the rate (15/35 = 43%) of mosaicism in these 35 cases to the 111 NF2 founders with no constitutional *NF2* mutations found in their blood, we inferred 48 mosaic subjects (111×0.429). Adding the 10 mosaic cases detected directly in blood specimens, we estimate the rate of mosaicism to be 24.8% (58/233) in our cohort of 233 NF2 founders with bilateral vestibular schwannomas.

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder characterised by multiple benign tumours of the nervous system.[1–3] The hallmark of NF2 is the development of bilateral vestibular schwannomas (BVS). Although a number of criteria exist, in the absence of affected relatives, the diagnosis of NF2 is based on the presence of BVS. NF2 is the result of inactivation of a classical tumour suppressor gene, *NF2*, on chromosome 22.[4 5] According to the population based study of Evans *et al*,[6] about half of the patients have clinically unaffected parents and thus are the result of sporadic mutations.

Since the identification of the *NF2* gene, a variety of mutations have been found in blood DNA of affected subjects.[7–11] Using exon scanning technology, mutations have been detected in up to 66% of all affected subjects studied.[9] Although large deletions, promoter and intronic mutations, and locus heterogeneity have been proposed as mechanisms to elude detection by exon scanning, others have suggested that somatic mosaicism, caused by postzygotic mutations in the early stage of embryo development, may account for a high percentage of such cases.[12–14] Only a subpopulation of the normal cells of a mosaic subject carries the constitutional mutation. Therefore, screening of a non-tumour tissue such as leucocytes may result in failure of detection when the mutation level of that tissue is low. Somatic mosaicism is of clinical interest because it may ameliorate the course of the disease, confound linkage testing of offspring, and decrease genetic risk to the next generation.[13 15] To date there have been no direct studies of the frequency of mosaicism in NF2 and the extent to which it may account for the failure of exon scanning technology to detect mutations. In this study we sought to determine the frequency of mosaicism in a large series of NF2 founders with BVS by screening tumour specimens.

METHODS

Patients and samples

This study included 233 patients with a diagnosis of NF2 and bilateral vestibular schwannomas (BVS) based on MRI.[3] One hundred and thirty-two of them were referred to V Mautner, NF Clinic Hamburg and 101 to the NF research program at Massachusetts General Hospital (M MacCollin). All patients were felt to be the founder by one of the authors or by the referring physician, with clinically unaffected parents. Patients meeting expanded criteria for NF2[16] with unilateral vestibular tumours and other NF2 related features were excluded from analysis.

Genomic DNA was extracted from peripheral blood leucocytes or from Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines. Tumour tissue was collected at the time of surgery and flash frozen in liquid nitrogen before extraction of genomic DNA, as previously reported.[17 18] When frozen tissue was unavailable, paraffin blocks were retrieved from pathology department archives of treating institutions. Pathology reports were reviewed to identify blocks containing predominantly a single tumour when more than one tumour was removed at operation. Genomic DNA was extracted from frozen tumours and paraffin blocks using a Qiagen DNA extraction kit (Qiagen, Valencia, CA).

This study was approved by the Institutional Review Boards of the participating institutions and informed consent was obtained from all subjects.

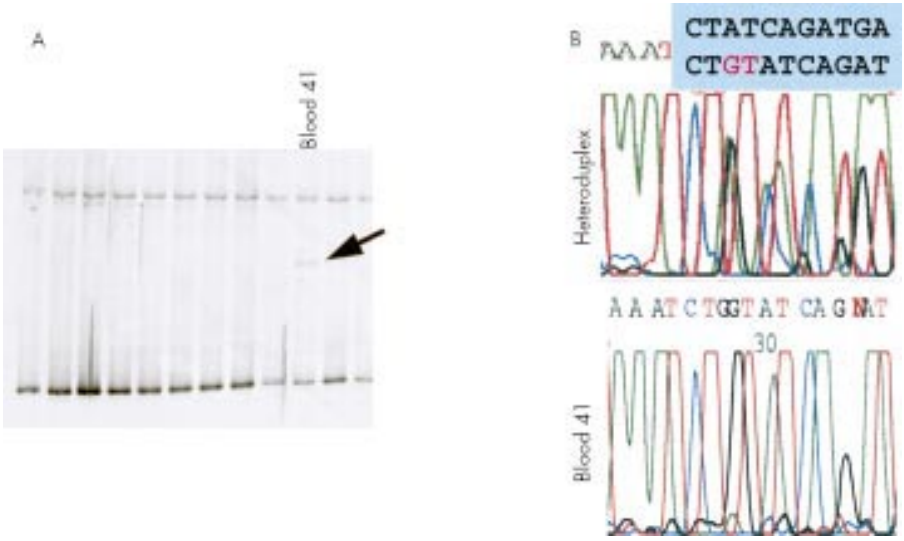

Figure 1 Mosaicism shown by exon scanning of blood specimens. (A) TGGE gel of exon 6 PCR products shows an aberrant band (arrow) in the blood of patient 41 which is lower in intensity than the wild type bands, suggesting mosaicism. (B) Sequencing of the amplified fragment from the aberrant band showed a 2 bp deletion. This deletion was not apparent in the blood specimen.

Mutational analysis

Coding exons 1 to 15 of the *NF2* gene were amplified from genomic DNA and scanned using single strand conformation polymorphism (SSCP) analysis or temperature gradient gel electrophoresis (TGGE) as previously described.[17 18] Aberrant mobility of single or double stranded fragments was identified by comparison with known positive and negative controls. When aberrations were detected by these methods, sequencing was performed bidirectionally using a Big Dye sequencing kit (Applied Biosystems, Foster City, CA) or by manual sequencing using a radiolabelled terminator cycle sequencing kit (USB, Cleveland, OH). In the case of weak mutation bands, the amplified fragment was cloned using a vector with T overhang (pGEM, Promega Life Sciences). The same fragment was then amplified from each clone again and resubjected to mutation analysis. Clones showing the same mutation pattern as the original sample were sequenced to determine the exact sequence of the change. Alternatively, the heteroduplex bands were excised from the TGGE gels. Corresponding exons were reamplified from DNA extracted from these bands, reanalysed on TGGE, and sequenced.[14] When a mutation was detected in a tumour specimen, the blood of the patient was examined again for the corresponding exon, by means of SSCP, TGGE, or sequencing. For quantitative estimation of mutation level in patients 24405 and 28165, T vector cloning was performed as above. Colonies were picked directly from plates and amplified using exon 6 primers.

Microsatellite marker analysis

Loss of the *NF2* region on chromosome 22 was determined using a panel of flanking and intragenic microsatellite markers. *CRYB2* and D22S193 are centromeric to the *NF2* gene, D22S929 (*NF2CA3*) and *NF2tet* are intragenic, and D22S268 and D22S430 are telomeric. The total distance from the most centromeric marker (*CRYB2*) to the most telomeric (D22S430) is 5 Mb. Products were labelled with either ^{33}P or fluorescence dyes and separated on 6% polyacrylamide gels or on an automated Genetic Analyzer ABI310 as previously reported.[20] All tumour/blood pairs were analysed in a minimum of two informative markers.

RESULTS

During a period of eight years, we have screened 350 NF2 patients with bilateral vestibular schwannomas for mutations in the *NF2* gene; 117 of the 350 patients had an affected parent, or a parent whose medical status was unknown. The remaining 233 patients were included in this study as NF2 founders. Using exon scanning based SSCP or TGGE, *NF2* mutations were found in blood DNA of 122 out of the 233 (52%) screened NF2 founders[7–9 14 17 20 21] (unreported data). For 10 patients, SSCP or TGGE analysis showed that the mutant *NF2* allele was obviously less than the normal *NF2* allele (fig 1A), indicating somatic mosaicism in this tissue (table 1). In eight of the 10 cases, direct sequencing of the PCR product from a blood sample was inadequate to detect the mutation

Table 1 Mutations defined as mosaic during exon scanning using blood DNA

Patient	Location	Sequence change*	Identification [references]
20781	Exon 1	113A>T (SP)	Direct sequencing [9]
39	Exon 2	137-144del8bp (FS)	Cloning (3 out of 20 clones) [13]
150	Exon 4	431ins1bp (FS)	Analysis of heteroduplex from TGGE gel [13]
41	Exon 6	527del2bp (FS)	Analysis of heteroduplex from TGGE gel
196	Exon 8	784C>T (NS)	Analysis of heteroduplex from TGGE gel [13]
147	Exon 8	784C>T (NS)	Analysis of heteroduplex from TGGE gel [13]
28941	Exon 11	1021C>T (NS)	Direct sequencing
18773	Exon 11	1027del2bp (FS)	Cloning [18]
629	Exon 12	1228C>T (NS)	Cloning (1 out of 20 clones)
29230	Exon 13	1393G>T (NS)	Direct sequencing of tumour material

*Nucleotide positions are numbered according to the cDNA sequence with the first nucleotide of the ATG initiation codon as 1. Mechanism of mutation identification is followed by reference number if previously reported. Putative effect of sequence change is given as SP=splice site alteration, FS=frameshift, NS=nonsense.

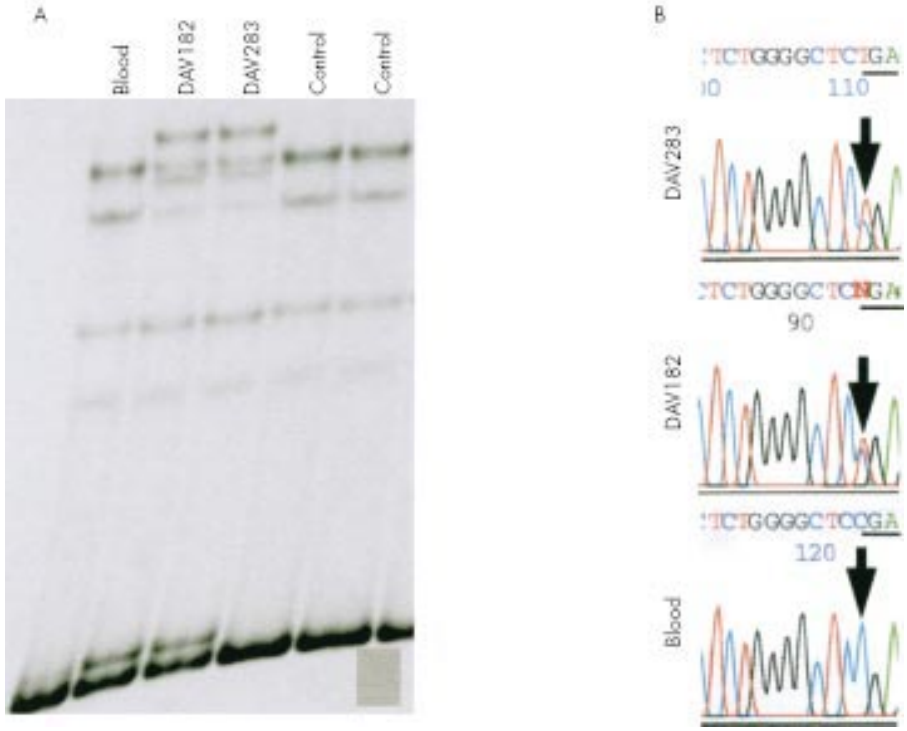

Figure 2 Mosaicism shown by finding constitutional mutations in tumours. (A) SSCP analysis of exon 2 of the *NF2* gene shows alteration in the patient's two tumour specimens (DAV182 and DAV283) which is not obvious at this level in the patient's blood specimen (G28350). Sequence analysis shows the basis of the SSCP shift to be a 169C>T transition (nonsense) in the tumour, not present at this level in the patient's blood.

because of low level of the mutant allele in this tissue (fig 1B). Mutations in these eight cases were characterised at a sequence level by T vector cloning, extraction of DNA from the heteroduplex band from the TGGE gels, or sequencing from an associated tumour.

One hundred and eleven out of the 233 screened classical NF2 founders did not show any alteration in the exons scanned at the level of their blood DNA. Adequate quality genomic DNA was available from an NF2 associated tumour for the complete exon scanning of the *NF2* gene in 35 of the 111 patients. Abnormal patterns on SSCP (fig 2A) or TGGE analysis were seen in 21 of these 35 tumour specimens. Sequence analysis (fig 2B) showed typical truncating mutations of the *NF2* gene including 12 nonsense, five frameshift, and two splice site mutations, in addition to one in frame deletion and one intronic deletion. Mutations were detected throughout exons 1-15 (table 2). Clear LOH was seen in 22 of the 35 tumours, including 10 of 14 tumours without mutations by exon scanning. In all but one case all informative markers tested were congruent with regards to loss or retention of alleles. In patient 17907 the proximal marker D22S193 and intragenic marker D22S929 both showed retention, while the distal markers D22S430 and D22S268 both showed loss. In one case (28364), LOH was subtle and eluded detection using radiolabelled primers on a denaturing acrylamide gel, but was apparent using fluorescence labelled primers on an automated sequencer.

Rescanning of corresponding exons of leucocyte DNA from the 21 specimens in which tumour mutations were identified showed very weak altered bands in three cases (143, 13, and 28165). These very weak alterations were not appreciated during the initial screening of the blood sample. Finding a low level of mutations in blood specimens additionally confirmed mosaicism of the mutations in non-tumour tissues of these three patients.

Additional anatomically distinct NF2 related tumours were available from 13 of the 21 patients. Identical mutations were detected in these tumours in nine of 13 cases supporting constitutional mosaicism for these changes (fig 2, table 2). In the other four cases, the mutation detected in the first tumour could not be detected in the second, indicating that it was tumour specific. In one of the four cases (28364), adequate quality genomic DNA was available from the second tumour for complete exon scanning and a different truncating mutation was seen in exon 11.

LOH was seen in 11 of these 13 second tumours including the second tumour from patient 28364. As with the primary tumours, all informative markers were congruent with regards to loss or retention at all informative markers with the single exception of patient 17907. In this patient's second tumour (a left vestibular schwannoma), the pattern was identical to that seen in the first tumour (a right vestibular schwannoma), with retention at proximal marker D22S193 and intragenic marker D222S929 and loss at distal markers D22S430 and D22S268.

In eight of the 21 cases in which *NF2* mutations were detected in the first tumours, no second tumour was available for analysis. In the six cases in which LOH of *NF2* was also detected, we were unable to distinguish between the possibility of mosaicism for these mutations and mosaicism for *NF2* deletion. In the other two cases (13772 and 28889) in which no LOH of *NF2* was detected, the mutations remain inconclusive for being constitutional or tumour specific.

A more detailed phenotypic and molecular examination of mosaicism of the nonsense mutation 586C>T in exon 6 of the *NF2* gene was possible in the two patients (28165 and 24405) because of our previous study of five unrelated patients carrying the same mutation at this warm spot at a non-mosaic level.[19] Although the mosaic patients had a delayed onset of symptoms, their eventual tumour load was similar to that of the non-mosaic patients and greater than the average seen in large clinical surveys (table 3). T vector cloning of blood samples from these two patients was completed to estimate the level of mosaicism in an unaffected tissue. DNA from a

Table 2 Mutations identified in tumour specimen from 21 patients

	First tumour			Second tumour		
Patient	Location	Sequence change*	LOH	Mutation†	LOH	Conclusion [ref]
29276	Exon 1	67insA (FS)	No	Detected	Yes	Constitutional for 67insA
28350	Exon 2	169C>T (NS)	Yes	Detected	Yes	Constitutional for 169C>T
143	Exon 3	352-354del3bp (in frame deletion)	No	Detected	No	Constitutional for 352-354del3bp
13	Exon 6	586C>T (NS)	Yes	Detected	Yes	Consitutional for 586 C>T [13]
24405	Exon 6	586C>T (NS)	No	Detected	No	Constitutional for 586C>T
28165	Exon 6	586C>T (NS)	Yes	Detected	Yes	Constitutional for 586C>T
74	Exon 6	586C>T (NS)	Yes	Detected	Yes	Consitutional for 586C>T [26]
17907	Exon 11	1021C>T (NS)	Yes‡	Detected	Yes‡	Constitutional for either 1021C>T or *NF2* deletion
28349	Exon 13	1396C>T (NS)	Yes	Detected	Yes	Constitutional for 1396C>T
28364	Exon 13	1396C>T (NS)	Yes	Exon 11: 1009 C>T (NS)	Yes	Tumour specific for both mutations; likely constitutional for *NF2* deletion
27255	Intron 12	IVS12+1G>A (SP)	No	Not detected	Yes	Tumour specific for IVS12+1G>A
28999	Exon 13	1396C>T (NS)	No	Not detected	Yes	Tumour specific for 1396C>T
191.3	Intron 14	IVS14+2T>C (SP)	No	Not detected	Yes	Tumour specific for IVS14+2T>C
72	Exon 2	169C>T (NS)	Yes	NA	–	Likely constitutional for 169C>T or *NF2* deletion [25]
358	Exon 2	193C>T (NS)	Yes	NA	–	Likely constitutional for 193C>T or *NF2* deletion [25]
28967	Exon 3	260del25bp (FS)	Yes	NA	–	Likely constitutional for 260del25bp or *NF2* deletion
608	Exon 3	293-294delC (FS)	Yes	NA	–	Likely constitutional for 293-294delC or *NF2* deletion
25974	Exon 4	439C>T (NS)	Yes	NA	–	Likely constitutional for 439C>T or *NF2* deletion
128	Intron 7	IVS7-33del30bp	Yes	NA	–	Likely constitional for IVS7-33del30bp or *NF2* deletion
13772	Exon 9	844delG (FS)	No	NA	–	Inconclusive (27)
28889	Exon 12	1199del2bp (FS)	No	NA	–	Inconclusive for 1199del12bp

*Nucleotide positions are numbered according to the cDNA sequence with the first nucleotide of the ATG initiation codon as 1. Positions for introns are according to the genomic sequence numbered from the closest exonic position.
†NA - no second tumour available.
‡Both tumours showed unusual pattern of LOH.
Conclusion of mutations is followed by reference number if previously reported.

Table 3 Mitigating effect of mosaicism on the nonsense mutation 586C>T in exon 6 of the *NF2* gene

	GUS24405 with mosaic 568C>T	GUS28165 with mosaic 568C>T	5 non-mosaic cases with 568C>T[19]	Survey of 63 unselected NF2 cases[2]
Age first symptom	43	29	10.2	20.3
Age hearing loss	67	31	13.5	25.7
Age diagnosis	67	59	14.2	28.3
Family history	No	No	0%	73%
Meningioma	Yes	Yes	80%	49%
Spinal cord tumour	Yes	Yes	100%	67%

non-founder NF2 patient with the same constitutional mutation was used as a control. Twelve of 22 clones tested in the control carried the 586C>T alteration (54%). In both mosaic patients, less than 10% of clones (two of 24 in patient 24405 and two of 22 in patient 28165) carried the mutation.

DISCUSSION

In this report, we define the extent to which somatic mosaicism may be detected in NF2 founders, and the value of tumour based mutational analysis in this syndrome. Mosaicism could be detected directly in a non-tumour tissue (blood lymphoblasts or lymphocytes) in 10 of 233 founders (4.3%). Exact molecular characterisation of these mutations was frequently arduous, involving T vector cloning or sequencing of altered heteroduplex bands extracted from gels. Among the 48% of founders in whom mutations could not be detected in blood specimens, detection of constitutional mutations could be facilitated by the analysis of tumour material. However, we were unable to obtain high quality tumour DNA from a majority (76 of 111, 68%) of the patients, primarily because of lack of availability of archived specimens or failure of older archived specimens to extract well. It is possible that this has introduced a bias away from more mildly affected patients who do not undergo as many surgical procedures.

Among the 35 founders in whom mutation could not be detected in blood specimens and from whom adequate tumour material was available for genetic analysis, constitutional mutations were confirmed in nine (25.7%) cases based on finding of identical mutations in different tumours in each patient. These nine patients were thus mosaic for the constitutional mutations and the failure to find these mutations in their blood specimens was not because of the limitation of the applied screening techniques. In six other patients from whom no second tumours were available, mosaicism was deemed highly likely based on mutations and LOH of the *NF2* gene region in each tumour (total of 15 of 35 patients or 42.9%). In each of these six cases, either the mutation in or the deletion of the *NF2* gene should be the constitutional alteration. Extrapolating these data to the 111 NF2 founders with no constitutional *NF2* mutations found in their

blood, we found 29 to 48 mosaic subjects (111 × 0.257 to 111 × 0.429). Adding the 10 mosaic cases detected directly in the blood specimens, we estimate the rate of mosaicism to be 16.7% to 24.8% (39/233 to 58/233) in our cohort of 233 NF2 founders with bilateral vestibular schwannomas.

Two different mutations were found in two unrelated tumours from patient 28364. However, both tumours had LOH of the *NF2* markers. One plausible explanation is that the deletion of the *NF2* gene is the constitutional genetic alteration and the two mutations are tumour specific "second hits". In the case of patient 17907, both the left and right vestibular schwannomas had the same mutation and both tumours exhibited allele loss of only the proximal marker D22S193 and intragenic marker D22S929, but not the distal markers D22S430 and D22S268. In this case, either the mutation or the microdeletion of the *NF2* gene could be the constitutional alteration.

The first report of mosaicism in the *NF2* gene was made shortly after the cloning of the gene itself.[23] These investigators specifically commented on the amelioration of the severe phenotype associated with the warm spot alteration 169C>T in a mosaic patient (estimated in 10% of blood cells) compared to a non-mosaic patient with an identical mutation. Their findings are thus quite similar to our observations in patients carrying the exon 6 mutation 586C>T. The confounding effects of mosaicism on linkage analysis were seen in a second study of a family carrying the exon 8 warm spot mutation 784C>T.[24]

Indirect evidence of mosaicism was seen in both our own large mutation survey[14] and that of Evans *et al*.[13] In both studies, the detection rate of mutation in founders was significant lower (51% in the study of Kluwe *et al*[14] and 34% in the study of Evans *et al*[13]) compared to that in non-founders (81% and 54%, respectively). In addition, mutations in blood samples were found more frequently in patients with a severe phenotype than in patients with a mild phenotype, consistent with the hypothesis that mosaicism may ameliorate the phenotype.[14] Recurrence risks to offspring of potential mosaics were significantly lower than the 50% expected for an autosomal dominant disease.[13] Mosaicism for *NF2* has rarely been documented molecularly in association with phenotypes other than BVS, such as unilateral VS with other NF2 related tumours[12] and schwannomatosis.[22]

Among the 15 mosaic cases identified by tumour analysis in this study, nine (60%) were recurrent mutations, including C>T changes at CpG islands in exons 2 (three cases), 6 (four cases), and 13 (two cases). For comparison, 37% of the mutations in 340 unrelated NF2 subjects in the database (http://neuro-trials1-mgh.harvard.edu/nf2) were nonsense mutations. It is not clear if tumour specific mutation is more common at these sites or if mosaicism for these mutations is more likely to be associated with the BVS phenotype that was studied. Mutations were not detected in 14 of 35 tumours (40%), which is comparable to the detection rate seen in large surveys of the disease using exon scanning and may partially reflect the inability of this methodology to detect large deletions or other alterations which elude exon scanning.

In summary, we have shown direct evidence that mosaicism is frequent in NF2 founders with bilateral vestibular schwannomas and have shown the value of molecular analysis of tumour material in detecting somatic mosaicism. Similarly, mosaicism may be common in other human autosomal dominant diseases, such as neurofibromatosis 1[15] and von Hippel-Lindau disease,[28] and needs to be studied in the future because of its implication in diagnosis, prognosis, and genetic counselling. For NF2, further work is in progress to define the spectrum of phenotypes associated with mosaicism, the exact genetic risks to offspring of founders, and optimal methods to detect mosaicism when tumour tissue is unavailable.

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