ORIGINAL ARTICLE

Mosaicism in neurofibromatosis type 2: an update of risk based on uni/bilaterality of vestibular schwannoma at presentation and sensitive mutation analysis including multiple ligation-dependent probe amplification

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Background: Neurofibromatosis type 2 (NF2) is almost unique among inherited disorders in the frequency of mosaicism in the first affected generation. However, the implications of this on transmission risks have not been fully elucidated.
Methods: The expanded database of 460 families with NF2 and 704 affected individuals was analysed for mosaicism and transmission risks to offspring.
Results: 64 mosaic patients, with a projected mosaicism rate of 33% for sporadic classical NF2 with bilateral vestibular schwannoma at presentation and 60% for those presenting unilaterally, were identified. Offspring risks can be radically reduced on the basis of a sensitive mutation analysis of blood DNA including multiple ligation-dependent probe amplification (MLPA, which detects 15% of all mutations), but even MLPA cannot detect high levels of mosaicism.
Conclusion: The chances of mosaicism in NF2 and the resultant risks of transmission of the mutation to offspring in a number of different clinical situations have been further delineated. The use of MLPA in this large NF2 series is also reported for the first time.

Neurofibromatosis type 2 (NF2) is characterised by the development of schwannomas, meningiomas and ependymomas, with the hallmark of bilateral involvement of the eighth cranial nerve.[1] Over 50% of patients have no family history, and have de novo mutations in the NF2 gene.[1] Furthermore, pedigree analysis and mutation studies in blood and tumour specimens have indicated that a minimum of 25–30% of these new cases of NF2 are mosaic, with the mutation often detected only in tumour material and not in lymphocyte DNA.[2 3] We have shown previously that the risk of transmission is low if a mutation is undetectable in blood.[4] We have now analysed our expanded dataset of over 700 patients with NF2 to study transmission risks in mosaic NF2.

METHODS

Analysis of NF2 database

We undertook an updated analysis of our NF2 database, which now includes 704 patients with NF2. Patients are either referred to us for mutation analysis or have been seen as part of our specialist service. Family trees are drawn to identify deceased cases, and information from hospital records is sought to verify their diagnosis. All patients on the database fulfil Manchester criteria[1] or have an NF2 germline or proven mosaic mutation (identical mutation in two separate tumours). Age at onset and laterality of vestibular schwannoma (VS) at presentation were examined. Patients who presented with a unilateral vestibular schwannoma (UVS), in which no contralateral VS was identified on scan, but other NF2 tumours were present, had been removed, or occurred subsequently before the presence of a contralateral VS, were identified.

Mutation analysis

Mutation analysis was carried out on DNA extracted from blood lymphocytes and, where possible, tumour material, as described previously,[4–6] by screening all 17 exons of the *NF2* gene. For this, we used either single-strand confirmation polymorphism or, since 2000, direct sequencing of all exons. Since 2000, we have also used an approach to detect exon duplications and deletions[5]; more recently, we have been using multiple ligation-dependent probe amplification (MLPA) for this purpose.

Offspring risk

The risk to offspring was assessed by analysing available pedigrees.

RESULTS

Analysis of NF2 database

Of the 704 patients on the NF2 database, 282 have been seen by at least one of the authors (DGRE). The database also includes 48 deceased affected relatives who have been identified from family trees. Age at onset or laterality of VS at presentation was not available for 44 of 704 patients, and 49 of the remaining 660 (7%) patients were yet to develop VS (18 of these were presymptomatic diagnoses with genetic testing). Only 12 out of the 49 were aged >20 years. Of the remaining 611 patients, 16 patients did not have information on the presence of VS, of whom six were inferred deceased carriers of NF2. Of the remaining 595 patients, 142 (24%) patients presented initially with UVS. In the subgroup seen by us, the ratio was very similar (64/282, 23%). Many of these patients (n = 30) were children or young adults undergoing a first asymptomatic screen. Out of 142 patients, 110 were known to be the first patients affected with NF2 in the family. Of the 103 patients presenting with UVS for whom we had lymphocyte DNA, 27 (26%) had a mutation detected in blood (including five in whom it was

Abbreviations: FISH, fluorescence in situ hybridisation; MLPA, multiple ligation-dependent probe amplification; NF2, neurofibromatosis type 2; UVS, unilateral vestibular schwannoma; VS, vestibular schwannoma

mosaic) and 18 (17%) were identified as mosaics from analysis of tumour DNA. The proportion of mosaics detected among patients with analysed tumours was 18/24 (75%). If a similar proportion of the patients with untested tumours (52) were mosaic, this would suggest that at least 39 further patients were mosaic. This would mean that at least 62/103 (60%) of those presenting with UVS were mosaic.

We also analysed the proportion of sporadic patients with NF2 presenting with VS bilaterally, who had detectable mutations. Of patients who presented bilaterally, 137/217 (63%) had a detectable mutation in blood (19 were mosaic), and a further 13 mosaic mutations were detected only in tumour. These 13 patients were from the 20 (65%) in whom tumour analysis was possible. If the 65% detection rate of mosaicism from tumour analysis was extrapolated to the remaining 60 patients, a further 39 mosaic patients would be identified. This implies that a minimum of 71/217 (33%) patients would be mosaic if they presented as *de novo* bilateral cases (table 1).

Forty-one isolated patients with NF2 were excluded, as information on laterality at presentation was insufficient or they had not yet developed VS.

We identified a mutation in 84 out of 92 (91%) second-generation families (table 2). We specifically tested affected members from the second or later generation of a family, as a mosaic mutation can be missed in analysis of blood DNA from the founder.[2 4] There was no testing bias as all available multigenerational families had had testing in the second or later generation. A mutation is assumed to be present in full form in a subsequent generation if individuals in that generation inherit the mutation from either the egg or the sperm of the affected parent. The sensitivity of our techniques is therefore >90%.

However, 20/92 (22%) NF2 defects were detected on MLPA. MLPA is only able to distinguish dosage between two copies and one or three copies of an exon. It would not therefore detect even relatively high-level mosaicism. An assessment of mutation detection and miss rates is presented in table 3.

Of the 92 multigenerational families, we have reliable information on VS for 58 founders. Out of 58 patients, 6 (10%) patients presented with UVS (three were mosaic, two had full mutations and patient 6006/201 discussed below was untested). Three founders had no VS at the time of death. In all, 7 of 34 (21%) founders that we were able to test were mosaic. In a further two families, including a previously reported family[7] and patient 6006/201, mosaicism in the founder appeared likely, owing to the vastly worse phenotype in the second generation. Patient 6006/201 had a UVS at age 24 years and was noted to have a cutaneous tumour at age 45 years. His son, who has developed classical NF2, has an exon 1/intron 1 deletion, but it has not been possible to obtain a blood sample from the father. The mean age at onset of symptoms in the seven definite mosaic founder patients was 33.9 years (median 36 years; range 23–43 years) and that in the eight affected children was 17 years (median 19 years; range 1–30 years).

In total, we have identified 64 definite mosaic patients. In addition to the 55 mosaic patients from the laterality analysis (23 with UVS, 32 with bilateral VS at presentation), there were two extra sporadic mosaic cases with no VS and seven mosaic founders from multigenerational families. Among the 64 mosaic patients, 36 have had 72 children. Of the 26 patients with a detectable mosaic point mutation in blood, 10 have had 26 children, of whom five have inherited the point mutation (two from one parent) and developed NF2 as reported previously.[3] Of 11 parents mosaic for an MLPA-detectable deletion/duplication (found in tumour or in the child), eight have had 14 children. Out of 14 children, three children have inherited the MLPA abnormality. MLPA was not able to detect mosaicism reliably in blood from any of the 11 de novo mosaic parents. In the three patients with NF2 with affected children, the mosaicism has been inferred after confirmation of an MLPA abnormality in the child. One mother (patient 158/201) who presented with bilateral VS at age 31 years had a splice mutation (on direct sequencing) and MLPA deletion of the whole NF2 gene on tumour analysis. Analysis of her second tumour demonstrated only the MLPA deletion, which was later shown in full form in an affected daughter. This was confirmed on fluorescence in situ hybridisation (FISH) analysis, but none of the 30 maternal lymphocytes showed the deletion. The second mosaic MLPA patient (149/101) who presented at age 56 years with UVS and six cranial meningiomas (four spinal tumours were also found on scan) had an exon 7 duplication identified in her son affected with classical NF2. This was not present on MLPA analysis in her own blood sample, and we have to assume that she was mosaic for the duplication. The third mosaic patient (3602/201) had bilateral VS at age 38 years, and was noted to have a cutaneous tumour at age 40 years. His son who has developed classical NF2 (bilateral VS and a spinal tumour aged 20 years) has an intron 1/exon 1 deletion on MLPA, which could not be confirmed in the father's blood sample, but the son had hemizygosity for the intron 1 CA repeat, having not inherited a paternal copy.

Twenty-four mosaic patients were identified with a point mutation that was detected in two tumours but not detectable in blood. Seventeen of these patients have had 34 children. None of the 34 children were affected (95% CI of 0/34: 0% to 8.4%), and the mutation has been excluded from 26 of them (0/26; 95% CI: 0% to 10%). None of the three patients with ring 22 have had children.

If a mutation is not detected in blood in a de novo patient, then there are four possibilities:

1. A mutation exists in all cells but is not detectable (9% miss rate in second generation).
2. A mosaic point mutation is present at a concentration too low to be detected in blood, which is detectable on tumour analysis.
3. A large-scale rearrangement detectable by MLPA is present in mosaic form but not detectable in blood (this could be identified in tumour).

Table 1 Mutations detected on the basis of laterality at presentation

	Detection in blood (mosaic) (%)	Mosaic from tumour analysis (%)	Extrapolated mosaic disease (%)
Sporadic case bilateral VS at presentation	137/217 (63)[19]	13/20 (65)	33
Sporadic case unilateral at presentation	28/103 (27)[5]	18/24 (75)	60

VS, vestibular schwannoma.

Table 2 Mutations identified in 460 families with neurofibromatosis type 2 (includes patients not in laterality analysis)

Type of mutation	Detection in second generation No (%) (n = 92)	Detection in sporadic non-mosaic patients (% non mosaic)	Mosaic mutations (% of mosaic) No (%)	Total*
Splice site	28 (30)	35 (22)	3 (5)	66 (15)
MLPA positive	20 (22)	21 (14)	11 (17)	52 (11)
FSD	14 (15)	31 (19)	16 (25)	61 (13%)
Nonsense	11 (12)	57 (35)	24 (37.5)	92 (20)
Missense	6 (6.5)	4 (2.5)	1 (1.5)	11 (2.5)
FSI	3 (3)	11 (7)	3 (5)	17 (4)
IFD	1 (1)	1	3 (5)	5 (1)
Ring 22	0	0	3 (5)	3
Not found	8 (9)	208 (56.5)		152/460 (33)
Total	84/92 (91)	160/368 (43.5)	64/368 (17)	308/460 (67)

FSD, frame shifting deletion; FSI, frame shifting insertion; IFD, intrinsic factor deficiency; MLPA, multiple ligation-dependent probe amplification.
*Mosaic patients are already counted under the previous two columns, and therefore the denominator is the addition from columns 2 and 3.

4. A mutation missed by our detection techniques is present in mosaic form.

Extrapolating the data from our analysis, the estimates of mosaicism for presentation with UVS or bilateral VS are presented in table 3. These are based on a number of assumptions, which are explained in the footnotes.

In table 4, we summarise the possible findings of DNA analyses on lymphocyte and tumour, their interpretation, and what test can then be used to exclude NF2 in the offspring.

DISCUSSION

We have further clarified the contribution of mosaicism to the causation of de novo NF2. The original reports suggesting that 25–30% of *de novo*[3 4] cases are mosaic were a minimum estimate, depending on the ability to ''prove'' mosaicism by identifying an identical mutation in two tumours, or at least excluding the presence of either of the two mutational hits from tumour in a blood sample. Our improved mutation detection techniques have led to detection of a higher proportion of mutations in tumours, boosting the minimum mosaicism rate in classical (bilateral VS) presentation of NF2 to 33%, and that in individuals with UVS presentation to 60%. All these patients fulfilled the Manchester criteria, which, while being more sensitive than other criteria, retain the specificity.[8 9] These results have implications for transmission of the NF2 mutation to the offspring. In contrast to a recent report,[10] there is a real transmission risk of a mutation from parents presenting with UVS to offspring, even when the mutation is not detectable in blood. It is not clear whether our findings reflect a greater sample number (110 vs 44) or the inclusion of patients who did not fulfil at least the Manchester criteria in the US report.[11] If a point mutation is detected at mosaic level in blood, an estimate of transmission risk can be made, although for an accurate risk a sample of sperm (or rarely ova) can clarify the proportion of zygotes carrying the mutation. The main question a geneticist will ask after a negative blood mutation analysis is ''what does this mean for offspring risk?'' While we have addressed this in the current study, the figures will depend on the type of mutation testing used, and on whether the mutation-detection

Table 3 Estimates of the proportion of different mutation types and mutation-negative de novo cases and potential offspring risks

	Sporadic case bilateral VS at presentation (proportion of unfound mutations in blood)	Sporadic case unilateral VS at presentation (proportion of unfound mutations in blood)
Point mutation in blood non-mosaic (actual)	94/217 (43)	28/103 (27)
MLPA mutation in blood (actual)	24/217 (11)	1/103 (1)
Point mutation detected in blood mosaic (actual)	19/217 (9)	5/103 (5)
(A) Point mutation detectable from tumour (estimate)	43/217 (20) [43/80–54%]	46/103 (45) [46/69–67%]
(B) Undetectable mutation in blood (estimate)	11/217 (5) [11/80–14%]	3/103 (3) [3/69–4%]
(C) MLPA detectable abnormality in mosaic form (estimate)	19/217 (9) [19/80–24%]	14/103 (14) [14/69–20%]
(D) Undetectable mosaic mutation (estimate)	6/217 (3) [7/80–9%]	6/103 (6) [6/69–9%]
(E) Offspring risk if mutation negative in blood	2.7%+7%+2.3%+0.9% 13% (1 in 8)	3.3%+2%+2%+1% 8.3% (1 in 12)

MLPA, multiple ligation-dependent probe amplification; VS, vestibular schwannoma.
(A) The proportion of point mutations that would be detectable if tumour material were available from all patients.
(B) Mutations that are present in full form but refractory to current mutation detection techniques (9% in multigenerational families). The assumption is that an additional 9–10% of detectable mutations in blood would be undetectable. For UVS 29+1/103 detectable in full form, assume 3 undetectable.
(C) Estimate of the number of MLPA-detectable abnormalities present in mosaic form. Assumptions are that 20–25% of missed mutations will be MLPA-detectable mosaic mutations.
(D) The number of mosaic mutations that would not be detected using current techniques. Again, the assumption is that 9% of non-detectable mosaic mutations will be in this category.
(E) The offspring risk is calculated from the following. The risk from row (A) is minimal, as shown from 0/34 children (95% CI for 0/34: 0% to 8.4%—assume 5%); (B) 50% offspring risk if present in all cells; (C/D) assume 10% (7/70 children of proven mosaics have developed NF2) as the proportion of mutations in blood is not estimable.

Table 4 Outcomes of tumour analysis after negative blood analysis in determining mosaicism and utility in testing offspring

Hits in tumour	Mosaicism confirmed	Test in offspring after single tumour analysis	Second tumour	Test in offspring for confirmed mosaic mutation in affected patient
Point mutation (A)+LOH	YES—need second tumour*	MLPA+point mutation (A)	Point mutation (A)	Point mutation (A)
Point mutation (A)+LOH+MLPA normal	YES—confirms mitotic recombination	Point mutation (A)	Not required	Point mutation (A)
Point mutation (A)+point mutation (B)	YES—need second tumour*—ensure at full dosage, as could be multifocal	Point mutation (A)+point mutation (B)	Point mutation (A)	Point mutation (A)
Point mutation (A) no second hit no LOH	NO—need second tumour*	No definitive test to exclude NF2	Point mutation (A)	Point mutation (A)
Point mutation (A), no second hit, no LOH	NO—need second tumour*	No definitive test to exclude NF2	Point mutation (A) not present	No definitive test to exclude NF2
Point mutation (A), no second hit, no LOH	NO—need second tumour*	No definitive test to exclude NF2	Point mutation (A) not present, but LOH	Test for inheritance of lost allele: patient mosaic for mutation on retained allele
Point mutation (A)+MLPA whole gene deletion and LOH	YES—need second tumour*	MLPA+point mutation (A)	No point mutation (A), but MLPA+LOH	MLPA–patient mosaic for whole gene deletion
Point mutation (A)+MLPA exon 1–4 deletion and LOH for intragenic marker only	YES—need second tumour*	MLPA+point mutation (A)	No point mutation (A), but MLPA exons 1–4	MLPA–patient mosaic for exon 1–4 deletion
No point mutation, but LOH	NO—need second tumour*	Test for inheritance of lost allele and MLPA, patient mosaic for mutation on retained allele		Patient may be mosaic for MLPA-detectable deletion or a point mutation on the retained allele
None identified	NO—need second tumour*	No test available	Point mutation (A)	No test available
None identified	NO—need second tumour*	No test available	Point mutation (A)+LOH	MLPA+point mutation (A): first tumour may have been contaminated with normal material and patient mosaic for point mutation or deletion

LOH, loss of heterozygosity; MLPA, multiple ligation-dependent probe amplification.
*Need second tumour to confirm which abnormality the patient is mosaic for.

techniques have a similar sensitivity to ours. Our combined use of sequencing and MLPA has a 91% sensitivity to detect a mutation in the second generation of an NF2 family. It is not reasonable to assume that the same sensitivity would apply to *de novo* cases. The mutation spectrum is different, with protein-truncating point mutations (nonsense and frameshift mutations) predominating in sporadic new mutation cases.[12–15] In our current analysis, 99 of 161 (61%) detectable mutations were protein truncating in the germline of de novo cases, compared with 28 of 84 (33%) in the second generation of a family with NF2. This reflects the increased severity of protein-truncating mutations on NF2 phenotype, and its resultant effect of reducing genetic fitness.[12–16] If anything, this higher proportion of point mutations in the exons would mean a higher sensitivity in detecting mutations in sporadic *de novo* patients in whom the mutation is non-mosaic. However, we still do not know the mechanism for the missed mutations. These could be deep intronic splicing variants or gene-silencing mutations, which would require RNA analysis.

Not withstanding these reservations, a fairly robust estimate of the frequency of different reasons for a negative blood mutation analysis can be made. From this, an estimate of offspring recurrence risk can be deduced. The very high mosaicism rate in patients presenting with UVS means that the risk of transmission to offspring will be considerably reduced from the expected 1 in 2 for a fully penetrant mutation to a figure of around 1 in 12. Even in patients presenting bilaterally with no identified mutation in blood, the risk is likely to reduce to around 1 in 8.

We have presented for the first time an analysis of our large NF2 series using MLPA. These 52 patients amount to about half of all reported large exonic deletions/duplications in the literature.[17] Our present study highlights the importance of a detection technique for large exonic alterations. In all, 15% of all detectable abnormalities were detected by MLPA, amounting to 11% of NF2 families analysed. This is identical with the result in a smaller study of 188 families, although the spectrum of alterations was different.[18] Failure to use a technique to detect these changes would result in a major loss of sensitivity of the testing process. As such, the reduction in recurrence of risk to offspring would be substantially smaller.

Our assessment of mosaicism in the first generation of a multigenerational family with NF2 is higher than the previous finding of only 3 of 27 (11%) testable founders as mosaic[4]. This is because we have now been able to identify a further three families using the previously unavailable MLPA analysis. The figure should be viewed with caution, nonetheless. The reason we have not been able to examine some founder individuals is that they died young from fairly classical NF2, and were thus unavailable for testing and hence unlikely to be mosaic. In contrast, the mildly affected mosaic cases may have near-normal life expectancy, and may therefore substantially boost the number of cases available for testing.

Tumour analysis does enable the testing of offspring to exclude NF2. In our experience, the ability to detect changes in the tumour after a normal complete mutation analysis of blood is high, reflecting the high sensitivity of our techniques and high rates of mosaicism for a detectable abnormality. Even if a common change is not detectable in two tumours, it is still possible to exclude NF2 in many scenarios, as shown in table 4. Even if a tumour shows only LOH, NF2 can still be excluded, as the patient is mosaic either for an undetectable mutation on the retained allele or for a large gene deletion.[4 6 19] As such, linkage analysis still has a place in excluding NF2, although the reverse situation (a high-risk result) should be interpreted with caution if testing the second generation of an NF2 family.[20] Inheritance of the high-risk allele may not be associated with increased risk if the parent is mosaic for a mutation on that allele.[20] Although it is possible to confirm NF2 if only a single point mutation is identified in a tumour, a negative test for this in the offspring is uninformative.

The difficulty in assessing mosaicism from tumour analysis arises if a tumour shows both a point mutation and a large-scale rearrangement detectable by MLPA (this could be a whole gene deletion). It is possible to exclude high-level mosaicism in blood for the point mutation (single-strand confirmation polymorphism detects this at the 5% level, and sequencing at 10–20%), but not the change detectable by MLPA. One patient with an affected child was clearly mosaic for an exon 1–16 deletion that was present in the child and in her own tumour DNA, but not detectable in blood. If an MLPA abnormality is detected in a tumour and only one tumour is available for analysis, then chromosome FISH may reveal whether the deletion is present in a significant number of lymphocytes. The fact that this patient had <3% of lymphocytes affected shows that there is a risk of transmission even below the 5% level. Approximately half of our MLPA deletions are likely to result in an abnormality detectable by FISH (table 3). In the clinical setting, the approach to who/what tissues to send for mutation testing depends on the presentation. In isolated/founder cases, mosaicism should be suspected in those presenting initially with UVS, in those with ⩾2 NF2-related tumours but falling short of diagnostic criteria, including those who might be classified as schwannomatosis,[4 21] or in those with meningiomatosis.[22] In families with two or more affected members, a further clue may be mild disease in the first generation, with more severe disease in subsequent generations.[7] In familial cases, initial mutation testing should be undertaken in the affected offspring rather than in the founder. In de novo cases, ideally, mutation testing using tumour DNA is the first step. Given the high frequency of mosaicism, it is essential that a part of all tumours removed (including two parts of a multifocal tumour) is sent for DNA analysis. When counselling a de novo mosaic case, the possibility of more severe disease in a non-mosaic offspring must be discussed.

NF2 is almost unique among genetic conditions in that mosaic disease represents a large proportion of classical disease. The proliferation of CNS tumours in NF2 will lead to the diagnosis as long as at least one tumour has the symptoms (others will be detected on scan). Mosaic disease could be under-recognised in other tumour-prone disorders such as familial polyposis, where mutation detection rates are still reduced in the first generation. However, in von Hippel Lindau disease and NF1, detection rates in sporadic patients are as high as 90–95% in blood.[22] NF2 therefore presents an unusual counselling situation for a monogenic disease, where failure to identify a mutation in blood is more likely to mean that a patient has a low risk of transmission to his or her offspring, rather than that the sensitivity of the detection techniques is reduced.

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