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Genetic findings in people with schwannomas who do not meet clinical diagnostic criteria for *NF2*-related schwannomatosis

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Abstract

Background: Most schwannomas are isolated tumours occurring in otherwise healthy people. However, bilateral vestibular schwannomas (BVS), or multiple non-vestibular schwannomas indicate an underlying genetic predisposition. This is most commonly *NF2*-related schwannomatosis, but when BVS are absent, this can also indicate *SMARCB1*- or *LZTR1*-related schwannomatosis.

Methods: We assessed the variant detection rates for the three major schwannomatosis genes (*NF2*, *LZTR1* and *SMARCB1*) in 154 people, from 150 families, who had at least one non-vestibular schwannoma, but who did not meet clinical criteria for *NF2*-related schwannomatosis at the time of genetic testing.

Results: We found that 17 (11%) people from 13 families had a germline *SMARCB1* variant and 19 (12%) unrelated individuals had a germline *LZTR1* variant. Nineteen people had an *NF2* variant, but 18 of these were mosaic and 17 were only detected when two tumours were available for testing. The overall detection rate was 25% using blood alone, but 36% when tumour analysis was included. Another 12 people had a germline variant of uncertain significance (VUS).

Conclusions: There were similar proportions of *LZTR1*, *SMARCB1*, or mosaic *NF2*. However, since an *NF2* variant was detected in tumours from 103 people, it is likely that further cases of mosaicism would be detected if more people had additional tumours available for analysis. In addition, if further evidence becomes available to show that the VUSs are pathogenic, this would significantly increase the proportion of people with a genetic diagnosis. Our results indicate the importance of comprehensive genetic testing and improved variant classification.

Key Words: Schwannomatosis, schwannoma, *NF2*, *LZTR1*, *SMARCB1*

What is already known on this topic?

The three major forms of schwannomatosis, *NF2*-, *LZTR1*- and *SMARCB1*-related schwannomatosis (SWN), share significant clinical phenotypic overlap. *NF2*-related SWN is the most common form, but it is unclear what proportion of people with schwannomatosis, not meeting clinical criteria for *NF2*-related SWN, have each form of schwannomatosis.

What this study adds:

We show similar proportions of *LZTR1*, *SMARCB1*, or mosaic *NF2*-related SWN and a high proportion of people with no identified predisposing variant in our cohort. However, there is also a high proportion of additional *LZTR1* variants that are classified as variants of uncertain significance and a high proportion of people with only one tumour who may have mosaic *NF2*-related SWN.

How this study might affect research, practice or policy:

Our results indicate the importance of comprehensive clinical genetic testing, particularly for low-level mosaic *NF2* variants, and improvements in variant classification, particularly for *LZTR1* variants.

Introduction

Over one in 500 people in the general population may develop a schwannoma during their lifetime[1]. Up to 75% of these arise from the vestibular nerve[2], often causing deafness and balance problems. The remaining schwannomas arise from non-vestibular cranial, spinal or peripheral nerves. The majority of schwannomas occur as isolated tumours in otherwise healthy people. However, when bilateral vestibular schwannomas occur, or multiple non-vestibular schwannomas, this is often shown to be associated with a genetic predisposition to tumour development. The most common schwannoma predisposition syndrome is *NF2*-related schwannomatosis (NF2; MIM# 101000), a neurogenetic disorder caused by heterozygous pathogenic variants in the *NF2* gene (NM_000268.4) and UK data suggests a prevalence of approximately 1 in 61 332 people [3]. Affected individuals are predisposed to develop multiple schwannomas, meningiomas and ependymomas[4] and in particular, there is a high risk of developing bilateral vestibular schwannomas. Non-*NF2* related schwannomatosis is much rarer and includes multiple sub-types. UK data suggests that it occurs in approximately 1 in 103,700 people and that schwannomatosis-causing germline variants in *LZTR1* (NM_006767.4) and *SMARCB1* (NM_003073.5) occur in 1 in 527,000 and 1.1 million people, respectively[3].

Schwannomas that occur in *SMARCB1*- or *LZTR1*-related schwannomatosis tend not to be located on the vestibular nerve (not reported at all so far for *SMARCB1*), but mainly occur on spinal and peripheral nerves, as well as, less commonly, other cranial nerves[5]. In addition, unilateral vestibular schwannomas are seen in approximately 5% of *LZTR1*-related schwannomatosis patients [6]. This means that there is significant clinical overlap between these syndromes and that genetic testing can be critical for diagnosis in uncertain cases. The importance of genetic testing is reflected in the updated diagnostic criteria [4]. Here, we have examined the genetic findings in our cohort of patients who do not meet clinical diagnostic criteria for *NF2*-related schwannomatosis, but who have at least one non-vestibular schwannoma that has undergone genetic testing, in order to determine the rates of pathogenic/likely pathogenic (P/LP) variant detection.

Materials and Methods

Patient materials

We identified a total of 154 patients from 150 families in the genetics database at the Manchester Centre for Genomic Medicine who had at least one non-vestibular schwannoma with genetic testing, but who did not meet clinical diagnostic criteria for *NF2*-related schwannomatosis at the time of genetic testing (up to 30th June 2023). Fifty three people had two tumours tested. The cohort included 72 females and 84 males and the range of ages at genetic testing was 9 months to 78 years (age distribution of the cohort is shown in supplementary figure 1). Ethical approval for the use of anonymised samples from our DNA archive was obtained from the North West 7–Greater Manchester Central Research Ethics Committee (reference 10/H1008/74).

DNA sequence analysis

All individuals underwent genetic analysis of lymphocyte DNA to screen for pathogenic variants in *NF2, LZTR1* and *SMARCB1*. In addition, tumour DNA from at least one non-vestibular schwannoma was assessed for somatic *NF2* variants. Somatic *LZTR1* and *SMARCB1* variants were also assessed when sufficient DNA was available. Samples were screened using a combination of Sanger sequencing and targeted next generation sequencing (NGS) panels to sequence coding exons and flanking intron/exon boundaries. Analysis of the coding region of *NF2*, included at least +/-15 bases flanking each exon and known intronic likely pathogenic/pathogenic variants using an Agilent SureSelect custom gene panel and Illumina

NGS. The custom panel includes *NF2* (NM_ 016418.5), *LZTR1* (NM_006767.3), and *SMARCB1* (NM_003073.3).

For optimum somatic variant detection, a neoplastic cell content of ≥20% is required. NGS sequences were covered at an average read depth of 1000x and identified P/LP variants were confirmed using Sanger sequencing. Patients were considered to have mosaic *NF2*-related schwannomatosis when a P/LP variant was identified in lymphocyte DNA at below 30% of the total allele count, or when identical *NF2* variants were detected in two anatomically distinct tumours.

Variant calling was carried out using a custom in-house bioinformatic analysis pipeline validated to detect single nucleotide variants (SNVs) and small insertion/deletion variants (<40bp) at ≥5% variant allele frequency (VAF). Variants are named according to the human genome variation society (HGVS) recommendations (varnomen.hgvs.org/). Variant classification followed the Association for Clinical Genomic Sciences (ACGS) Best Practice Guidelines for Variant Classification in Rare Disease 2020 & 2024 framework (https://www.acgs.uk.com/media/12533/uk-practice-guidelines-for-variant-classification-v12-2024.pdf) and Cancer Variant Interpretation Group UK (CanVIG-UK) guidance [7]. The ACGS guidelines follow the original guidance of Richards et al. (2015), and include updates from other groups, such as recommendations for the points-based system[8], loss-of-function predictions[9] and predicted and observed splice effects[10].

Copy number and loss of heterozygosity analysis

Multiple ligation-dependent probe amplification (MLPA) was used to detect copy number variants (CNVs), using probe sets, P043-NF2, P258-SMARCB1 and P455 LZTR1 (MRC-Holland, Amsterdam, the Netherlands) and microsatellite analysis was used to detect loss of heterozygosity (LOH).

RNA analysis

RNA containing the *LZTR1* c.263+5G>T variant was isolated from whole blood collected in a PAXgene tube. A lymphoblastoid cell line was available for the *NF2* c.1532A>G variant and the cultured cells were treated with puromycin before harvesting to prevent nonsense-mediated decay. RNA isolated from each sample was treated with DNase1 before being reverse transcribed to cDNA with oligo(d)T primers, using the Superscript IV cDNA first strand synthesis kit (Invitrogen, Carlesbad, CA, USA). Full-length transcripts were amplified in the

patient samples and seven control RNA samples using custom primers and VeriFi HotStart 2X Mastermix (PCRBio). The amplified transcripts were analysed by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and an ABI 3730xl DNA Analyzer (ABI, Life Technologies) to detect aberrant transcripts.

Results

Cohort summary

We assessed the genetic findings reported in matched blood and tumour DNA of 154 people, from 150 families to determine the rate of detection of schwannomatosis-associated variants. Each person had at least one non-vestibular schwannoma, but did not meet diagnostic criteria for *NF2*-related schwannomatosis at the time of testing. The cohort included 72 females (47%) and 84 males (53%) and the average age at initial genetic testing was 38.8 years (range: 9 months to 78 years). Genetic predisposition variants identified in the cohort are summarised below and listed in supplementary table 1.

Germline DNA analysis

In blood DNA, we found that one person (1/154; 0.65%) had a non-mosaic *NF2* P/LP variant. This patient underwent genetic testing at 6 years of age and subsequently developed bilateral vestibular schwannomas within two years of genetic testing. There were 17 (11%) people from 13 families who had a germline *SMARCB1* P/LP variant and 19 (12%) unrelated individuals who had a germline *LZTR1* P/LP variant. The remaining 117 people had no germline P/LP variant detected on initial analysis in blood of *NF2*, *SMARCB1* or *LZTR1*. Overall, there was a 25% detection rate for P/LP variants using blood DNA alone. However, 11 additional people had a variant of uncertain significance (VUS) in *LZTR1* (mainly missense variants) and one had a VUS in *NF2* (missense). A summary of the results is shown in Figure 1 and a list of predisposing variants is shown in supplementary table 1.

Schwannoma DNA analysis

Genetic testing of schwannoma DNA showed that 107/154 (69%) had LOH involving chr 22. Of these, 94/107 (88%) also had an *NF2* variant detected on the retained allele. In schwannomas without identified LOH, only 9/47 (19%) had an identified *NF2* variant. In total 116/154 (75%) non-vestibular schwannomas had at least one *NF2* variant identified in tumour DNA. *NF2* variants found in tumours are summarised in Figure 2a. Parallel blood and tumour analysis for one patient showed that the *NF2* variant identified in tumour could be seen in 3%

of reads in the matched blood samples, indicating a low level of mosaicism, below the validated limit of detection of the NGS panel (5% mutant allele frequency). One person with no variant identified in blood, but both an *NF2* variant and LOH identified in a single non-vestibular schwannoma, also had a *SMARCB1* variant and copy number gain of *LZTR1* in the same tumour that were also not visible in blood.

A second, anatomically distinct, tumour was available for testing from 53 people. Forty of these additional tumours had detectable LOH and 35 had a detectable P/LP variant. Of the tumours with identified variants, 31 had both inactivating *NF2* variants identified (30 had an inactivating variant in *NF2* and LOH of the trans allele, while one tumour had two SNVs and no LOH). For 17 of the additional tumours, the same *NF2* variant was identified in the second tumour as was seen in the first tumour, confirming a diagnosis of mosaic *NF2*-related schwannomatosis. None of these mosaic variants were visible in the germline DNA and were thus present at below the limit of detection of the NGS panel in blood. The proportions of second tumours with *NF2* variants identified are summarised in Figure 2b.

Five people with analysis of a second tumour had a germline *SMARCB1* variant and eight had a germline *LZTR1* variant. However, no mosaic or somatic *SMARCB1* or *LZTR1* variants were detected and the remaining 23 people with two tumours tested had no detectable germline variant. Overall, 17/40 (43%) people with no identified germline P/LP variant in blood, had low-level mosaic *NF2*-related schwannomatosis. This leaves 23/53 people who had two tumours available for testing and who had no detected germline variant and either non-identical somatic *NF2* variants (11/23), or no *NF2* variants (12/23) identified in the two tumours. Therefore, the addition of tumour analysis increased the detection rate of P/LP variants from 25% to 36%.

Unilateral vestibular schwannomas associated with an LZTR1 variant

Three of 19 people (16%) with a P/LP *LZTR1* variant had a unilateral VS. All three of these people had two tumours tested and all tumours had identifiable LOH of chromosome 22. One person had non-identical inactivating *NF2* variants in each tumour, one person had an inactivating *NF2* variant identified in only one of two tumours, and the third person had no inactivating *NF2* variants identified on the retained allele in either tumour. Therefore, mosaic *NF2* variants were not found for any of these patients.

Confirmation of splice variants

Two of the variants identified in the study were initially classified as VUS, but were re-classified following RNA analysis. These were a non-canonical *LZTR1* splice region variant, c.263+5G>T, found in the germline of one patient and an apparent *NF2* missense variant, c.1532A>G p.(Asp511Gly), identified in the tumour of a patient with no detected germline variant. Both variants were predicted to affect splicing using the SpliceAI [11] algorithm (spliceailookup.broadinstitute.org). RNA analysis of the *LZTR1* c.263+5G>T variant showed that it causes the insertion of 33 nucleotides of intron 2, resulting in the introduction of an in-frame termination codon immediately after the end of exon 2, i.e. p.Gly88GlyTer1 (Figure 3.). The somatic *NF2* c.1532A>G variant had been seen previously in the germline of a patient with *NF2*-related schwannomatosis and RNA analysis found it to cause an out-of-frame deletion of 43 nucleotides of exon 14, resulting in a frame-shift and the introduction of a premature stop codon, i.e. p.Asp511GlufsTer24 (supplementary figure 2.). Therefore, both of these variants result in truncated transcripts and are expected to undergo nonsense-mediated decay. Indeed, the aberrant transcript produced by the *LZTR1* c.263+5G>T variant, which was assessed in RNA from a PaxGene tube and which was therefore not treated with puromycin before extraction, was observed at a very low level, indicating that the transcript had degraded. The *LZTR1* c.263+5G>T variant has been observed three times in gnomAD v4.1.0 (gnomad.broadinstitute.org) [12], but the *NF2* c.1532A>G variant was not observed in the gnomAD cohort.

Discussion

Overall, our study of people with at least one non-vestibular schwannoma, who did not meet diagnostic criteria for *NF2*-related schwannomatosis at the time of genetic testing, identified similar proportions of people with a germline P/LP variant in *NF2*, *SMARCB1*, or *LZTR1*, although four of the people with a *SMARCB1* variant were from families represented by more than one family member. In addition, the majority of predisposing *NF2* variants were low-level mosaic variants that were not detected in blood, but were confirmed to be mosaic due to their presence in two anatomically distinct tumours. Only one person (1/154; 0.65%) not meeting criteria for *NF2*-related schwannomatosis had a non-mosaic *NF2* variant. However, this patient underwent genetic testing at a young age and subsequently developed bilateral vestibular schwannomas within two years of initial genetic testing.

Excluding the 13 people with two tumours tested who were known to have a germline P/LP variant in either *SMARCB1* or *LZTR1*, 17/40 (43%) had a mosaic *NF2* variant. In addition, three of 19 people with a germline P/LP *LZTR1* variant had a vestibular schwannoma, meaning that their diagnosis would be unclear without genetic testing. However, all three of these people also had two tumours available for analysis and none were found to have identical *NF2* variants in each tumour. These factors demonstrate the importance of genetic testing for schwannomatosis diagnosis and the testing of multiple tumours to increase diagnostic power.

Interestingly, one person with no germline variant detected and only one tumour available, had a multifocal schwannoma and three different single nucleotide P/LP *NF2* variants were identified in three samples taken from different regions of the same tumour. Another person with no variant identified in blood and only one non-vestibular schwannoma with genetic testing, had biallelic somatic *NF2* variants detected in tumour, as well as a somatic *SMARCB1* variant and somatic copy number gain of the whole of *LZTR1*. The lack of a second tumour for testing means that this person remains at risk of mosaicism for any of the three variants. Another person with an *LZTR1* VUS in blood and who had only one tumour available for analysis, had an *LZTR1* nonsense variant and an *NF2* frameshift deletion detected in tumour, but no LOH was detected. Therefore, only three of the potential four mutational events could be detected. No other *SMARCB1* or *LZTR1* variants were seen in tumours that were not also detected in blood.

For the people who had no P/LP variant identified, it is still possible that they have a very low-level mosaic variant that was not detected by standard clinical testing methods, but this would require a more sensitive technique to determine. Similarly, for people who only had one tumour tested, there remains a possibility that any of the variants that were detected in that tumour could be mosaic, but this would also need to be confirmed by a more sensitive method of detection or by testing of a second tumour if it becomes available.

Another issue that may hinder detection of additional variants is that many schwannoma DNA samples were isolated from formalin-fixed paraffin-embedded tissue blocks, which can produce poor quality DNA due to cross-linking during the fixing process. It is not normally possible to tell which of these samples is likely to provide acceptable quality DNA for testing and it has been shown that samples that have been stored for many years can still provide DNA that is of acceptable quality for genetic analysis[13]. However, it is known that differences in tissue fixing protocols can have a significant impact on DNA integrity[14]. In addition, the

tumour cell content of schwannoma samples can vary considerably and DNA from non-tumour cells can hinder the detection of somatic variants. This may be a particular issue for copy number variants, which have a lower sensitivity of detection. Prior estimation of tumour cell content and tissue macro dissection to increase the tumour cell content can improve variant detection[15].

In our current cohort, there was one person with a germline *NF2* variant of uncertain significance (missense) and 11 people with a germline *LZTR1* variant of uncertain significance (10 missense variants and one whole gene duplication). The high frequency of *LZTR1* loss-of-function variants and even higher frequency of missense variants in the general population, as well as the known incomplete penetrance of disease in *LZTR1*-related schwannomatosis families makes it particularly difficult to classify *LZTR1* variants as pathogenic[16]. This can hinder a definitive diagnosis in many cases. If further evidence becomes available that confirms more of the *LZTR1* missense variants to be definitively pathogenic, then the proportion of people diagnosed with *LZTR1*-associated schwannomatosis would be significantly increased in this cohort. This indicates the importance of comprehensive, sensitive testing and a robust variant pathogenicity classification system for genetic diagnosis. In addition, we acknowledge that there may have been a bias towards testing of schwannomas in people without an identified P/LP variant in blood. Thus, the detection frequency of P/LP variants for *LZTR1* in particular may be underestimated for a cohort of mainly sporadic schwannomatosis patients.

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Figure Legends

Figure 1. Pie chart indicating the proportions of pathogenic, likely pathogenic and variants of uncertain significance in *NF2*, *SMARCB1* or *LZTR1*, identified in people not meeting criteria for *NF2*-related schwannomatosis at the point of testing.

Figure 2. Pie charts indicating a) the proportions of 1st tumours or b) 2nd tumours, containing loss of heterozygosity and an *NF2* variant, loss of heterozygosity only, an *NF2* variant only or no identified *NF2* variant.

Figure 3. Results of splice-analysis using RNA extracted from a PaxGene blood tube from a patient with the *LZTR1* variant, c.263+5G>T. Chromatograms indicate low-level expression of an aberrant *LZTR1* transcript containing an insertion of 33 nucleotides of intron 2.

Supplementary table 1. Table of pathogenic or likely pathogenic *SMARCB1*, *LZTR1* and *NF2* variants identified as germline, predisposing variants in blood and/or tumour DNA.

Supplementary figure 1. Age distribution of the study cohort at the time of genetic testing.
Supplementary figure 2. Results of splice-analysis using RNA extracted from a puromycin-treated lymphoblastoid cell line of a patient with the *NF2* variant, c.1532A>G. Chromatograms indicate: a) the deletion of the last 43 nucleotides of exon 14 (p.Asp511GlufsTer240) and a low-level transcript including the deletion of the last 43 nucleotides of exon 14 combined with deletion of the whole of exon 15, and b) the control samples also contained a low-level expression of the deletion of exon 15 without the deletion of the last 43 nucleotides of exon 14.