Mutations in *LZTR1* add to the complex heterogeneity of schwannomatosis

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ABSTRACT

Objectives: We aimed to determine the proportion of individuals in our schwannomatosis cohort whose disease is associated with an *LZTR1* mutation.

Methods: We used exome sequencing, Sanger sequencing, and copy number analysis to screen 65 unrelated individuals with schwannomatosis who were negative for a germline *NF2* or *SMARCB1* mutation. We also screened samples from 39 patients with a unilateral vestibular schwannoma (UVS), plus at least one other schwannoma, but who did not have an identifiable germline or mosaic NF2 mutation.

Results: We identified germline *LZTR1* mutations in 6 of 16 patients (37.5%) with schwannomatosis who had at least one affected relative, 11 of 49 (22%) sporadic patients, and 2 of 39 patients with UVS in our cohort. Three germline mutation–positive patients in total had developed a UVS. Mosaicism was excluded in 3 patients without germline mutation in *NF2*, *SMARCB1*, or *LZTR1* by mutation screening in 2 tumors from each.

Conclusions: Our data confirm the relationship between mutations in *LZTR1* and schwannomatosis. They indicate that germline mutations in *LZTR1* confer an increased risk of vestibular schwannoma, providing further overlap with NF2, and that further causative genes for schwannomatosis remain to be identified. ***Neurology*® 2015;84:141–147**

GLOSSARY

LOH = loss of heterozygosity; ***LZTR1*** = leucine-zipper-like transcription regulator 1; **MLPA** = multiplex ligation-dependent probe amplification; **NF2** = neurofibromatosis type 2; **SIFT** = sorting intolerant from tolerant; ***SMARCB1*** = SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1; **SNP** = single nucleotide polymorphism; **UVS** = unilateral vestibular schwannoma.

Schwannomatosis is a member of the neurofibromatosis family of neurogenetic disorders, which predispose to benign tumors throughout the nervous system. Within this group, neurofibromatosis type 2 (NF2) and schwannomatosis share clinical overlap and can be difficult to distinguish, particularly in cases of mosaic disease.[1] The main tumor type seen in both NF2 and schwannomatosis is the schwannoma, although the location of these tumors differs somewhat between these 2 syndromes, with bilateral vestibular schwannomas being almost universal in patients with classic NF2,[2,3] and with both intradermal and nonintradermal schwannomas also frequently seen. In contrast, schwannomatosis-associated schwannomas tend to be nonintradermal and nonvestibular, although rare cases of unilateral vestibular schwannomas (UVS) have been observed.[4] Both conditions also lead to meningiomas, although there is a much higher incidence in NF2 (>50%) than in schwannomatosis

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(approximately 5%). In either disease, meningiomas may be the only tumors that develop.[5–7] Ependymomas and cataracts are also confined to individuals with NF2.[2,3]

Genetic characterization is an important tool for distinguishing between diseases, because germline mutation of the *NF2* gene is reported in more than 90% of individuals with nonmosaic NF2,[8] but is not present in patients with schwannomatosis, although somatically acquired *NF2* mutations are usually found in tumors.[9] Germline mutation of the SWI/SNF chromatin remodeling complex gene, *SMARCB1*, is responsible for approximately 20% of patients with schwannomatosis disease, with a much higher detection rate in familial patients (~50%) than sporadic patients (~10%),[10] indicating genetic heterogeneity. Recently, leucine-zipper-like transcription regulator 1 (*LZTR1*), which lies approximately 3 Mb centromeric to *SMARCB1* on chromosome 22, was identified as a second causative gene for schwannomatosis, with loss of function mutations identified in 80% of the study's *SMARCB1* mutation–negative schwannomatosis cohort, all of whom had somatic loss of chromosome 22 in their tumors.[11]

We investigated the frequency of *LZTR1* mutations in our own cohort of 16 families with multiple affected members with schwannomatosis and 49 individuals with sporadic schwannomatosis and no known germline *SMARCB1* or *NF2* mutations, with or without known somatic loss of chromosome 22 in their tumors, to determine the proportion of patients with genetically uncharacterized schwannomatosis whose disease is accounted for by this new gene.

METHODS

Patient material. We analyzed genomic DNA from peripheral lymphocytes from familial and sporadic patients meeting clinical diagnostic criteria for schwannomatosis,[12,13] who had previously tested negative for constitutional mutations in both *NF2* and *SMARCB1* genes using Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) probesets P258-B1 and P044 (MRC-Holland, Amsterdam, the Netherlands).[10] DNA extracted from paraffin-embedded or fresh-frozen tumors was used to confirm mutations and screen for second somatic mutations.

Standard protocol approvals, registrations, and patient consents. Ethical approval for use of anonymized samples from a historical retrospectively collected archive in this study was obtained from the North West 7–Greater Manchester Central Research Ethics Committee (reference 10/H1008/74). Written consent was obtained from prospectively collected individuals.

Exome sequencing. Whole-exome targeted enrichment and sequencing were performed on lymphocyte DNA extracted from 7 unrelated individuals with a family history of schwannomatosis. Enrichment was performed using the SureSelect Human All Exon Kit v.1 (Agilent, Santa Clara, CA) for the Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA). Sequence data were mapped to the human reference sequence hg19 (build GRCh37) with the Burrows-Wheeler aligner (BWA v0.6.2).[14] The genome analysis tool kit (GATK v2.4.7)[15] was used for base quality score recalibration and indel realignment before variant calling using the unifiedGenotyper. Single nucleotide polymorphisms (SNPs) with ≥5× coverage and indels were annotated to genes using Ensembl v68, and the functional consequences were defined. Between 85% and 98% of the targeted exome was covered at least 20× for each sample. Additional annotation was provided from OMIM and Genomic Evolutionary Rate Profiling (35 species alignment) as well as population frequencies from 1000 Genomes Project (phase 1 release), NHLBI Exome Sequencing Project (v6500), and our own in-house frequencies. PolyPhen-2 and sorting intolerant from tolerant (SIFT) predictions were also included to help determine pathogenicity. All candidate mutations were verified by Sanger sequencing.

Sanger sequencing. Primers were designed to amplify each of the 21 *LZTR1* exons, including all of the coding regions and approximately 50 to 100 bases of flanking intronic sequence per exon. Each fragment was amplified by PCR, using the GoTaq Green Master Mix (Promega, Southampton, UK). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, Danvers, MA). Sequencing was performed using BigDye Terminator v3·1 Cycle Sequencing Kit (ABI, Life Technologies, Foster City, CA). Sequencing PCR products were purified using Agencourt CleanSEQ beads (Beckman Coulter Genomics) and sequence analysis was performed using the ABI 3730*xl* DNA Analyzer (ABI, Life Technologies).

Design and application of an *LZTR1*-specific MLPA probeset. The genomic sequence of the 21-exon human *LZTR1* gene (NM_006767) was downloaded from the University of California Santa Cruz genome browser (www.genome.ucsc.edu). A total of 8 MLPA probes were placed such as to target exons 1, 3, 5, 7, 10, 15, 18, and 20. Probes were designed according to criteria provided by MRC-Holland at www.mlpa.com. Seven reference probes targeting physically distinct genomic regions were derived from previously established probesets (unpublished). Oligonucleotides for MLPA probes were from Biolegio (Nijmegen, the Netherlands). MLPA reactions utilized reagents from MRC-Holland, and products were visualized on a LICOR4200 (LICOR Biosciences, Lincoln, NE). Relative MLPA signals were calculated as described previously.[16]

RESULTS

We undertook exome sequencing analysis on lymphocyte DNA samples from 7 patients with familial schwannomatosis who had previously been found negative for germline *SMARCB1* and *NF2* mutations. We identified novel heterozygous loss of function mutations in the *LZTR1* gene in 3 of these patients (shown in table 1 and figure 1, A–C). The mutations were confirmed by Sanger sequencing and segregated with all affected family members available for testing (table 1). Of note, one clinically unaffected father in family 2 carried a pathogenic mutation

Table 1 *LZTR1* mutations identified in individuals with schwannoma disease

Patient	Exon	DNA alteration	Protein alteration	Mutation type	Tumor LOH	Clinical features (age, y)
Family 1a	6	c.570delT	p.(Phe190Leufs10)	Frameshift	Not tested	UVS (37), 3 PNS (23), 1 SpS (30)
Family 1b	6	c.570delT	p.(Phe190Leufs10)	Frameshift	Not tested	6 PNS (16), 1 SpS (37)
Family 2a	19	c.2284C>T	p.(Gln762*)	Nonsense	Not tested	2 PNS (20), 1 SpS
Family 2b	19	c.2284C>T	p.(Gln762*)	Nonsense	Not tested	>50 schwannomas (17)
Family 2c	19	c.2284C>T	p.(Gln762*)	Nonsense	Not tested	3 PNS (25)
Family 2d	19	c.2284C>T	p.(Gln762*)	Nonsense	Not tested	Asymptomatic
Family 3a	7	c.605T>G	p.(Met202Arg)	Missense	Yes	4 PNS (17), 3 SpS (44), 1 CNS (28)
Family 3b	7	c.605T>G	p.(Met202Arg)	Missense	Not tested	1 PNS (23)
Family 4	9	c.964G>T	p.(Glu322*)	Nonsense	Not tested	4 SpS (38)
Family 5a	14	c.1483dupG	p.(Glu495Glyfs*174)	Frameshift	Yes	2 SpS (53)
Family 5b	14	c.1483dupG	p.(Glu495Glyfs*174)	Frameshift	Not tested	1 PNS (25), 1 SpS (25)
Family 6	11	c.1175C>T	p.(Ala392Val)	Missense	Not tested	5 PNS (20)
Sporadic 1	5	c.401-2A>G	p.(?)	Splice-site	Not tested	1 PNS (60), 2 SpS (60)
Sporadic 2	5	c.509G>A	p.(Arg170Gln)	Missense	Not tested	4 PNS (19), 1 CNS (39)
Sporadic 3	5	c.509G>A	p.(Arg170Gln)	Missense	Yes	66 schwannomas
Sporadic 4	9	c.842delC	p.(Pro281Argfs*70)	Frameshift	Not tested	1 PNS (52), 2 SpS (51)
Sporadic 5	9	c.856G>A	p.(Gly286Arg)	Missense	Not tested	4 PNS (40), 2 SpS (51)
Sporadic 6	12	c.1353+1G>A	p.(?)	Splice-site	Yes	2 SpS (15)
Sporadic 7	14	c.1583T>G	p.(Leu528Arg)	Missense	Yes	2 PNS (26), 2 SpS (43)
Sporadic 8	16	c.1893delG	p.(Lys632Serfs*20)	Frameshift	Not tested	2 PNS (28), 2 SpS (28)
Sporadic 9	17	c.1961A>G	p.(Asp654Gly)	Missense	Yes	1 SpS (45), 1 CNS (45)
Sporadic 10	17	c.2002G>T	p.(Asp668Tyr)	Missense	Yes	1 PNS (15), 1 SpS (22)
Sporadic 11	17	c.2062C>T	p.(Arg688Cys)	Missense	Not tested	5 PNS (16)
UVS + 2	1	c.27delG	p.(Gln10Argfs*15)	Frameshift	Not tested	3 PNS (48), UVS (32)
UVS + 3	15	c.1785+2delT	p.(?)	Splice-site	Yes	9 PNS (15), 6 SpS (30), UVS (43)
Somatic mutation not present in the germline						
UVS + 4	14	c.1615G>T	p.Gly539Cys	Missense	No	2 PNS (21), UVS (31)

Abbreviations: CNS = cranial nerve schwannoma; LOH = loss of heterozygosity; PNS = peripheral nerve schwannoma; SpS = spinal nerve schwannoma; UVS = unilateral vestibular schwannoma.

(figure 1B). This may indicate nonpenetrance in this individual, although he has not undergone full-body MRI and may therefore harbor undetected tumors. Sanger sequencing of probands from 9 additional schwannomatosis families identified 3 further germline loss-of-function mutations (figure 1, D–F).

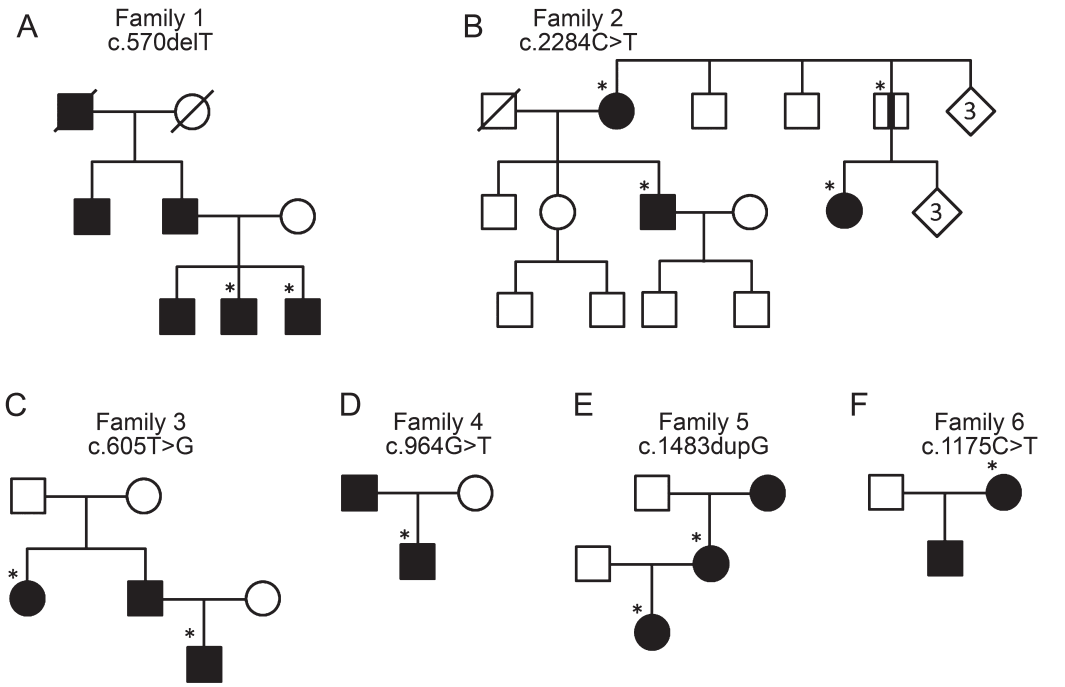
Tumor DNA for 2 familial patients, in whom no germline mutation had been detected, showed a reduced signal for known SNPs on one allele in comparison to heterozygous signals in germline DNA on sequencing chromatograms, suggestive of loss of heterozygosity (LOH).

We next sequenced *LZTR1* in 49 patients with sporadic schwannomatosis and identified 11 (22%) additional germline point mutations (listed in table 1). Matched tumor DNA was available for 4 of the mutation-positive patients and all showed evidence of loss of the wild-type allele (table 1).

To determine whether larger single- or multiexon deletions of *LZTR1* were present in the samples negative for point mutations, we developed an MLPA assay containing 8 probes spanning the *LZTR1* gene. Lymphocyte DNA of sufficient quantity and quality was available to perform MLPA analysis on 9 familial patients and 30 sporadic patients. The tumor from the familial patient with an exon 7 missense mutation and 4 tumors from mutation-negative sporadic patients were also tested.

No significant copy number changes were seen in any germline DNA samples. Loss of the wild-type allele was seen for the tumor from the patient with an exon 7 mutation. Three of the 4 tumors from sporadic patients with no detected germline mutation showed a reduced copy number of the entire *LZTR1* gene at levels indicating loss of one allele with some nontumor cell contamination in the sample. The remaining tumor

Figure 1 Pedigrees of *LZTR1* mutation-positive families



(A) Family 1; (B) family 2; (C) family 3; (D) family 4; (E) family 5; and (F) family 6. Asterisks indicate family members screened for the mutation. Black stripe indicates an asymptomatic mutation carrier.

showed no evidence of LOH. No point mutations were identified by Sanger sequencing in this tumor.

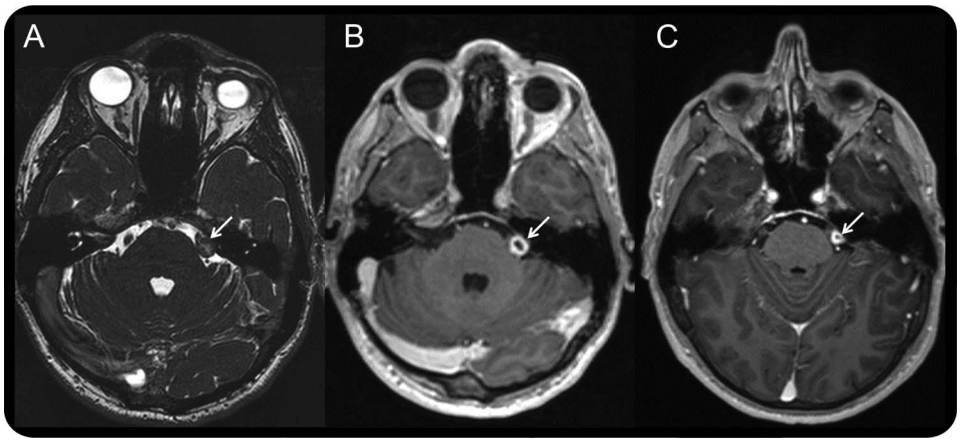
None of the mutations identified in our cohort were seen on dbSNP137 or on the ESP6500. The variant c.2062C>T, identified in one individual in our study, has been previously associated with schwannomatosis disease.[11] In silico analysis of all the missense mutations by PolyPhen2, SIFT, Align GVGD, and MutationTaster predicted 5 of 8 different mutations to be damaging to the protein by all 4 algorithms, 2 more were predicted to be damaging by 3 of 4 algorithms, and one was predicted to be damaging by 2 of 4 and likely to be damaging by a third (table e-1 on the *Neurology*® Web site at Neurology.org). This mutation was also found in 2 unrelated individuals in our cohort and was retained in the matched tumor, available for one of these individuals, in conjunction with loss of the wild-type allele, further suggesting that it is pathogenic. Evolutionary conservation of the affected amino acids (table e-1) shows that the missense mutations occurred at conserved residues.

These results determine an overall detection rate of *LZTR1* mutations in 6 of 16 (38%) familial patients and 11 of 49 (22%) sporadic patients in our cohort without germline *SMARCB1* mutations.

None of the *LZTR1* mutation carriers identified in the schwannomatosis cohort had meningiomas, ependymomas, intracutaneous schwannomas, or cataracts. Two patients had facial nerve schwannomas and one a lower cranial nerve schwannoma, which was initially thought to be a vestibular schwannoma. Schwannomas typically occurred at deep locations, including spinal nerve roots with pain as the predominant symptom.

One individual with an inherited *LZTR1* mutation (family 1, table 1) had a schwannoma removed at 37 years of age, which was clearly identified at surgery as emanating from the vestibular nerve during cochlear nerve–preserving surgery in Germany. Because of this result, we screened 39 additional lymphocyte samples from individuals with a UVS and at least one additional schwannoma, but who had no identifiable *NF2* mutation in blood or proven mosaic mutation in tumors. Tumors from 7 of these individuals were also sequenced. Most of these patients met Manchester criteria for NF2 with at least 2 nonvestibular schwannomas or meningiomas in addition to a UVS.[2] We detected germline mutations in 2 of these individuals and one somatic mutation, not present in blood, in a third individual (table 1). The exon 1 frameshift mutation, c.27delG, p.(Gln10Argfs*15), previously associated with schwannomatosis,[11] was identified in one of these individuals. This mutation has subsequently been added to the Exome Variant Server, seen in 59 of 11,848 alleles. It is difficult to surmise that this mutation would not lead to total loss of protein product by nonsense mediated decay, but a mechanism for reinitiation of RNA sequence has been described recently for *SMARCB1*.[17] Two of these last 3 individuals had a definite UVS whereas the third had a cerebellopontine angle tumor

Figure 2 Cerebellopontine angle tumor in an *LZTR1* mutation carrier



MRIs showing (A) contact image sensors image taken in 2006, (B) postcontrast image taken in 2006, and (C) postcontrast image taken in 2013 showing cystic degeneration around the tumor. White arrows indicate the location of the tumor.

identified on MRI with a location consistent with involvement of the eighth nerve (figure 2). In total, *LZTR1* mutations were identified in 3 of 39 individuals (8%) with a UVS and at least one other schwannoma, but without a germline *NF2* mutation.

The results of schwannoma tumor analysis from patients with schwannomatosis without known *NF2* mosaicism are shown in table 2. All 12 tumors from *SMARCB1* mutation carriers had the typical 22q loss including LOH for *NF2* and a point mutation. All 11 tumors from *LZTR1* carriers had LOH, but in 4 cases this was due to mitotic recombination. A substantial proportion of patients with typical *NF2* involvement in their tumors, including 4 with typical different *NF2* point mutations in different schwannomas, had no identifiable *LZTR1* or *SMARCB1* mutation. Three of these patients whose lymphocyte DNA showed no mutation of the *LZTR1*, *SMARCB1*, or *NF2* gene, had 2 tumors tested for somatic mutations. None of these patients had 2 identical point mutations in both tumors in any of these 3 genes.

DISCUSSION In contrast to the 80% of individuals identified with a germline *LZTR1* mutation in the initial disease gene discovery publication linking *LZTR1* to schwannomatosis,[11] we identified novel germline mutations in *LZTR1* in only 26% of our cohort of 65 patients with *SMARCB1* mutation–negative familial and sporadic schwannomatosis. Unlike the initial report, we did not confine our analysis to patients with proven involvement of the chromosome 22q locus. However, even in patients with 22q involvement in tumor, but no proven *NF2* or *SMARCB1* mosaicism, we found only 28.5% with germline *LZTR1* mutations. It is therefore likely that a further schwannomatosis gene exists on chromosome 22q.

Among the mutation-positive cases, we identified both truncating and nontruncating mutations across the length of *LZTR1* (8 missense, 3 splice-site, 2 nonsense, and 4 frameshift), consistent with the hypothesis that at least some of these mutations will produce hypomorphic protein products similar to the predicted effect of schwannomatosis-associated *SMARCB1* mutations.[18]

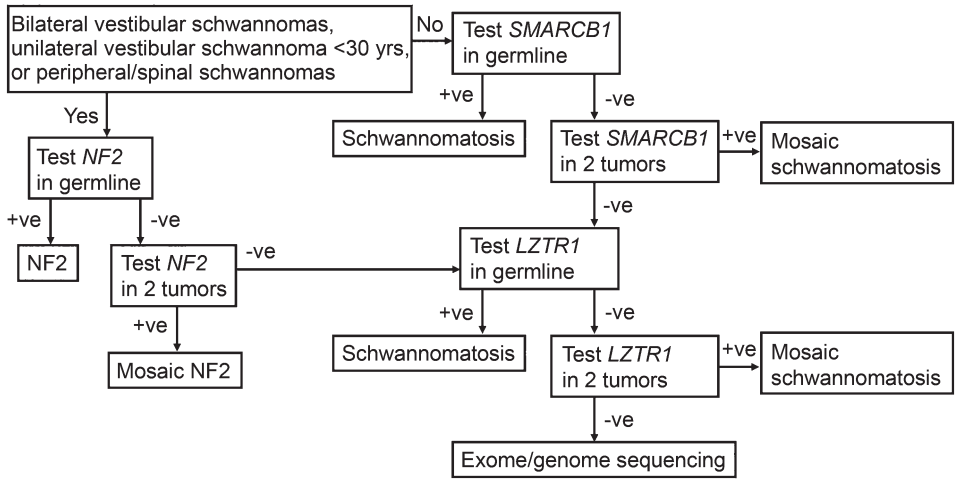
We identified an *LZTR1* mutation in 4 individuals with a UVS plus at least one other schwannoma (table 1): one diagnosed clinically to have familial schwannomatosis, one who met Manchester criteria

Table 2 *NF2* mutational hits in schwannomas from patients with a *SMARCB1* germline mutation, an *LZTR1* germline mutation, or no identified mutation who have been genetically determined not to have mosaic NF2

	No. of schwannomas	*NF2* point mutation or single-exon deletion	LOH	Mutational hit and LOH	Mitotic recombination
SMARCB1	12	12/12 (100)	12/12 (100)	12/12 (100)	0/12
LZTR1	11[a]	9/11 (82)	10/11 (91)	8/11 (73)	4/10 (40)
No germline mutation	29[a]	17/29 (58)	18/29 (62)	16/18 (89)	3/18 (17)

Abbreviation: LOH = loss of heterozygosity.
Data are n (%). Germline *LZTR1* mutations were found in 6 of 21 patients (28.5%) with typical *NF2* point mutations and LOH with no proven NF2 mosaicism or *SMARCB1* mutation.
[a] Two tumors analyzed from 4 patients with *LZTR1* mutations and 5 with schwannomatosis with no identifiable germline mutation.

Figure 3 Flow diagram for schwannomatosis mutation screening



The diagram indicates an ideal mutation screening strategy for schwannomatosis mutations, assuming availability of all samples.

for a diagnosis of NF2, and 2 with a clinically uncertain disease status because mutation analysis in *NF2* was negative in DNA extracted from lymphocytes. The identification of *LZTR1* mutations in these individuals supports our previous assertion that UVS can occur in the context of schwannomatosis,[4] and further suggests that *LZTR1* mutations may confer a greater risk of vestibular schwannoma than *SMARCB1* mutations, because there have been no reports of *SMARCB1* mutation–positive schwannomatosis patients with a proven vestibular schwannoma. Vestibular schwannomas are still considered exclusion criteria for schwannomatosis in published criteria,[12] although limiting this restriction to patients with bilateral tumors has been proposed.[19] In light of our results, a UVS should not be considered an exclusion criterion for schwannomatosis, and *LZTR1* mutation analysis should be considered in patients with a UVS and other painful schwannomas, who do not have other typical NF2 features, or a proven germline or mosaic *NF2* mutation.

The *LZTR1* gene encodes a member of the BTB-Kelch superfamily of proteins, exclusively localized in the Golgi network. The cellular function of *LZTR1* is not clear, although mutations in this gene have recently been implicated in the development of glioblastomas.[20] *LZTR1* lies on chromosome 22, approximately 3 megabases centromeric to *SMARCB1* and 9 megabases centromeric to *NF2*. Three patients whose lymphocyte DNA showed no mutation of the *LZTR1*, *SMARCB1*, or *NF2* gene had 2 tumors tested for somatic mutations (a fourth had no DNA left for *LZTR1* analysis). One of these patients has been reported previously to have LOH at the *NF2* locus in one tumor without a change in copy number, resulting from mitotic recombination and with a breakpoint occurring downstream of both *LZTR1* and *SMARCB1*.[21] None of these 3 patients had identical mutations in both tumors in any of the 3 genes. This shows that the schwannomas in these patients are not due to mosaic disease caused by any of the 3 genes and suggests that there are further causative genes still to be identified.

In the context of our analysis, we present a flow diagram of the ideal screening protocol for mutation analysis of schwannoma disease, assuming availability of relevant samples (figure 3). This will evolve as further genes are discovered and sequencing of panels of genes relevant to schwannoma disease become available and in time exome and/or genome sequencing become routine first-line diagnostics. Currently, we would recommend that next-generation sequencing panels for patients with NF2 without bilateral vestibular schwannoma and those with schwannomatosis should include the *NF2*, *SMARCB1*, and *LZTR1* genes for both blood and tumor analysis and that, where possible, 2 tumors should be analyzed from patients with no known family history.

AUTHOR CONTRIBUTIONS

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