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Expanding the mutational spectrum of *LZTR1* in schwannomatosis

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Schwannomatosis is characterized by the development of multiple non-vestibular, non-intradermal schwannomas. Constitutional inactivating variants in two genes, *SMARCB1* and, very recently, *LZTR1,* have been reported. We performed exome sequencing of 13 schwannomatosis patients from 11 families without *SMARCB1* deleterious variants. We identified four individuals with heterozygous loss-of-function variants in *LZTR1*. Sequencing of the germline of 60 additional patients identified 18 additional heterozygous variants in *LZTR1*. We identified *LZTR1* variants in 43% and 30% of familial (three of the seven families) and sporadic patients, respectively. In addition, we tested LZTR1 protein immunostaining in 22 tumors from nine unrelated patients with and without *LZTR1* deleterious variants. Tumors from individuals with *LZTR1* variants lost the protein expression in at least a subset of tumor cells, consistent with a tumor suppressor mechanism. In conclusion, our study demonstrates that molecular analysis of *LZTR1* may contribute to the molecular characterization of schwannomatosis patients, in addition to *NF2* mutational analysis and the detection of chromosome 22 losses in tumor tissue. It will be especially useful in differentiating schwannomatosis from mosaic Neurofibromatosis type 2 (NF2). However, the role of *LZTR1* in the pathogenesis of schwannomatosis needs further elucidation.

INTRODUCTION

Schwannomatosis (MIM 162091) is a tumor predisposition syndrome characterized by the development of multiple intracranial, spinal and peripheral schwannomas, without involvement of the vestibular nerve, which is pathognomonic of Neurofibromatosis type 2 (NF2; MIM 101000).[1,2] NF2 and schwannomatosis patients share common clinical features; however, germline *NF2* gene mutations are not identified in schwannomatosis patients. In 1996, schwannomatosis was recognized as a distinct clinical entity from NF2[3] by the molecular analysis of tumors from schwannomatosis patients: schwannomatosis-associated schwannomas frequently harbor inactivating variants of the *NF2* gene and loss of heterozygosity (LOH) of 22q, exclusively in tumors. Therefore, the hallmark of schwannomatosis is different somatic variants in multiple tumors from the same patient.[2]

Schwannomatosis is a genetic condition, but for poorly understood reasons its occurrence does not follow common inheritance patterns. The majority of cases are sporadic with 15–25% of cases being inherited from an affected parent.[4] The transmission risk can be assumed to be 50% in an individual with proven family history, but the risks for sporadic cases are less clear. In addition, non-penetrance has been described.[4]

In 2007, germline variants in the *SMARCB1* gene, located on 22q centromeric to *NF2*, were identified in schwannomatosis patients,

implicating it as the first predisposing gene for schwannomatosis.[5] However, genetic studies demonstrated that constitutional variants in this gene occur only in 40–50% of familial cases and in 8–10% of sporadic cases.[6–9] Very recently, germline variants in a novel gene, *LZTR1*, were identified in 100% of familial cases and in about 70% of sporadic patients.[10] *LZTR1* is also located on 22q, centromeric to *SMARCB1*.

In *SMARCB1*- and *LZTR1*-related schwannomatosis, tumorigenesis follows the 4-hits/3-step model: the germline mutated *SMARCB1* or *LZTR1* gene, as well as the somatic *NF2* variant, are retained in the tumor, whereas the other chromosome 22 (or at least a segment containing wild-type *SMARCB1*, *LZTR1* or *NF2*) is lost.[11]

To further assess the clinical indications for *LZTR1* molecular screening in medical genetics practice, we evaluated its involvement in the pathogenesis of a series of *SMARCB1*-unrelated schwannomatosis patients.

MATERIALS AND METHODS

Patients

Samples from schwannomatosis patients were collected at the Medical Genetics Unit of the University of Florence (Italy) and at the Department of Genome Analysis, Academic Medical Center, Amsterdam (the Netherlands). Clinical data of patients are shown in Supplementary Tables 1 and 2. Informed consent

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Received 17 April 2014; revised 21 August 2014; accepted 16 September 2014; published online 22 October 2014

was obtained from all subjects. The study was approved by the ethical committee of Careggi Hospital (protocol no. 0062368/2011).

Data of the study (variants and phenotypes) were submitted at the gene variant database (URL: http://lovd.nl/3.0/home) with patient IDs 17623–17625, 17628–17630 and 17632–17646.

Exome sequencing

DNA was extracted from blood and fresh tumor tissues, and sequence libraries were generated following standard Illumina (San Diego, CA, USA) protocols established in the UCLA Clinical Genomics Center for Clinical Exome Sequencing. The sample libraries were uniquely tagged and hybridized with capture oligonucleotides from the Agilent SureSelect 50 Mb All Exon capture kit to enrich for exons (Agilent, Santa Clara, CA, USA), and then sequenced on an Illumina HiSeq 2500.

Bioinformatic analysis

First, reads that pass Illumina quality filters were converted from qseq to fastq file format. Novoalign, using an index of the NCBI human genome reference build 37(HG37), produced a binary SAM (bam) file of aligned reads. PCR duplicates for all files were marked using Picard's MarkDuplicates command and ignored in downstream analysis.

Genome analysis toolkit (GATK) was used for variant calling.[12,13] Quality scores generated by the sequencer were recalibrated to more closely represent the actual probability of mismatching the reference genome by analyzing the covariation among reported quality score, position within the read, dinucleotide and probability of a reference mismatch. Then, local realignment around small insertion/deletions (indels) was again performed, this time using GATK's indel realigner to minimize the number of mismatching bases across all the reads. This removes alignment artifacts that can cause false-positive single-nucleotide variations (SNVs). Statistically significant non-reference variants, SNVs and indels, were identified using the GATK unified genotyper. Using the GATK variant annotator, each identified SNV and indel was annotated with various statistics, including allele balance, depth of coverage, strand balance and multiple quality metrics. These statistics were then used to identify likely false-positive SNPs using the GATK variant quality score recalibrator (VQSR). Variants with a VQSR score under a chosen threshold were assumed to be false and filtered out, leaving a set of likely true variants.

After the variants were identified, an in-house variant annotator based on ENSEMBL (ensembl.org) was used to additionally annotate each variant with information including dbSNP ID, gene names and accession numbers, variant consequences, protein positions and amino-acid changes, conservation scores, HapMap frequencies, tissue-specific expression, Polyphen and SIFT predictions on the effect of the variant on protein function and clinical association.[14] Novel (non-dbSNP) variants predicted to be protein damaging by Polyphen or SIFT were included for further analyses. Potentially causative variants were identified by filtering for damaging, heterozygous variants found in the affected patients and absent in unaffected family members.

High-resolution melting analysis

Blood DNAs were analyzed by high-resolution melting analysis (HRMA), performed on a Rotor Gene_6000 Instrument (Corbett Research, Sydney, Australia), followed by direct sequencing of amplicons showing an abnormal melting curve.

Sanger sequencing

All variants of interest, and the entire coding sequences of *LZTR1*, *SMARCB1* and *NF2*, were sequenced with PCR and capillary sequencing. All primers were designed using Primer3Plus (http://primer3plus.com/web_3.0.0/primer3web_input.htm) and ordered from MWG-Biotech AG (Ebersberg, Germany). Primer sequences are available on request. Capillary sequencing was performed on Biosystems 3100 or 310 Capillary DNA Analyzer (Life Technologies, Carlsbad, CA, USA). Raw and analyzed sequence results were visualized on Sequence Scanner v1.0 (Life Technologies).

Variants were named according to the reference sequences NM_006767.3 (*LZTR1*) and NM_181832.2 (*NF2*). *LZTR1* exons were numbered as in NG_034193.1.

Microsatellite analysis

LOH on 22q was investigated using microsatellites D22S420, D22S1174, D22S315, D22S1154, D22S1163, D22S280, D22S277 and D22S1169 from the ABI PRISM Linkage Mapping set version 2.5 (Life Technologies). PCR products were analyzed on a model 310 automated sequencer (Life Technologies); after electrophoresis, data were analyzed using GeneMapper software (Life Technologies).

Multiplex ligation probe amplification

Copy number changes (deletions or duplications) of *SMARCB1, NF2* and *LZTR1* loci and flanking genes were analyzed by Multiplex Ligation-Dependent Probe Amplification (MLPA) when fresh tumor tissues were available. *SMARCB1, NF2* and 22q11 MLPA test kits (MRC-Holland, Amsterdam, The Netherlands; P044_B1, P258_C1 and P324_A2) were used and electrophoresis data were analyzed using GeneMapper software (Life Technologies).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (3 μm thick) were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed with Dako (Glostrup, Denmark) target retrieval solution (pH 9) for 25 min at 98 °C. Slides were treated with 3% H_2O_2 in dH_2O for 10 min and then incubated in 10% normal goat serum for 30 min. Incubation with the primary antibody against LZTR1 (NBP1-77121, Novus Biologicals, Littleton, CO, USA) was carried out for 1 h at room temperature (dilution 1/25) and with the secondary antibody (Biotinylated goat anti-rat, BA-1000, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature (dilution 1/2000). Biotin was detected using the Vectastain Elite ABC kit (PK-6100, Vector Laboratories) following the manufacturer's recommendations. Slides were counterstained with hematoxylin, dehydrated and then mounted with xylene-based liquid mounting media.

In silico analysis

The following tools designed to provide splicing prediction were used: Human Splicing Finder (http://www.umd.be/HSF/),[15] splice site prediction by neural network (NNSplice; http://www.fruitfly.org/seq_tools/splice.html)[16] and Automated Splice Site and Exon Definition Analysis (ASSEDA) server (http://splice.uwo.ca).[17]

The predicted effects of missense variants on LZTR1 function were assessed using the following open access software: PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/),[18] SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html),[19] Mutation Taster (http://www.mutationtaster.org/)[20] and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).[21–23]

RESULTS

To identify additional determinants of schwannomatosis, we performed exome-sequencing analysis in 4 sporadic and 9 familial cases from 11 unrelated families with *SMARCB1*-unrelated schwannomatosis. Exome sequencing identified four deleterious variants of the *LZTR1* gene in four unrelated families that were verified by Sanger sequencing (Table 1). The first variant identified in two affected members of family 176 was a frameshift variant in exon 13, c.1373dupG, which introduces a stop codon after 210 amino acids and is predicted to result in nonsense-mediated decay (NMD) of the corresponding mRNA (Figure 1). The second variant, c.791+1G>A, identified in patient 3857, affected the donor splice site of intron 8. No RNA was available for further analysis; however, the disruption of the donor splice site of intron 8 is predicted to cause the skipping of exon 8 with a frameshift and the gain of a stop codon in exon 9. By direct sequencing, we found the same variant in her affected brother, confirming the segregation of the variant with the disease. The third variant, a frameshift alteration in exon 4, c.352dupC, predicted to cause NMD, was found in the sporadic patient from family 467 and in her unaffected father; moreover, the wild-type allele was lost in three of the four tumors analyzed. The fourth variant, a frameshift alteration in exon 14, c.1602delA, was confirmed in a patient from family 307

and his unaffected father, but not in his affected brother (Figure 1). The lack of segregation of the c.1602delA variant in one of the affected, and its presence in the healthy father, might argue against its pathogenic role; however, the mutated *LZTR1* allele was retained in the two available tumors of II:2 (Figure 1). To rule out the possibility of non-paternity and to determine which paternal chromosome 22 was inherited by the two siblings, we performed microsatellite analysis in the family. The six microsatellites tested (D22S420, D22S1174, D22S1154, D22S1163, D22S277 and D22S1170) ruled out non-paternity and clearly demonstrated that the siblings inherited the two different chromosomes from the father. In addition, the microsatellite analysis of the brothers' tumors demonstrated that in both cases the paternal chromosome was retained in the tumors (Figure 1).

To further assess the involvement of *LZTR1* in schwannomatosis, we screened all coding sequence and exon/intron boundaries of *LZTR1* in 60 additional *SMARCB1*-unrelated schwannomatosis cases. Previously unidentified deleterious variants in *LZTR1* were found in 18 of these cases. In total, 22 distinct *LZTR1* variants (Table 1) were identified in 24 of 74 subjects from 70 unrelated families (30% and 43% of sporadic and familial cases, respectively). In five sporadic cases, for which parental DNA was available, the *LZTR1* variant was inherited from an unaffected parent.

The *LZTR1* variants found were nonsense or frameshift predicted to cause NMD in nine patients. In one patient (381), we found a frameshift variant that led to a new stop codon in the 3′-untranslated open reading frame, predicting an elongated protein. In patient 318 we identified an in-frame deletion of three nucleotides, predicted to cause deletion of valine 124 in the LZTR1 protein, which would disrupt the Kelch domain. Seven different missense variants were found in *LZTR1;* they involve amino acids highly conserved across species (Supplementary Table 3) and predicted to be deleterious by MutationTaster,[20] PolyPhen 2 (ref. 18) and SIFT.[19] Four of these missense variants involve amino acids (His71, Leu187, Arg284 and Met400) likely to disrupt the Kelch domain; the missense variants involving Ala465, Arg697 and Cys760 are likely to disrupt the first, the second and the last BTB domains, respectively.

To evaluate the presence of mosaicism in patients without *LZTR1* variants, we sequenced *LZTR1* in five tumors from three unrelated cases, but all were wild type.

We then checked loss on 22q by microsatellite and MLPA analyses (Supplementary Figure 1). LOH/MLPA analyses showed losses of 22q involving the *LZTR1* locus in 14 of 28 tumors from 11 unrelated patients. D22S420 was not informative in tumors from four patients; in each of these cases, sequencing analysis showed that the mutant *LZTR1* allele was retained in the tumors with reduction of the wild-type peak height, suggesting loss of the wild-type allele.

Table 1 Germline *LZTR1* variants in schwannomatosis families

Family	Sample	Inheritance	Affected	*LZTR1*[a] Variant	Effect
3701		De novo	Yes	c.212A>G	p.(His71Arg)
422	I:2		No	c.243T>G	p.(Tyr81*)
	II:1	De novo	Yes	c.243T>G	p.(Tyr81*)
467[b]	I:1		No	c.352dupC	p.(Arg118Profs*28)
	II:1	De novo	Yes	c.352dupC	p.(Arg118Profs*28)
318		De novo	Yes	c.373_375delGTC	p.(Val125del)
302		De novo	Yes	c.513delG	p.(Leu171Phefs*29)
268		De novo	Yes	c.555_556dupCA	p.(Lys186Thrfs*15)
244		De novo	Yes	c.560T>G	p.(Leu187Arg)
288		De novo	Yes	c.628C>T	p.(Arg210*)
3857[b]	II:1	Familial	Yes	c.791+1G>A	r.spl?
	II:2	Familial	Yes	c.791+1G>A	r.spl?
388		De novo	Yes	c.850C>T	p.(Arg284Cys)
325		De novo	Yes	c.1018C>T	p.(Arg340*)
476		De novo	Yes	c.1199T>G	p.(Met400Arg)
176[b]	I:1	De novo	Yes	c.1373dupG	p.(His459Profs*210)
	II:1	Familial	Yes	c.1373dupG	p.(His459Profs*210)
183		De novo	Yes	c.1394C>A	p.(Ala465Glu)
389		De novo	Yes	c.1486delG	p.(Ala496Profs*60)
307[b]	I:1		No	c.1602delA	p.(Lys534Asnfs*22)
	II:1	Familial	Yes	Wild type	
	II:2	Familial	Yes	c.1602delA	p.(Lys534Asnfs*22)
174		De novo	Yes	c.1779delA	p.(Gln593Hisfs*7)
148	I:1		No	c.1807delG	p.(Val603*)
	II:1	De novo	Yes	c.1807delG	p.(Val603*)
504		De novo	Yes	c.2089C>T	p.(Arg697Trp)
408	I:1		No	c.2278T>C	p.(Cys760Arg)
	II:1	De novo	Yes	c.2278T>C	p.(Cys760Arg)
259		De novo	Yes	c.2284C>T	p.(Gln762*)
381		De novo	Yes	c.2487dupA	p.(Asp830Argfs*21)

[a]Reference *LZTR1* sequence: NM_006767.3. Nucleotide numbering starts with the A of the ATG translation initiation codon as +1.
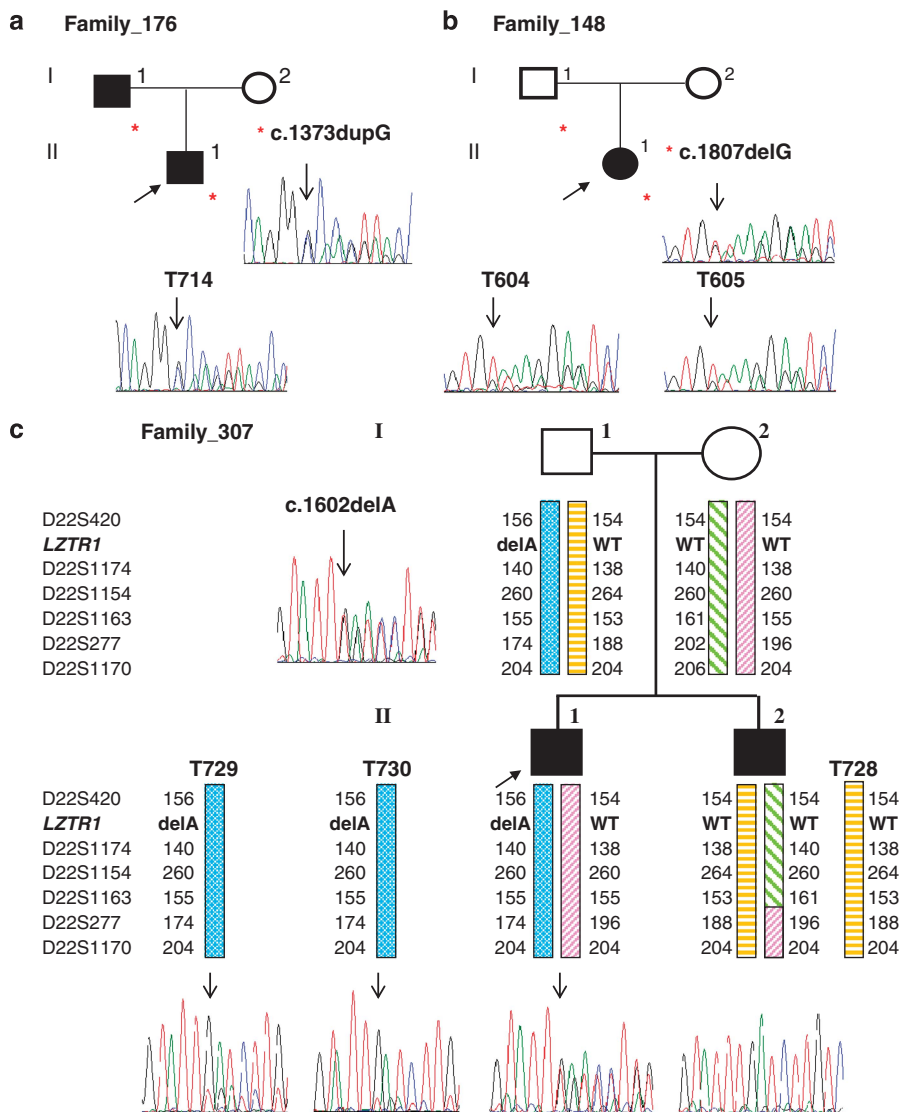[b]Variants identified by exome sequencing.

Figure 1 Sequence analysis of *LZTR1* in blood and tumors of mutated patients. Asterisks indicate subjects in the family who carry the variant. (**a**) The frameshift variant in exon 13 of *LZTR1* was detected in the genomic DNA from the proband (arrow) and his affected father. The variant was present in the proband's schwannoma T714, which retained both alleles. (**b**) The frameshift variant in exon 16 of *LZTR1* is present in the proband and her unaffected father and is retained in two schwannomas. (**c**) A frameshift variant in exon 14, c.1602delA (p.Lys534Asnfs*22), was identified in the proband from family 307 and his unaffected father, but not in the affected brother. The altered *LZTR1* allele was retained in two tumors of the brother. Haplotype analysis for chromosome 22 markers in the family clearly indicated that the affected siblings inherited the two different chromosomes 22 from their father.

In Supplementary Table 4, we summarize the somatic *NF2* variants in schwannomatosis-associated schwannomas from patients with germinal *LZTR1* variants.

Finally, we tested LZTR1 protein immunostaining in 22 tumors from nine unrelated patients with or without *LZTR1* deleterious variants (Supplementary Figure 2). We used seven vestibular schwannomas from seven unrelated NF2 patients as positive controls. Compared with NF2-associated schwannomas, tumors from patients with variants in *LZTR1* exhibited absent or reduced immunostaining. In contrast, tumors from *LZTR1*-unrelated schwannomatosis patients showed diffuse positive immunostaining, comparable with the NF2-associated tumors. Tumors from patients with nonsense or frameshift *LZTR1* variants had almost complete negative immunostaining, whereas patients carrying splicing or missense variants showed reduced immunostaining compared with the NF2-associated schwannomas.

DISCUSSION

Currently, germline deleterious variants in two tumor suppressor genes, *SMARCB1* and *LZTR1*, are involved in schwannomatosis predisposition.[5,10] In our study, we report our experience with *LZTR1* gene mutational screening in a large cohort of *SMARCB1*-negative schwannomatosis patients. We found 24 patients, from 22 unrelated families, carrying germline variants of *LZTR1*. Unlike glioblastomas, where somatic variants are preferentially located in the Kelch domains of the LZTR1 protein,[24] our data confirm that, in schwannomatosis patients, germline *LZTR1* variants occur along the entire sequence of the gene. However, we identified *LZTR1* variants in a lower proportion of patients compared with the earlier report:[10] 43% *versus* 100% and 30% *versus* 73% of familial and sporadic patients, respectively. One explanation might be our mutation-detection strategy, as we used HRMA to screen the entire coding sequence of the gene instead of direct sequencing; however, HRMA has an overall sensitivity of 99.3%

to detect heterozygous variants[25] and, in our experience, it is more sensitive than direct sequencing in detecting *NF2* mosaic alterations.[26] Another explanation might be the patient selection criteria. Piotrowski *et al*[10] only included patients with a molecularly confirmed diagnosis of schwannomatosis by the finding of different *NF2* variants in multiple tumors of the patients. In contrast, we analyzed patients with a confirmed clinical diagnosis according to the current schwannomatosis diagnostic criteria,[4] and we performed molecular diagnosis on multiple schwannomas only in 15 patients (1 familial and 14 sporadic). If we focus on the 14 sporadic patients with a confirmed diagnosis of tumors, we found variants in 10 of them (71%), a result comparable to that of the previous report.[10] Our lower detection rate in the entire cohort of our patients may be because some might actually be mosaic NF2, and, consequently, the diagnostic criteria for schwannomatosis should be reevaluated in the light of these new discoveries.

Our work confirms that *LZTR1* is the most prevalent gene causing schwannomatosis. However, a fraction of schwannomatosis cases, mainly the sporadic ones, remain genetically unsolved. For these unexplained cases we cannot exclude the presence of unrecognized large deletions or duplications of the *LZTR1* gene that are not detected by Sanger sequencing; however, genomic rearrangements are usually rarer than point variants. To explain the *LZTR1*- and *SMARCB1*-negative patients, Piotrowski *et al*[10] hypothesized the presence of somatic mosaicism for *SMARCB1* or *LZTR1* variants. However, we demonstrated the absence of mosaicism in at least three *SMARCB1*- and *LZTR1*-negative patients by the analysis of multiple tumors of the patients, which did not carry alterations in *SMARCB1* or *LZTR1* but showed different somatic *NF2* variants. Furthermore, multiple tumors from two of these patients showed positive LZTR1 immunostaining, suggesting the lack of involvement of *LZTR1* in schwannoma development. Considering that we did not find *SMARCB1* or *LZTR1* deleterious variants in six familial schwannomatosis patients analyzed, our findings suggest the involvement of an as yet unidentified schwannomatosis gene(s) in a subset of cases.

The mutational analysis of *LZTR1* in sporadic schwannomatosis patients reveals some inconsistences. First, variants were inherited from an unaffected parent in nine sporadic patients (from this and Piotrowski *et al*[10] reports) and there were no additional affected members in their families. Incomplete penetrance might be one explanation, but the lack of families with multiple affected members prevents the verification of this hypothesis.

In addition, we found a family (307) with two affected siblings; only one of them carried the *LZTR1* deleterious variant that was inherited by the unaffected father, whereas the other sibling had wild-type *LZTR1* and *SMARCB1* sequences; furthermore, the two affected brothers inherited two different chromosomes 22 from the father. This suggests that more than one gene could be involved in the same schwannomatosis family.

Currently, there is no evidence regarding protein interactions between LZTR1 and SMARCB1, nor that they are part of a common signaling pathway. *LZTR1* encodes a member of the BTB-kelch superfamily; the protein localizes exclusively to the Golgi network where it may help stabilize the Golgi complex.[27] Recent studies also suggest that LZTR1 is an adaptor in CUL3 ubiquitin ligase complexes.[24] The tumor suppressor *SMARCB1* is a core component of the SWI/SNF chromatin remodeling complex;[28] the complex is involved in nucleosome mobilization and makes compacted DNA accessible to transcription factors and repair enzymes. In the future, schwannomatosis research will be focused on understanding how proteins with so dissimilar functions could cause the same disorder.

In conclusion, our study demonstrated that molecular analysis of the *LZTR1* gene in clinical genetics practice may contribute to molecular characterization of schwannomatosis patients, especially in distinguishing schwannomatosis with mosaic NF2. However, the role of *LZTR1* in the pathogenesis of schwannomatosis needs to be further clarified. In addition, the assessment of genomic DNA from *LZTR1*- and *SMARCB1*-negative schwannomatosis patients and their tumors should also be carried out to discover other genes contributing to the pathogenesis of this disorder.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)