Phenotypic and genotypic overlap between mosaic NF2 and schwannomatosis in patients with multiple non-intradermal schwannomas

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Abstract
Schwannomatosis and neurofibromatosis type 2 (NF2) are both characterized by the development of multiple schwannomas but represent different genetic entities. Whereas NF2 is caused by mutations of the *NF2* gene, schwannomatosis is associated with germline mutations of *SMARCB1* or *LZTR1*. Here, we studied 15 sporadic patients with multiple non-intradermal schwannomas, but lacking vestibular schwannomas and ophthalmological abnormalities, who fulfilled the clinical diagnostic criteria for schwannomatosis. None of them harboured germline *NF2* or *SMARCB1* mutations as determined by the analysis of blood samples but seven had germline *LZTR1* variants predicted to be pathogenic. At least two independent schwannomas from each patient were subjected to *NF2* mutation testing. In five of the 15 patients, identical somatic *NF2* mutations were identified (33%). If only those patients without germline *LZTR1* variants are considered (n=8), three of them (37.5%) had mosaic NF2 as concluded from identical *NF2* mutations identified in independent schwannomas from the same patient. These findings imply that a sizeable proportion of patients who fulfil the diagnostic criteria for schwannomatosis, are actually examples of mosaic NF2. Hence, the molecular characterization of tumours in patients with a clinical diagnosis of schwannomatosis is very important. Remarkably, two of the patients with germline *LZTR1* variants also had identical *NF2* mutations in independent schwannomas from each patient which renders differential diagnosis of *LZTR1*-associated schwannomatosis versus mosaic NF2 in these patients very difficult.

Introduction

Schwannomatosis and neurofibromatosis type 2 (NF2) are tumour suppressor syndromes that follow an autosomal dominant pattern of inheritance and predispose affected individuals to multiple schwannomas. Nevertheless, both disorders have been considered to be separate entities. Whereas NF2 is caused by mutations of the *NF2* gene on chromosome 22q12.2 (Rouleau et al. 1993; Trofatter et al. 1993), patients with schwannomatosis lack germline *NF2* mutations (Jacoby et al. 1997; MacCollin et al. 2003). Instead, schwannomatosis patients have been shown to harbour germline mutations within *SMARCB1* or *LZTR1*, both of which are also located on 22q (Hulsebos et al. 2007; Boyd et al. 2008; Hadfield et al. 2008; Sestini et al. 2008; Rousseau et al. 2011; Smith et al. 2012, 2015; Hutter et al. 2014; Piotrowski et al. 2014; Paganini et al. 2015; Asai et al. 2015). However, 50-60% of sporadic patients with schwannomatosis and 15-30% of those patients with a family history of schwannomatosis, do not harbour identifiable germline mutations within either *SMARCB1* or *LZTR1* (reviewed by Kehrer-Sawatzki et al. 2017). Hence, further schwannomatosis-causing genes may well exist but as yet they have not been identified. Even though patients with schwannomatosis lack germline *NF2* mutations, the tumours of these patients frequently show somatic, tumour-specific *NF2* mutations and the loss of the second *NF2* allele (Boyd et al. 2008; Sestini et al. 2008; Hadfield et al. 2008, 2010; Piotrowski et al. 2014; Paganini et al. 2015; Smith et al. 2017). According to the 4-hit/3-step model of tumorigenesis, in schwannomas from patients harbouring *LZTR1* (or *SMARCB1*) germline mutations, the chromosome 22 is retained that harbours the germline *LZTR1* mutation and an *NF2* allele with a somatically acquired tumour-specific mutation. The other copy of chromosome 22 is lost or partially deleted, including the wildtype alleles of *LZTR1* (or *SMARCB1*) and *NF2* (Figure 1). These observations indicate that *NF2* inactivation is important for schwannoma growth in patients with NF2 as well as in patients with schwannomatosis.

In terms of their clinical manifestations, overlap is apparent between schwannomatosis and NF2 (Supp. Tables S1 and S2). Peripheral nerve schwannomas are common in both disorders, although more prevalent in schwannomatosis than in NF2. Non-vestibular intracranial schwannomas are also associated with both disorders but occur less commonly in schwannomatosis as compared with NF2 (reviewed by Kehrer-Sawatzki et al. 2017). By contrast, bilateral vestibular schwannomas in patients younger than 70 are pathognomonic for NF2 and have never been observed in patients with schwannomatosis as yet. Unilateral vestibular schwannomas (UVS) are common in patients with NF2 and approximately 40% of patients with UVS and NF2 develop a contralateral tumour later in life (Smith et al. 2017). Patients with UVS and two non-intradermal schwannomas fulfil the clinical diagnostic criteria for NF2 (Supp. Table S2). Smith et al. (2017) showed that among 50 patients with UVS and two non-intradermal schwannomas who did not develop a contralateral vestibular schwannoma, nine (18%) had NF2 due to a germline *NF2* mutation whilst three (6%) of the 50 patients had mosaic NF2. Remarkably, five (10%) of the 50 patients had germline *LZTR1* mutations (Smith et al. 2017). Indeed, other studies confirmed that UVS in combination with two or more intradermal schwannomas are observed in patients with schwannomatosis and *LZTR1* germline mutations (Mehta et al. 2016; Gripp et al. 2017). Even though UVS is much less common in the context of schwannomatosis than in NF2, these findings are clearly indicative of a degree of clinical overlap between schwannomatosis and NF2. However, the extent of this overlap between these disorders, in particular between schwannomatosis and mosaic NF2, has not yet been fully investigated. Patients with multiple non-intradermal schwannomas in the absence of bilateral vestibular schwannoma may be affected either by

schwannomatosis or by mosaic NF2. Somatic mosaicism in NF2 is not uncommon since it has been detected in 33% of sporadic NF2 cases with bilateral vestibular schwannomas (BVS) and in up to 60% of patients with UVS (Moyhuddin et al. 2003; Evans et al. 2007; Halliday et al. 2017). Patients with mosaic NF2 often present with a mild clinical phenotype (Halliday et al. 2017). In patients with mosaic NF2, the 'first-hit' *NF2* gene mutation may be present at a low level in blood cells or is only detected in tumour tissue but not in blood cells (Evans et al. 1998a, 2007; Kluwe and Mautner, 1998; Kluwe et al. 2003; Paganini et al. 2014; Spyra et al. 2015; Smith et al. 2017). To distinguish between schwannomatosis and mosaic NF2 confined to tumour tissue and not detectable in blood, *NF2* mutation testing is recommended to be performed in more than one tumour from a given patient. If an identical *NF2* mutation (first-hit) is observed in two independent schwannomas from a given patient in addition to tumour-specific (second-hit) inactivation of the other *NF2* allele, mosaic NF2 is assumed to be the underlying cause of the disease. Indeed, by means of this approach, Castellanos et al. (2015) identified mosaic NF2 in a patient considered to have segmental schwannomatosis but without either an *LZTR1* or a *SMARCB1* germline mutation.

The aim of the study presented here was to investigate the overlap between mosaic NF2 and schwannomatosis in patients without vestibular schwannomas but with spinal and peripheral nerve schwannomas. To this end, we analysed 15 patients who fulfilled the clinical diagnostic criteria for schwannomatosis and in whom two locally distinct, independent schwannomas could be analysed for the presence of an identical *NF2* gene mutation, that would imply mosaic NF2. All 15 patients were negative for germline mutations in *NF2* and *SMARCB1* but seven of them exhibited germline *LZTR1* mutations. Remarkably, five (33%) of the 15 patients initially diagnosed with schwannomatosis harboured identical *NF2* mutations in independent schwannomas indicative of a considerable overlap between mosaic NF2 and schwannomatosis.

Patients and methods

Fifteen sporadic patients who fulfilled the current clinical diagnostic criteria for schwannomatosis but not NF2 were analysed. The current diagnostic criteria for NF2 and schwannomatosis are listed in Supp. Tables S1 and S2. None of the 15 patients had unilateral vestibular schwannomas as determined by MRI and none of them had ocular anomalies such as subcapsular cataracts or retinal hamartomas as determined by ophthalmological investigation. The patients provided written informed consent and the study was approved by the ethics committee of the Ärztekammer Hamburg.

Mutation analysis

The presence of germline mutations of *SMARCB1*, *LZTR1* and *NF2* in these patients was investigated by Sanger sequencing using DNA isolated from blood samples. DNA derived from at least two different schwannomas from each of the 15 patients was subjected to *NF2* mutation testing by Sanger sequencing. The tumour DNA samples were also analysed by multiplex ligation-dependent probe amplification (MLPA) to detect loss of heterozygosity (LOH) of the *NF2* gene using the SALSA MLPA P044 NF2 probemix (MRC Holland, The Netherlands). The *LZTR1* variants identified in the blood of patients 4075 and 3617 were also detected in the schwannomas of these patients by means of PCR and sequence analysis using the primers listed in Supp. Table S3. The SALSA MLPA probemix P455-A1 *LZTR1* ((MRC Holland, The Netherlands) was used to analyse the tumours of patients 4075 and 3617.

Evaluation of variants detected

The allele frequencies of the *LZTR1* variants identified were extracted from the Exome Aggregation Consortium and the genome Aggregation Database (ExAC/gnomAD) (Lek et al. 2016). The latter includes all variants identified in 126,216 exomes and 15,136 whole-genome sequences analysed as part of various disease-specific and population genetic studies. Individuals known to be affected by severe pediatric disease and their first-degree relatives were removed from the database. According to Lek et al. (2016), most ExAC individuals were ascertained for biomedically important disease; however, the inclusion of both cases and controls for several known polygenic disorders indicates that the database is likely to contain some disease-associated variants. It nevertheless represents the most comprehensive database available for the assessment of variant frequencies. Allele frequencies were also evaluated by the analysis of the NHLBI Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS).

Combined Annotation Dependent Depletion (CADD) analysis was performed in order to assess the potentially deleterious character of missense variants (http://cadd.gs.washington.edu/score) (Kircher et al. 2014). The likely impact of missense variants was separately evaluated by means of PolyPhen-2 (Adzhubei et al. 2010), SIFT (Ng and Henikoff, 2003) and MutationTaster2 (Schwarz et al. 2014). Intronic splice site variants were evaluated by means of the Human Splicing Finder (HSF 3.1) tool which combines 12 different algorithms to identify and predict a given mutations' effect on splicing-relevant motifs (Desmet et al. 2009).

Results

Germline *SMARCB1* or *NF2* gene mutations were not detected in the blood of any of the 15 patients analysed, who fulfilled the clinical diagnostic criteria for schwannomatosis, and from whom two different schwannomas could be analysed in order to investigate the occurrence of identical *NF2* mutations.

Germline *LZTR1* mutations

Seven of the 15 patients exhibited germline *LZTR1* variants (Figure 2). Five of the seven variants were protein truncating (Table 1). In the following, the features of two of these germline *LZTR1* variants are presented in greater detail since the patients harbouring these variants also exhibited identical *NF2* mutations in independent schwannomas and hence the nature of these germline *LZTR1* variants are of particular interest. Importantly, the *LZTR1* variants in patients 3617 and 4075 were detected in blood and in the tumour samples of the patients implying that these variants were constitutional (Supp. Figures S1 and S2).

LZTR1 missense variant c.1792T>C in patient 3617

The *LZTR1* missense variant c.1792T>C (p.Cys598Arg) was detected in the blood of patient 3617. This missense variant was predicted to be 'probably damaging' with a score of 1.00 according to PolyPhen-2, as 'deleterious' by SIFT (Score: 0.02) and as 'disease-causing' by MutationTaster2 (score: 0.999). The *LZTR1* variant c.1792T>C has a combined annotation-dependent depletion (CADD) score of 27.1 (Kircher et al. 2014) indicating that the missense variant is among the top 1% of variants in the human genome in terms of its likelihood of being deleterious. The *LZTR1* variant c.1792T>C identified in patient 3617 is not listed in the gnomAD database. Nor has it been reported as a variant by the NHLBI Exome Sequencing Project (ESP) or in previously studied patients with schwannomatosis or Noonan syndrome

(Yamamoto et al. 2015; Johnston et al. 2017). The gnomAD database does however report a missense variant, c.1793G>A (p.Cys598Tyr), which affects the same amino acid as the variant observed in patient 3617, namely the cysteine at position 598 of the LZTR1 protein. This variant (c.1793G>A) is very rare since it has only been observed once among 245,892 alleles investigated.

LZTR1 truncating variant c.628C>T in patient 4075

The protein truncating *LZTR1* variant identified in patient 4075 (c.628C>T; p.R210*) has been reported previously in a patient with schwannomatosis (Paganini et al. 2015) and in the father of four children with autosomal recessive Noonan syndrome (Johnston et al. 2017). The father has been investigated by contrast spinal MRI and schwannomas or meningiomas were not identified. However, non-contrast whole body MRI identified a possible schwannoma at the base of the neck/upper chest region, near the brachial plexus (Johnston et al. 2017). *LZTR1* variant c.628C>T; p.R210* has an allele frequency of 6.88e-5 (observed 19-times among 276,182 alleles investigated) according to the gnomAD database (Table 1). This CG>TG transition may result from recurrent mutation as a consequence of methylation-mediated deamination of 5mC.

NF2 mutation analysis of schwannomas

Remarkably, patient 4075 with the protein truncating *LZTR1* variant c.628C>T (p.R210*) exhibited an identical *NF2* mutation (c.586delC; p.R196Efs*13) in two independent schwannomas (Table 1). One of these schwannomas was located in the abdominal wall around the navel; it was well circumscribed and 2-3cm in diameter. The other tumour was an intraspinal, intradural schwannoma in the region of the seventh and eighth thoracic vertebrae. The tumours were clearly independent and not part of a single internal tumorous mass.
This proved not to be an isolated case since patient 3617, with the *LZTR1* missense variant c.1792T>C (p.Cys598Arg), also had an identical *NF2* mutation (c.169C>T; p.R57*) in three independent schwannomas which derived from different anatomical locations. One schwannoma was an intraspinal tumour, and the other two were located in the plexus brachialis and the elbow, respectively (Table 3). The truncating *NF2* mutation p.R57* has been identified in many previous studies as a recurrent *NF2* germline variant (Bourn et al. 1994; MacCollin et al. 1994; Evans et al. 1998b; Kluwe et al. 1996; Parry et al. 1996; Baser et al. 2006; Piotrowski et al. 2014) and as a somatic mutation in the blood of a patient with mosaic NF2, as a tumour-specific variant in NF2 patients (Irving et al. 1994; Moyhuddin et al. 2002; Piotrowski et al. 2014) and in a sporadic UVS (Lee et al. 2012). Hence, this CG>TG transition is likely to result from methylation-mediated deamination of 5mC leading to recurrent mutation.
In contrast to these two patients, the other five patients with germline *LZTR1* mutations exhibited different *NF2* mutations (or no detectable *NF2* mutation) in at least two schwannomas of each patient.
In five of the eight patients without germline *LZTR1*, *SMARCB1* and *NF2* mutation, the analysis of at least two independent tumours did not indicate an identical *NF2* mutation rendering mosaic NF2 in these patients unlikely. However, in three of the eight patients without germline *LZTR1*, *SMARCB1* and *NF2* mutation, an identical *NF2* mutation was identified in independent tumours of each patient indicating that these patients had mosaic NF2 instead of schwannomatosis (Table 2).

Taken together, *NF2* mutation analysis of at least two independent tumours from each patient suggested mosaic NF2 in five (33%) of the 15 patients investigated. The clinical features observed in these five patients and tumour locations are summarized in Table 3. Mosaic NF2 could be unambiguously diagnosed in three of the eight patients, who did not exhibit *LZTR1* germline variants (Table 2). By contrast, a clear and unambiguous distinction between mosaic NF2 and *LZTR1*-associated schwannomatosis is difficult to make in the remaining two patients (3617 and 4075), since they harboured germline *LZTR1* variants that were predicted to be pathogenic in addition to identical somatic *NF2* mutations identified in independent schwannomas (Figure 2).

Analysis of the 4-hit/3-step model of tumorigenesis in schwannomas of patients 3617 and 4075

The 4-hit/3-step model of tumorigenesis in patients with germline *LZTR1* mutations implies that the *LZTR1* germline mutation (first hit) is retained in the schwannoma and the wildtype allele is inactivated by somatic loss of heterozygosity (LOH) as the second mutational hit (Figure 1). LOH is commonly caused by a large deletion or mitotic recombination event, which also encompasses one copy of *NF2* located approximately 8-Mb distal to *LZTR1*. This loss of one *NF2* allele represents the third mutational hit. According to this model, the second *NF2* allele is inactivated by a somatic, intragenic *NF2* mutation which represents the fourth hit (Figure 1). These four mutational hits are mediated by three steps, since the large deletion or mitotic recombination event causes the loss of *LZTR1* and *NF2*. To investigate whether this model of tumorigenesis applies to the schwannomas of patients 3617 and 4075, *LZTR1* mutation analysis and MLPA for *LZTR1* and *NF2* was performed. We observed that the germline *LZTR1* variants identified in blood of patients 3617 and 4075 were retained in the respective schwannoma samples. Sequence analysis of *LZTR1* exon 7 amplified by PCR from schwannomas T2568,1 and T2716 of patient 4075 with the *LZTR1* variant c.628C>T indicated a much higher allele peak size of the mutant allele (T) than the wildtype allele (C) (Supp. Figure S1) which implies the loss of the wildtype allele in the majority of tumour cells in both schwannomas as confirmed by MLPA. In both tumours, MLPA analysis also indicated the loss of one copy of *NF2* in addition to the mutation of the other *NF2* allele (summarized in Table 1). These findings indicate that the 4-hit/3-step model of tumorigenesis, characterized by biallelic inactivation of *LZTR1* and *NF2*, is applicable to the schwannomas of patient 4075.The germline *LZTR1* variant c.1792T>C was detected in all three schwannomas of patient 3617 analysed. In tumours T2396 and T2718 of this patient, analysis of the allele peak size at position c.1792 indicated low amounts of the wildtype *LZTR1* allele suggestive of its loss in the majority of tumour cells and hence biallelic inactivation of *LZTR1* (Supp. Figure S2). Taken together with the biallelic inactivation of the *NF2* gene observed in these tumours (Table 1), we conclude that the 4-hit/3-step model of tumorigenesis is also applicable to these schwannomas.

However, one schwannoma (T2687) of patient 3617 exhibited equal amounts of the wildtype and the germline *LZTR1* variant at position c.1792 indicating that the wildtype *LZTR1* allele is not deleted (Supp. Figure S2). Likewise, the second *NF2* allele is not deleted in this tumour as determined by MLPA (Table 1). Nevertheless, a 4-hit mutational mechanism may also be underlying the growth of this schwannoma even though more than three steps would have been necessary to inactivate both alleles of *NF2* and *LZTR1*.

Discussion

Clinically, schwannomatosis is distinguished from NF2 by the absence of bilateral vestibular schwannomas and ependymomas (Merker et al. 2012; Smith et al. 2017). However, non-intradermal schwannomas, including spinal schwannomas, are common in both disorders although less frequent in NF2 as compared with schwannomatosis. If patients present with multiple non-intradermal schwannomas, in the absence of vestibular schwannoma or multiple meningiomas, the differential diagnosis of mosaic NF2 versus schwannomatosis based solely upon clinical criteria, is not possible (Evans et al. 1997; Murray et al. 2006; Plotkin et al. 2013).

In the study presented here, we analysed tumour tissue from 15 patients who had multiple non-intradermal schwannomas and fulfilled the clinical diagnostic criteria for schwannomatosis. Seven of these 15 patients had germline *LZTR1* mutations but none of them had germline mutations in *NF2* or *SMARCB1*. The analysis of at least two independent schwannomas from each patient indicated that five of the 15 patients exhibited identical *NF2* mutations in independent schwannomas (33%). None of the patients had ocular anomalies such as subcapsular cataracts or retinal hamartomas as determined by ophthalmological investigation. The type and the location of the tumours of these patients are summarized in Table 3. Three of the five patients did not have a germline variant in *LZTR1* and the finding of identical *NF2* mutations in independent schwannomas in these cases points to a diagnosis of mosaic NF2 instead of schwannomatosis (Figure 2; Table 2). These findings clearly indicate the high value of mutation analysis of independent schwannomas in order to identify patients with mosaic NF2. However, two of the five patients with identical *NF2* mutations in independent schwannomas had germline *LZTR1* variants predicted to be pathogenic (patients 3617 and 4075; Table 1). In these cases, a clear distinction between mosaic NF2 and *LZTR1*-associated schwannomatosis is extremely difficult to make. *LZTR1* exhibits a loss-of-function intolerance (pLI) factor of 0 (Lek et al. 2016) indicating that loss-of function variation is tolerated. Indeed, some *LZTR1* variants have been shown to be associated with reduced penetrance; thus, to date, ten unrelated and clinically unaffected *LZTR1* mutation carriers have been identified who had relatives harbouring the same mutation and were affected by schwannomatosis (Piotrowski et al. 2014; Paganini et al. 2015; Smith et al. 2015). Although the *LZTR1* variants of patients 3617 and 4075 analysed here were not among those variants reported as being associated with reduced penetrance, this does not exclude the possibility that these variants might be of reduced penetrance. Siblings or offspring of patients 3617 and 4075 with germline *LZTR1* variants and identical *NF2* mutations in schwannomas were not available for analysis which could have helped to distinguish between NF2 and schwannomatosis in these cases. In order to distinguish between mosaic NF2 and schwannomatosis in patients with germline *LZTR1* variants, it is also important to evaluate the type of somatic *NF2* mutation. In patient 3617 with the germline *LZTR1* variant c.1792T>C (p.Cys598Arg), the truncating *NF2* mutation c.169C>T (p.R57*) was identified in independent tumours. This CG>TG transition has been previously described as a recurrent *NF2* mutation (Evans et al. 1998b; Kluwe et al. 1996; Parry et al. 1996; Lee et al. 2012; Piotrowski et al. 2014). It may be argued that the somatic CG>TG transition could have occurred independently in spatially different schwannoma precursor cells in patient 3617 and hence these mutations might represent independent events. However, since this mutation was detected in three independent schwannomas from this patient, this postulate appears unlikely. Taken together, on the basis of what is currently known about *LZTR1* and the variants identified in patients 3617 and 4075, it is not possible to distinguish between mosaic NF2 and

LZTR1-associated schwannomas in these patients. Nevertheless, the identification of mosaic NF2 in three patients (3730, 4474 and 4175), who were initially diagnosed with schwannomatosis by means of clinical criteria and who did not harbour *LZTR1* germline variants, indicates the value of the analysis of independent tumours from the same patient. Two of the five patients with identical *NF2* mutations in independent tumours had meningiomas. These two patients (patients 3730 and 4175) did not exhibit a germline *LZTR1* variant. NF2 is known to confer an increased risk of meningioma, and intracranial meningiomas have been reported to occur in approximately 50% of NF2 patients (Smith et al. 2011). By contrast, meningiomas are much less common in schwannomatosis, being observed in only 5% of patients (Merker et al. 2012). Smith et al. (2017) reported that none of the 64 *LZTR1* mutation carriers identified by them had a meningioma. Hence, the occurrence of meningiomas in patients with non-intradermal schwannomas, in the absence of vestibular schwannomas and without germline *SMARCB1*, *LZTR1* and *NF2* mutations, could be suggestive of mosaic NF2 instead of schwannomatosis.

Schwannomatosis differs from NF2 not only in terms of the much lower prevalence of vestibular schwannomas and meningiomas but also in terms of the frequent occurrence of frequent chronic and severe pain. Merker et al. (2012) investigated 87 patients with schwannomatosis, 50 of whom (57%) presented with pain, including 40 patients (46%) experiencing pain not associated with a specific tumour and 10 patients (11%) with tumour-associated pain. Chronic neuropathic pain was the most common symptom (68%) in patients with schwannomatosis, and it persisted despite both surgical and medical attempts to manage it (Merker et al. 2012). The neuropathic pain in patients with schwannomatosis has been associated with tumour burden but not location, and a genetic contribution to the underlying pain syndrome is hypothesized (Merker et al. 2012; Jordan et al. 2018). Two of the five patients investigated by us, who had identical *NF2* mutations in independent schwannomas, suffered from generalized pain (patients 4075 and 4175). Whilst patient 4075 had a germline *LZTR1* variant, such an alteration was not identified in patient 4175. Further studies are needed to investigate the genetic causes underlying neuropathic pain in these patients.

Taken together, our findings serve to emphasize the point that *NF2* mutation testing of independent tumours from the same patient is necessary to distinguish between mosaic NF2 and schwannomatosis in patients with multiple non-intradermal schwannomas who fulfil the clinical diagnostic criteria for schwannomatosis. However, even when this approach is applied, a differential diagnosis may still be difficult in a subgroup of patients as exemplified by patients 3617 and 4075 with germline *LZTR1* variants predicted to be pathogenic and identical *NF2* mutations in independent schwannomas.

Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Legends

Figure 1: Schwannomagenesis according to the 4-hit/3-step model. This model of tumorigenesis in patients with germline *LZTR1* mutations implies that the *LZTR1* germline mutation (first hit; (1)) is retained in the schwannoma while the wildtype *LZTR1* allele is inactivated by loss of heterozygosity (LOH) as the second mutational hit (2). LOH is caused by a large deletion or mitotic recombination event, which also encompasses one copy of *NF2* located approximately 8-Mb distal to *LZTR1*. This loss of one *NF2* allele represents the third mutational hit (3). According to this model, the second *NF2* allele is inactivated by an intragenic *NF2* mutation (fourth hit; (4)). These four mutational hits are mediated by three steps, since the large deletion or mitotic recombination event causes the loss of both, *LZTR1* and *NF2*. Chr.22: chromosome 22.

Figure 2: Results of the *NF2* mutation testing in at least two independent schwannomas of 15 patients who were negative for germline *SMARCB1* and *NF2* mutations. UVS: unilateral vestibular schwannoma.