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ORIGINAL ARTICLE

Molecular characterisation of sporadic endolymphatic sac tumours and comparison to von Hippel–Lindau disease-related tumours

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Abstract

Aims: Although inactivation of the von Hippel–Lindau gene (*VHL*) on chromosome 3p25 is considered to be the major cause of hereditary endolymphatic sac tumours (ELSTs), the genetic background of sporadic ELST is largely unknown. The aim of this study was to determine the prevalence of *VHL* mutations in sporadic ELSTs and compare their characteristics to VHL-disease-related tumours.

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Methods: Genetic and epigenetic alterations were compared between 11 sporadic and 11 VHL-disease-related ELSTs by targeted sequencing and DNA methylation analysis.

Results: *VHL* mutations and small deletions detected by targeted deep sequencing were identified in 9/11 sporadic ELSTs (82%). No other cancer-related genetic pathway was altered except for *TERT* promoter mutations in two sporadic ELST and one VHL-disease-related ELST (15%). Loss of heterozygosity of chromosome 3 was found in 6/10 (60%) VHL-disease-related and 10/11 (91%) sporadic ELSTs resulting in biallelic *VHL* inactivation in 8/10 (73%) sporadic ELSTs. DNA methylation profiling did not reveal differences between sporadic and VHL-disease-related ELSTs but reliably distinguished ELST from morphological mimics of the cerebellopontine angle. VHL patients were significantly younger at disease onset compared to sporadic ELSTs (29 vs. 52 years, $p < 0.0001$, Fisher's exact test). VHL-disease status was not associated with an increased risk of recurrence, but the presence of clear cells was found to be associated with shorter progression-free survival ($p = 0.0002$, log-rank test).

Conclusion: Biallelic inactivation of *VHL* is the main mechanism underlying ELSTs, but unknown mechanisms beyond *VHL* may rarely be involved in the pathogenesis of sporadic ELSTs.

KEYWORDS
DNA methylation, endolymphatic sac tumour, *TERT* promoter mutation, *VHL*, von Hippel–Lindau disease

INTRODUCTION

Endolymphatic sac tumours (ELSTs) are low-grade neuroectodermal tumours arising from the endolymphatic sac in the temporal bone. Histopathological architecture is variable with predominantly papillary or cystic appearances, and the presence of follicular structures containing colloid-like secretions or clear cells [1]. ELSTs can be locally aggressive, infiltrating the petrous bone and labyrinth. They may even expand into the posterior fossa representing a rare differential diagnosis for tumours of the cerebellopontine angle; e.g., Roche et al. [2] found one ELST for every 300 vestibular schwannomas. They may be misdiagnosed as reactive and inflammatory processes, plexus tumours or metastases from thyroid, lung or clear cell renal carcinomas [1, 3–5].

ELST can occur sporadically; however, about one third of cases arise in von Hippel–Lindau (VHL) disease, a cancer susceptibility syndrome characterised by genetic alterations in the *VHL* tumour suppressor gene at chromosome location 3p25.3 [1]. Molecular data on ELSTs are sparse, due to the rarity of the disease; only single tumours or small series have been subjected to genetic analysis [3, 6–10]. So far, sequencing approaches exclusively focused on the detection of genetic alterations in *VHL*. A germline alteration of *VHL* and loss of the remaining wild-type allele was shown as the disease mechanism in several ELSTs from patients with VHL-disease (in agreement with the Knudson two-hit model of carcinogenesis) [1, 8, 9]. In sporadic ELSTs, however, analysis of *VHL* status revealed *VHL* mutations in only 5/10 cases (50%), possibly due to technological limitations of previous sequencing approaches or indicating that biallelic *VHL* inactivation is not essential in sporadic ELST development [7, 10, 11].

Genomic analysis, beyond *VHL*, and epigenetic analysis of ELST have not been conducted so far, and the molecular features associated with aggressive behaviour are unknown. Furthermore, clinical, pathological and genetic differences between sporadic and germline tumours are largely unknown. To determine the contribution of biallelic alteration of *VHL* to ELST formation, we applied genome-wide DNA methylation analysis, copy number variation analysis and targeted next-generation sequencing of 90 cancer-related genes to compare 11 sporadic and 11 VHL-disease-related ELSTs. Our results show that *VHL* deficiency is the disease mechanism in most ELSTs together with *TERT* promoter mutations, but *VHL* wild-type status in single sporadic ELSTs argue for another, unknown process of tumour formation in these rare cases.

MATERIALS AND METHODS

Patient samples

Twenty-two ELST cases diagnosed between 1998 and 2020 were retrieved from the archives of the Departments of Neuropathology in Berlin, Freiburg, Hannover, Heidelberg, Münster and Tübingen. Clinical data were retrospectively determined and de-identified. Written informed consent was not required for this retrospective study. The study was approved by the ethical committee of the Charité—Universitätsmedizin Berlin (certificate number: EA2/248/18). All tumours were confirmed histopathologically to be ELST according to the criteria of the 2017 WHO Classification of Head and Neck Tumours. The extent of haemorrhage was graded

as no bleeding residuals (–), low (+, few siderophages), medium (++, <50% of tumour tissue affected) and pronounced haemorrhagic alterations (+++, >50%). The presence of clear cells was evaluated for each case and scored as positive when present at least focally. Immunohistochemistry was performed on a Benchmark XT autostainer (Ventana Medical Systems) with standard antigen retrieval methods (CC1 buffer, pH8.0) using monoclonal mouse anti-MIB1 (Ki-67, 1:100, Dako M7240) and monoclonal mouse AE1/AE3 (1:200, DAKO M3515). Primary antibodies were applied and developed by using the iVIEW DAB Detection kit (Ventana Medical Systems) according to the manufacturer's instruction.

DNA extraction

Genomic DNA was extracted from formalin-fixed, paraffin embedded tissue using the Maxwell DNA FFPE Kit (Promega). In one case (ELST #14), the DNA concentration was too low for further analyses. The tumour cell content was estimated based on haematoxylin and eosin (H&E) and cytokeratin-stained slides by two neuropathologists. For all samples, we selected areas with the highest available tumour cell content (based on histological estimation 20%–60%).

Targeted next-generation sequencing

For targeted sequencing of 90 cancer-related genes, 100-ng genomic DNA was sheared on a Covaris M220 (Covaris). DNA integrity and fragment size were determined on a Fragment Analyzer™ 5200 (Advanced Analytica). Paired-end library preparation was conducted using Illumina v2 protocols. The Intracranial Tumour Panel (ICT v1.0, for gene list see Table S1) was specifically designed for the neuropathological spectrum of tumours and is based on a customised hybrid-capture/enrichment approach (adopted TruSeq DNA Exome workflow protocol). Sequencing was performed on an Illumina® MiSeq® system with an average coverage of 550-fold (range 159× to 1084×). Bioinformatics data analysis was performed as previously described with the following adjustments: alignment against hg19, filtering for variants with mutant allele frequency >5% and variants in coding and splice site regions [12]. Variants were annotated using ClinVar as well as applying the CancerVar script (https://github.com/WGLab/CancerVar). Variants were classified into the three categories 'likely benign/benign', 'uncertain significance' and 'likely pathogenic/pathogenic' according to the joint AMP-ASCO-CAP 2017 guidelines for cancer variant interpretation [13]. *VHL* and *TERT* were visually inspected in all cases in the integrated genomics viewer (IGV_2.5.1).

TERT Sanger sequencing

TERT promoter hotspot mutations (genomic position chr5:1295228 and chr5:1295250) were analysed by Sanger Sequencing (forward primer: GGATTCGCGGGCACAGAC; reverse primer: CAGCGCTGCCTGAAACTC; details on PCR conditions is available upon request). Sequencing was performed at Eurofins Genomics, Ebersberg, Germany.

DNA methylation array analysis

Genome-wide DNA methylation profiles were obtained using Infinium HumanMethylation450 (450 k) BeadChip or Infinium MethylationEPIC (850 k) BeadChip array (Illumina) according to the manufacturer's instruction. Methylation array data were processed in R (version 3.5.0) applying Bioconductor packages minfi (version 1.28.4), RtSNE (version 0.15), methyAnalysis (version 1.24.0), ComplexHeatmap (version 1.20.0) and NbClust (version 3.0) with default settings unless otherwise indicated. *t*-distributed stochastic neighbour embedding (tSNE) plots were calculated using the 32,000 probes with most variant beta-values (perplexity 15, iterations 1000, theta 0, pca = FALSE, seed = 1). We used the following reference tumour classes from the CNS classifier cohort (GSE90496) [14]: hemangioblastoma (n = 25), plexus tumour paediatric A (n = 15), plexus tumour paediatric B (n = 46) and plexus tumour adult (n = 22). Additional tumour entities included: head and neck paraganglioma (GSE111336, n = 6) [15], as well as 24 previously published head and neck paragangliomas [16], clear cell renal cell carcinoma (GSE105288, n = 35), papillary thyroid carcinoma (GSE97466, n = 46), follicular thyroid carcinoma (GSE97466, n = 6) and lung adenocarcinoma (GSE94785, n = 68). ELSTs from patients with VHL disease were encircled in black in the tSNE plot. Copy number alterations were assessed using the R package conumee (version 1.16.0). Heatmaps were calculated using the R package ComplexHeatmap (version 2.0.0). Differentially methylated cg sites between VHL-disease-related and sporadic ELSTs were assessed applying the limma package (version 3.40.6). Beta-density plots were calculated with cola (version 3.12). To evaluate the *VHL* promoter methylation status, we additionally analysed normal tissue pons (n = 12; GSE90496). As evidence of epigenetic silencing, the *VHL* promoter was considered methylated if the beta value for probe cg15267345 within the CpG Island exceeded 0.2 as previously published [17, 18].

Statistics and survival analysis

Student's t test for age and Fisher's exact test for categorical variables were used to identify differences between VHL-disease-related and sporadic tumours. Disease onset was defined as date of magnetic resonance imaging (MRI) evidence of ELST. Recurrence was defined as the appearance of a new tumour or progression of a residual tumour on MRI. Survival analysis was performed using Kaplan–Meier estimation for survival curves and the log-rank test in R applying the packages 'survminer' (version 0.4.6) and 'ggpubr' (version 0.2.4). A p value of <0.05 was considered significant.

Review of literature

Published ELST cases between 1997 and 2020 were reviewed by screening the PubMed database for search terms 'endolymphatic sac tumour' AND 'VHL mutation', identifying a total of 66 publications. Publications were excluded if no information on the specific position of the genetic *VHL* alteration for ELST patients was provided, if they were not published in English, or the diagnosis of ELST was not confirmed histologically. This resulted in 19 publications. We additionally identified eight publications which were cross-referenced in the screened literature. All publications (n = 27) were searched for the genomic position of *VHL* gene alteration and somatic versus germline lineage information. Additionally, information on age at diagnosis, sex, additional VHL-disease-related tumours and the diagnostic institution were retrieved.

RESULTS

The majority of sporadic ELSTs demonstrate somatic *VHL* alterations and rarely carry *TERT* promoter mutations

To investigate the mutational landscape of sporadic ELST, we performed targeted next-generation sequencing of 90 genes, including *VHL*, in 11 sporadic ELSTs. Pathogenic *VHL* alterations were detected in 9/11 cases (82%) with seven of nine *VHL* alterations affecting Exon and Intron 1 (78%), whereas only one alteration was observed in exon 2 and exon 3, respectively (Table 1). The mean allele frequency of *VHL* alterations was 20% (range 2%–43%; coverage of cases with less than 20% mutant allele frequency: #1 – 159×, #2 – 1084×, #5 – 620×, #8 – 1075×, #11 – 811×). Frameshift deletions resulting in premature stop codons were the most common mechanism of *VHL* inactivation in sporadic ELSTs (5/9, 56%). Two cases (#7 and #11) demonstrated pathogenic splice site mutations at the exon–intron 1 boundary. Missense mutations in exon 1 were observed in two cases (#4 and #9) and located in the beta-domain of *VHL* before the HIF-binding site. Exon deletions, known to frequently occur in patients with VHL-disease-related ELSTs, were not detected in sporadic ELSTs. No pathogenic mutation in 90 cancer related genes was found in cases #3 and #6. We additionally found *TERT* promoter hotspot mutations (C228T) in two sporadic ELSTs by our next-generation panel sequencing (#7 and #9) which have not been described in ELSTs, so far. We therefore additionally investigated 10 VHL-disease-related ELSTs for *TERT* mutations by Sanger Sequencing and found one more case with a C228T *TERT* promoter mutation (#18). In total, 3/20 ELSTs (15%) carried a *TERT* promoter hotspot mutation.

Methylation analysis reveals a distinct epigenetic profile of ELST, but no epigenetic differences between VHL-disease-related and sporadic ELSTs

To investigate epigenetic differences between sporadic and VHL-disease-related ELSTs, we performed genome-wide DNA methylation profiling of 11 sporadic and 10 VHL-disease-related ELSTs. Methylation analysis revealed a distinct methylation profile of ELSTs based on tSNE analysis. ELSTs grouped separately

TABLE 1 *VHL* alterations in 11 sporadic ELSTs

ELST	*VHL* alteration (HGVS)	MAF	*TERT*	3p	*VHL* inactivation
#1	c.228delC;C77Afs*82	11%	WT	LOH	Biallelic
#2	c.160_184del; M54Cfs*5	2%	WT		
#3	not detected		WT	LOH	
#4	c.233A>G;N78S	43%	WT	LOH	Biallelic
#5	c.292_296delTACCC;Y98Nfs*32	14%	WT	LOH	Biallelic
#6	not detected		WT	LOH	
#7	c.341-2A>G	42%	C228T	LOH	Biallelic
#8	c.256C>T;P86S	13%	WT	LOH	Biallelic
#9	c.464_480del;V155Afs*13	20%	C228T	LOH	Biallelic
#10	c.394delC;Q132Kfs*27	20%	WT	LOH	Biallelic
#11	c.463+1G>T	16%	WT	LOH	Biallelic

Note: Pathogenic and likely pathogenic *VHL* mutations were detected in 9/11 ELSTs along with two *TERT* promoter hotspot mutations in case #7 and #9. In two cases (#3 and #6), no pathogenic mutations could be detected in 90 cancer associated genes. The low mutant allele frequency (MAF) in case #2 indicates a very low tumour cell content, possibly preventing the detection of a chromosome 3p loss by methylation profiling. Description of sequence variants according to the Human Genome Variation Society (HGVS) and the canonical *VHL* transcript NM_000551.3.

Abbreviations: 3p, chromosome 3p; C228T, *TERT* promoter hotspot mutation; LOH, loss of heterozygosity; MAF, mutant allele frequency; WT, wild type.

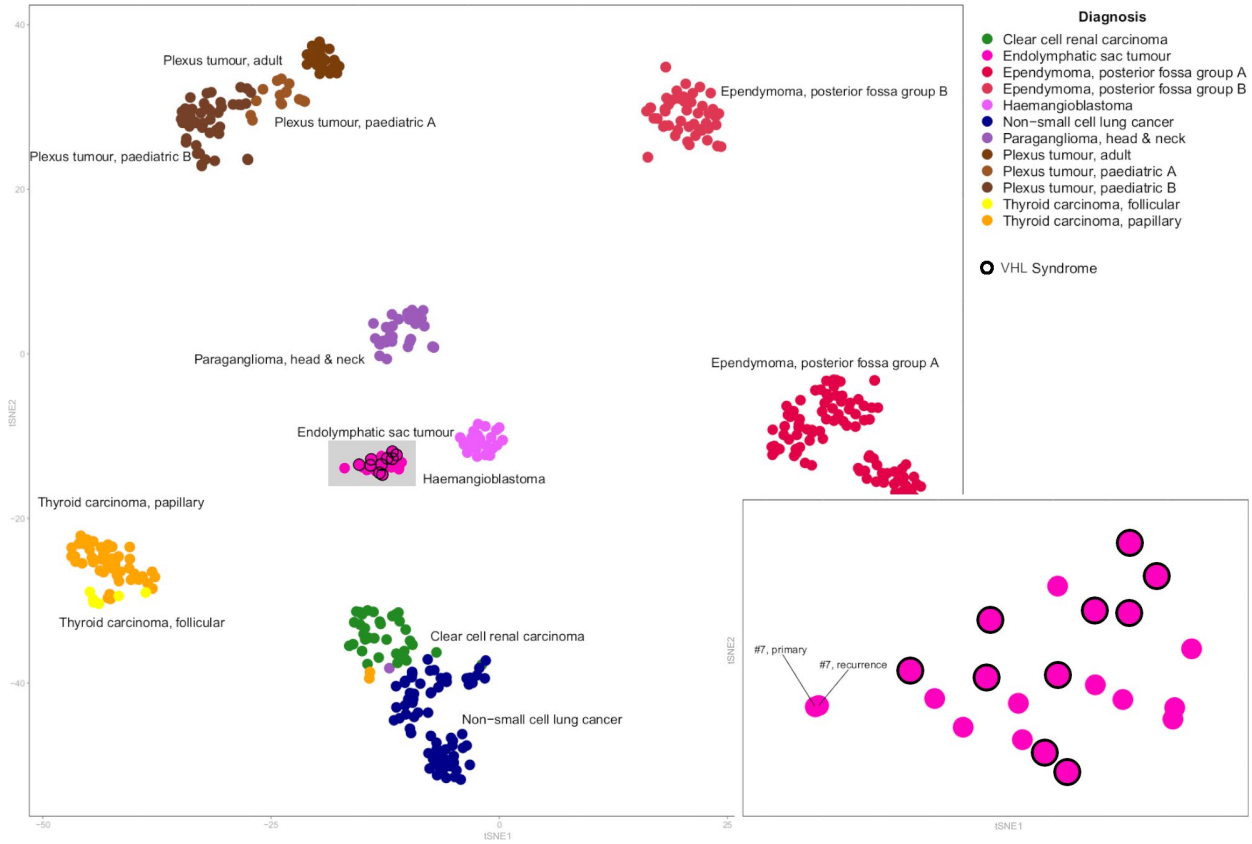

FIGURE 1 Comparison of the methylation profiles of VHL-disease-related and sporadic ELST. A *t*-distributed stochastic neighbour embedding (tSNE) plot shows that ELSTs clearly group apart from morphological mimics of the cerebellopontine angle. Inset: There is no evidence of subgroups within the ELST methylation class, especially not regarding VHL-disease status

from morphological mimics and previously established CNS tumour methylation classes (Figure 1). Compared to other entities, the mean beta-density of ELST (0.52) ranked in the lower third but did not significantly differ (Figure S1A and B). tSNE analysis did not indicate heterogeneity within ELSTs, in particular, there was no subgrouping according to VHL disease status (Figure 1, Inset) which was also not observed in unsupervised hierarchical clustering (Figure S1C). Furthermore, in the enhanced volcano plot analysis, there were only very few significantly differently methylated CpG sites between VHL-disease-related and sporadic ELSTs (Figure S1D), not enriched in cancer-related genes or pathways.

VHL promoter hypermethylation is rare in sporadic ELSTs and absent in VHL-disease-related ELSTs

Methylation analysis of cg sites in the *VHL* promoter region revealed similar methylation levels compared to normal pontine tissue in most ELST cases, except for one sporadic case (#6) showing hypermethylation at probe cg15267345 (ß value 0.34; mean: pons = 0.0675; ELST = 0.0648; Figure S2). Of note, none of the VHL-disease-related ELSTs showed increased *VHL* promoter methylation.

ELST frequently show chromosome 3p loss resulting in biallelic inactivation of *VHL* in most sporadic cases

Copy number variation plots were calculated from DNA methylation array data. Loss of chromosome 3p, resulting in a heterozygous loss of the *VHL* tumour suppressor gene, was the most common alteration in ELSTs (16/21, 76%; Figure 2). Chromosome 3p loss was more frequent in sporadic ELSTs (10/11; 91%) compared to VHL-disease-related (6/10; 60%), although not statistically significant (p = 0.15, Fisher's exact test). Biallelic inactivation of the *VHL* tumour suppressor gene by chromosome 3p loss and *VHL* mutation of the wild-type allele occurred in 8/11 sporadic ELSTs (73%; Table 1). Of note, the two sporadic ELSTs without *VHL* mutation (#3 and #6) both demonstrated chromosome 3p loss.

Chromosomal aberrations besides 3p loss are rare and not significantly different between sporadic and VHL-disease-related ELSTs

We additionally detected a loss of 9q and 16q in 3/21 cases (14%; two VHL-disease-related and one sporadic case, respectively; Figure 2). In three cases (#5, #8 and #21), the loss of chromosome

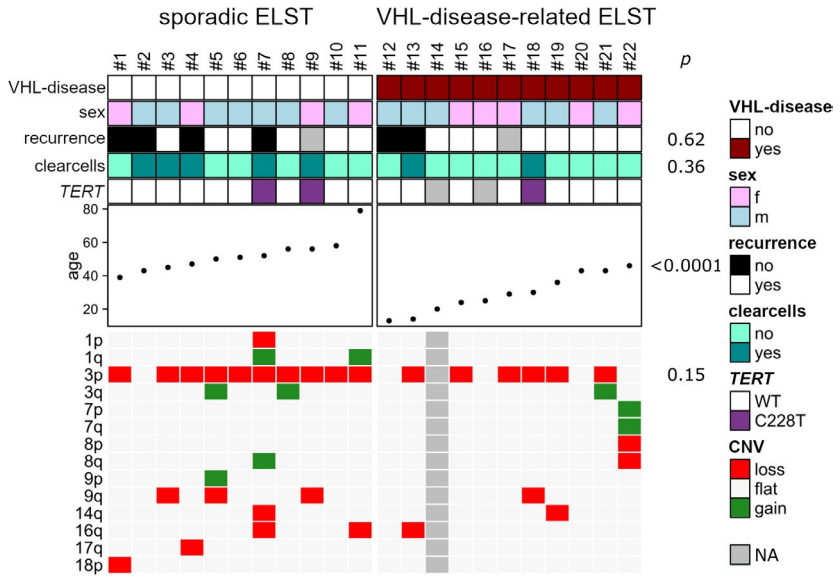

FIGURE 2 Clinico-pathological and molecular disease features in sporadic and VHL-disease-related ELSTs. Patients with VHL-disease-related ELSTs are significantly younger compared to sporadic ELST patients. Other parameters (e.g., gender, recurrence rate, clear cell phenotype and chromosome 3p loss) were not significantly enriched in either of the two subgroups. Fisher's exact tests were calculated for comparison of categorical variables

3p was accompanied by a gain of chromosome 3q (3/21; 14%). Amplification or homozygous deletions were not noted in any of the cases. There was no distinct pattern of chromosomal aberrations specific for VHL-disease-related or sporadic ELSTs. Only a few chromosomal gains and losses besides chromosome 3p loss were detected in ELSTs and occurred in 8/11 sporadic and 5/10 VHL-disease-related ELSTs (p = 0.387, Fisher's exact test). In total, there were 25 losses or gains of chromosome arms p and q observed in 11 sporadic ELSTs compared to 12 in 10 VHL-disease-related ELSTs (p = 0.19, Students t test). ELST #7 demonstrated an increased number of chromosomal losses 1p, 3p, 14q and 16q as well as gains of chromosome 1q and 8q. Upon recurrence of ELST #7, 2 years later, chromosomal alterations remained stable except for the initial chromosome 14q loss that was not preserved in the recurrent tumour (data not shown).

Clinical characteristics and differences between sporadic and VHL-disease-related ELSTs

Patients with VHL-disease-related ELSTs were significantly younger compared to patients with sporadic ELSTs (median age 29.4 vs. 52.4 years; $p < 0.001$, Student's t test, Figure 2). There was no significant difference in recurrence rate between patients with VHL syndrome (2/10, 20%) and patients with sporadic ELSTs (4/10, 40%; p = 0.62, Fisher's exact test). All tumours originated from the petrous temporal bone and extended variably into the mastoid or cerebellopontine angle. Metastatic disease was not observed in any of the cases. In patients with VHL syndrome, the most common differential diagnosis raised on MRI was ELST (n = 6), followed by haemangioblastoma in two cases with previous spinal and cerebellar haemangioblastomas (ELST #22; Figure 3A). In sporadic cases, ELST was suspected in 6/11 cases followed by paraganglioma in 3/11 cases and cholesterol granuloma (n = 2; NA = 3).

Comparison of the morphological features between sporadic and VHL-disease-related ELSTs

Papillary architecture was the most prevalent growth pattern in both sporadic (9/11; 82%) and VHL-disease-related ELSTs (9/11, 82%, Figure 3B), compared to a predominantly colloidal appearance in only 2/11 cases, respectively (18%; Figure 3C). Clear cells were observed at least focally in seven ELSTs in both tumours with papillary and colloidal growth pattern (Table S3). The presence of a clear cell phenotype was not significantly more prevalent in VHL-disease-related ELSTs (2/11, 18%, Figure 3D) compared to sporadic cases (5/11, 46%; p = 0.36, Fisher's exact test; Figure 2). *TERT* promoter mutations were only observed in ELST with a clear cell phenotype (3/7) and absent in those without (0/13, NA = 2; p = 0.03, Fisher's exact test). Evidence of prior bleeding (no or low vs. medium or high) did not significantly differ between VHL-disease-related and sporadic ELSTs (p = 1, Fisher's exact test, Table S2). Proliferative activity was low in all cases (Ki67 ranging from <1% to 5%, Table S3) and not significantly different in sporadic compared to VHL-disease-related ELSTs (p = 0.42, Fisher's exact test).

Clear cell phenotype is associated with shorter progression-free survival in ELSTs

Clinical outcome data were available for 20 patients. After a median observation period of 37 months, 14 patients were alive without evidence of tumour recurrence, whereas six patients experienced recurrences. All patients were alive at last follow-up. VHL disease status was not significantly associated with PFS in ELST patients (median observation period for VHL-disease-related tumours: 52 months, for sporadic cases: 34 months; log-rank p = 0.27; Figure 4A), nor was *TERT* promoter mutation (log-rank p = 0.88), presence of chromosome 3p loss (log-rank p = 0.97) or proliferative activity (≤1% vs. >1%, log-rank p = 0.51; data not shown). However, the presence of

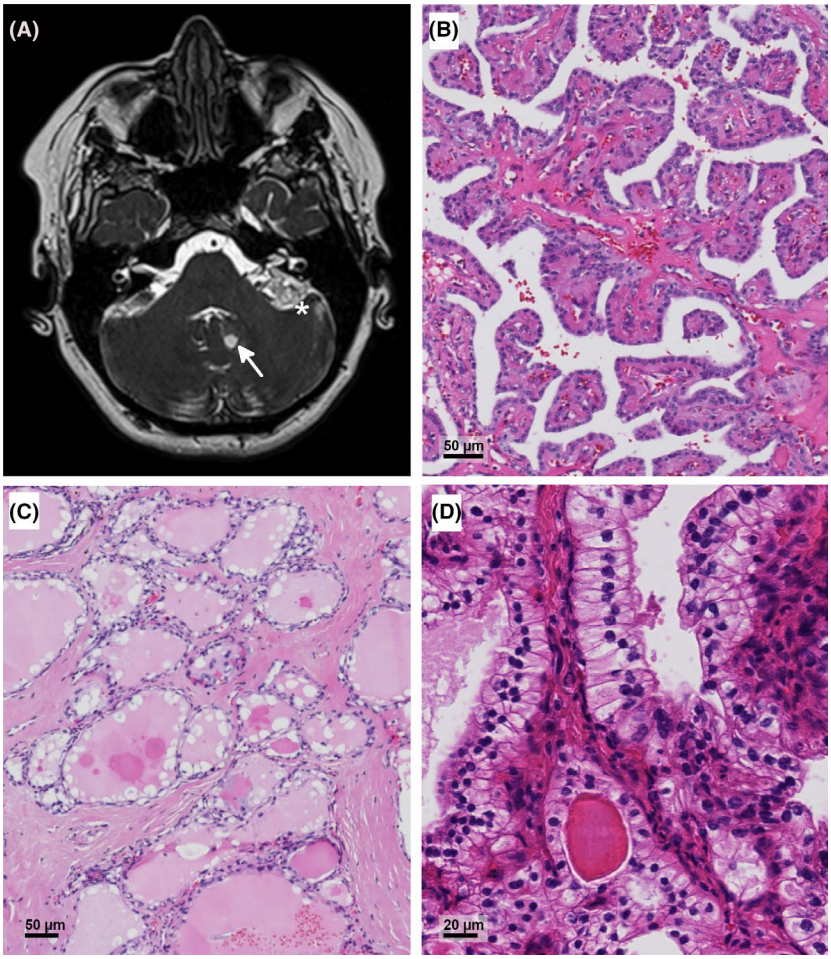

FIGURE 3 Radiological and morphological characteristics of ELST. (A) MRI of a patient with VHL syndrome shows a T2-weighted hyperintense, destructive temporal bone mass extending from the inner ear into the cerebellopontine angle (*). Please note also, the periventricular cerebellar lesion representing one of multiple haemangioblastomas, which was the initially suspected diagnosis (→). (B) Papillary differentiation was the predominantly observed growth pattern in most ELST cases, followed by a less prevalent follicular-colloidal growth pattern reminiscent of thyroid follicles (C). (D) Higher magnification of an ELST showing clear cell phenotype in cuboidal cells lined up along papillary structures

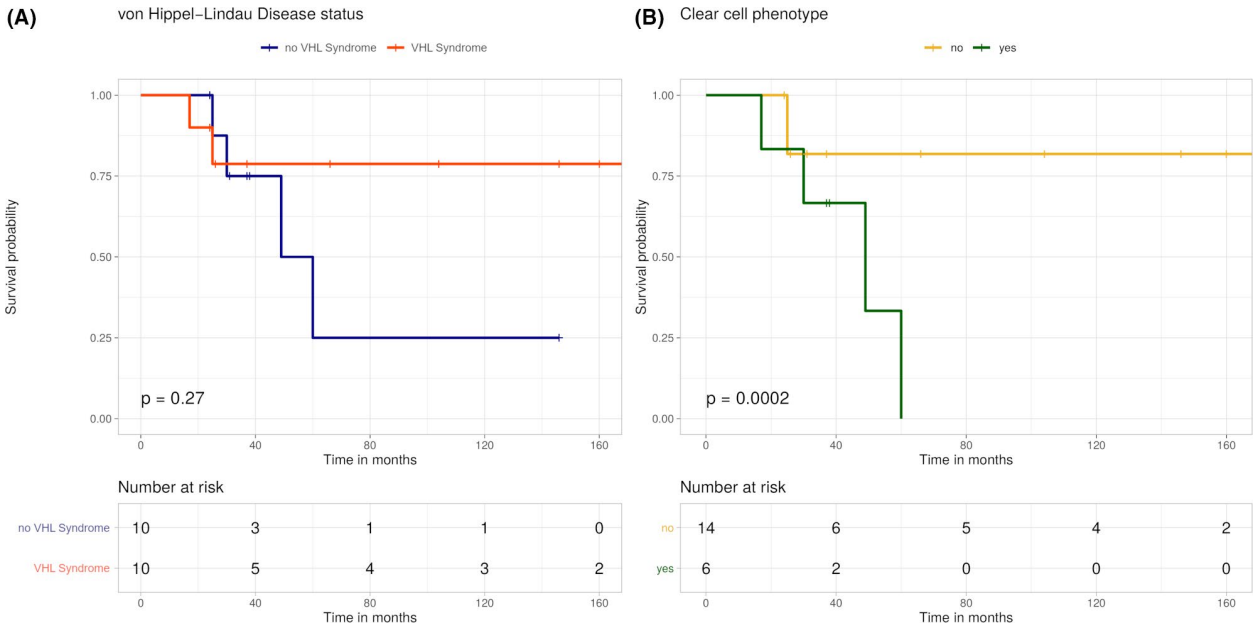

FIGURE 4 Kaplan–Meier plots of progression-free survival for ELSTs patients, with follow-up durations of up to 14 years after surgical resection. (A) VHL-disease status was not associated with progression-free survival; instead, clear cells phenotype (B) was significantly associated with shorter progression-free survival (p = 0.0002). Patients at risk are shown in the risk table below; p value was calculated by log-rank test

a clear cell phenotype was associated with shorter progression-free survival (log-rank $p = 0.0002$; Figure 4B).

Sporadic ELSTs predominantly carry mutations in exon 1, whereas VHL-disease-related ELSTs demonstrate a more even mutation distribution with a hotspot region in exon 3

Summarising information from 27 publications and our own data, genetic and clinical information of 158 VHL-disease-related ELSTs and 21 sporadic ELSTs was available (Table S4) [3, 6–11, 19–38]. VHL-disease-related ELSTs affected 31 females and 20 males (NA = 107), sporadic ELSTs 11 females and 10 males. The mean age at presentation of 21 sporadic cases was 46.8 years versus 32.1 in 40 VHL-disease-related cases (NA = 107; $p < 0.0001$, Student's *t* test). *VHL* alterations could not be detected in 3/147 VHL-disease-related ELST (2%; NA = 11) and 7/21 sporadic ELSTs (33.3%). In 43/147 VHL-disease-related ELSTs (29.3%, NA = 11), partial or complete deletions of the *VHL* gene were the disease-causing mechanism (exon 1: $n = 2$, exon 2: $n = 2$, exon 3: $n = 4$, exons 2–3: $n = 1$, whole gene deletion (exons 1–3): $n = 8$, not specified $n = 26$; see Figure 5). Compared to VHL-disease-related ELSTs, no *VHL* exon or whole gene deletion was discovered in sporadic ELSTs. In VHL-disease-related ELSTs, 43/101 mutations (42.5%) affected exon 1, 14/101 (13.9%) exon 2 and 44/101 (43.6%) intron and exon 3. In exon 3, *VHL* variants at position R167 in the Elongin C domain were identified most often (13/101; 12.9%) followed by splice site mutations affecting the donor splice site of exon 1/intron 2 (7/101; 6.9%). In sporadic ELSTs, *VHL* mutations were most commonly observed in exon 1 (8/14; 57.2%) compared to intron and exon 2 as well as intron and exon 3 (each 3/14; 21.4%). Genetic alterations resulting in a premature stop or substantially truncated protein (e.g., exon deletions, nonsense mutations, frameshift deletions and splice site mutations) were observed in 9/14 (64.3%) sporadic ELSTs and 80/147 (54.4%) of VHL-disease-related ELSTs ($p = 0.59$, Fisher's exact test). Missense mutations and in-frame deletions were reported in 5/14 (35.7%) and 64/147 (43.6%) of VHL-disease-related ELSTs. For 40 VHL-disease-related ELST patients, information on age at disease onset as well as mutation subtype was available. Mean age of onset for patients with truncating alterations (e.g., deletions, stop gains, frameshift and splice site alterations) was 34.2 years ($n = 24$; range 11–59 years) compared to 30.8 years ($n = 16$; range 8–54 years) in patients with missense mutations ($p = 0.43$, Student's *t* test).

DISCUSSION

Biallelic inactivation of *VHL* has been known as the disease mechanism in VHL-disease-related ELSTs for many years, whereas its relevance in sporadic ELSTs is unclear and has only been investigated in single cases. Morphological and clinical differences between sporadic and VHL-disease-related ELST have so far only been analysed once [39]. To identify differences and to evaluate the significance of *VHL* alterations in sporadic ELSTs, we compared VHL-disease-related and sporadic ELSTs using methylation profiling, CNV analysis and next-generation sequencing. Our findings suggest that in addition to biallelic inactivation of *VHL* in most sporadic ELSTs, *TERT* promoter mutations and currently unknown mechanisms beyond *VHL* may play roles in the pathogenesis of sporadic ELST.

According to our literature review, in total, eight sporadic ELSTs had previously been subjected to *VHL* sequencing by PCR-based single-strand conformational polymorphism (SSCP) analysis or Sanger sequencing and *VHL* alterations were demonstrated in only 3/8 cases (38%) [3, 7, 9, 11, 23, 29]. Therefore, it has been suspected that biallelic inactivation of *VHL* is not essential in sporadic ELST tumorigenesis, and other genetic alterations may be involved in tumour formation. By next-generation sequencing, however, we could identify *VHL* alterations in 9/11 sporadic ELSTs (82%), a substantially higher frequency than previously reported. *VHL* mutations were detected in two more sporadic ELSTs by next-generation sequencing [3, 11]. In total, in 85% of sporadic ELSTs (11/13), *VHL* alterations were detected by next-generation sequencing compared to 38% by direct sequencing. A higher detection rate of *VHL* alterations in VHL-disease-related tumours by targeted deep sequencing compared to direct sequencing has also been demonstrated for haemangioblastomas [40, 41]. The lower detection rate of *VHL* alterations by direct sequencing might be explained by the higher detection limits of classical sequencing approaches together with the low tumour cell content in some cases, reflected in the low mutant allele frequencies (mean 20%, ranging from 2% in #2 to 43% in #4) observed in our series of sporadic ELSTs.

In two sporadic ELSTs (#3 and #6), we did not find a genetic alteration of *VHL*, although both cases demonstrated a loss of chromosome 3p, indicating a sufficiently high tumour cell content in both cases. *VHL* promoter hypermethylation as second hit to inactivate the remaining *VHL* wild-type has been described for 3p-deficient sporadic haemangioblastomas without evidence of *VHL* mutations [41], as well as being mutually exclusive with *VHL* mutations in clear cell renal carcinomas [42]. ELST #6 demonstrated hypermethylation at cg15267345 within the CpG island of the *VHL* promoter compared to normal control tissue and other ELSTs. *VHL* promoter hypermethylation at cg15267345 has previously been described for sporadic renal clear cell carcinomas [17, 43]. However, because mRNA expression data were not available, it was not possible to show if the observed hypermethylation within the CpG island of the *VHL* promoter had a functional consequence and resulted in epigenetic silencing of *VHL* expression in ELST #6. Overall, the frequency of *VHL* hypermethylation in ELSTs is very low and seems to be restricted to sporadic cases, in line with what has been shown for haemangioblastomas and renal clear cell carcinomas [41, 42].

We did not observe *VHL* promoter hypermethylation in ELST #3 whose molecular pathogenesis remains elusive. Given heterozygous loss of chromosome 3p, mutations in other tumour suppressor genes on 3p may explain tumour aetiology, similar to clear cell renal carcinomas. Potential candidate genes include the tumour

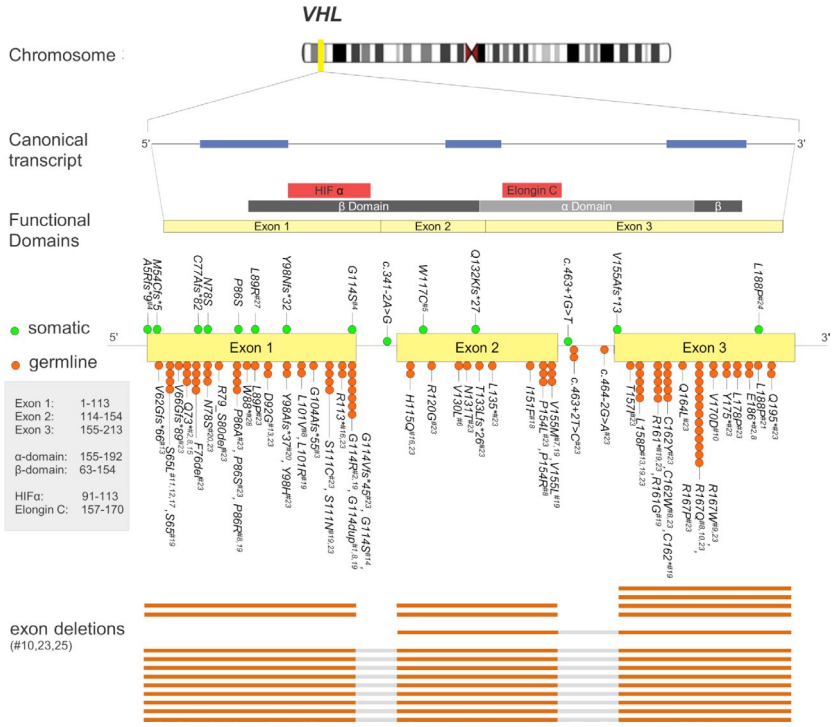

FIGURE 5 *VHL* mutations in ELST patients. *VHL* is located on chromosome 3p25.3. Functional α and β domains contain binding sites of the transcription factor HIF1-α and Elongin C. Germline mutations are shown in orange, somatic mutations in green. The circles represent individual patients. Most sporadic mutations are found in exon 1, germline mutations are distributed throughout the gene with a hotspot in exon 3. *VHL* exon and whole gene deletions were exclusively seen in VHL-disease-related tumours and are depicted as orange bars (intronic regions are coloured in grey). Every bar indicates an individual patient. The length of the bar corresponds to the length of exons 1–3 shown above in yellow. There were nine partial gene deletions (two exon 1, two exon 2, four exon 3 and one exon 2 + 3) as well as nine whole gene deletions (exons 1–3) documented in the literature. All genetic alterations described so far carry a superscripted number (Refs. [1–28]) that refers to the publication ID (Pub ID, first column in Table S4) of the literature review. Somatic mutations without a superscripted number were found in our analysis

suppressor and chromatin remodelling genes, *PBRM1* (3p21.1), *SETD2* (3p21.31) and *BAP1* (3p21.1), which are recurrently mutated in clear cell renal carcinomas (in about 38%, 13% and 11% respectively) [44–47], but were not covered by our next-generation sequencing panel. Furthermore, another possible disease mechanism might cause *VHL* inactivation but could not be detected by sequencing coding regions of *VHL* (e.g., deep intronic variants, variants in regulatory genetic regions upstream or downstream, or microRNA silencing).

Besides *VHL* mutations, sequencing demonstrated the absence of pathogenic mutations in 90 cancer-related genes, except for *TERT* promoter hotspot mutations in three ELSTs (15%). *TERT* promoter mutations occur at a similar frequency of 9%–12% in clear cell renal carcinomas and are associated with an aggressive disease course and poor outcome [48, 49]. Based on our outcome data, we did not find *TERT* promoter mutations to be associated with worse survival compared to wild-type tumours. However, ELST #7 displayed a *TERT* promoter mutation and showed recurrence and progressive disease. Moreover, very recently, an aggressive sporadic ELST with multiple recurrences and *TERT* promoter mutation has been described [11]. The prognostic relevance of *TERT* promoter mutations remains unclear and needs to be evaluated in larger series in the future. Instead, we found the clear cell phenotype to be associated with shorter progression-free survival in ELST. One shortcoming of our outcome analysis is the lack of the resection status for this series, which may also be an important factor.

Clinical and morphological differences between six sporadic and six VHL-disease-related ELSTs were first described by Nevoux et al. [39] in 2015. The authors noted that VHL-disease-related tumours more often appeared fibrous with little haemorrhagic change compared to sporadic ELSTs which seems to be more cystic in nature and heavily haemorrhagic. In our own series, though, we did not detect a difference in the prevelance of growth type (papillary-solid vs. colloidal-cystic) or extent of haemorrhagic transformation in sporadic versus VHL-disease-related tumours. We also did not identify a difference in recurrence rate whereas, Nevoux et al. [39] described more recurrences in sporadic (four in two patients) than in VHL-disease-related cases (one recurrence). In line with our own data, none of their cases showed histopathological signs of malignancy or distant metastasis. Interestingly, the authors did not find any difference in age at surgery between VHL-disease-related and sporadic ELSTs, in contrast to our own data, several other studies as well as our literature review, demonstrated a significantly earlier disease onset in VHL patients [19, 50].

Compared to missense mutations, truncating gene alterations are associated with even earlier manifestation and a more severe phenotype in some cancer syndromes. We therefore reviewed the available literature and analysed if truncating germline alterations are associated with earlier disease onset in VHL patients with ELST but could not detect a significant difference compared to patients with missense mutations. Poulsen et al. [33] investigated genotype–phenotype correlations in VHL patients with ELST to identify a high-risk group which may profit from intensified audiological surveillance strategies. However, they could not find evidence for an increased overall risk of ELST development related to VHL-disease subtype (type 1, without phaeochromocytoma; type 2, with phaeochromocytoma) or *VHL* gene alteration type (truncating vs. missense mutations).

The distribution of mutations in *VHL* in sporadic and VHL-disease-related ELST was different, although our analysis was limited by the low number of sporadic cases analysed so far. Mutations in sporadic cases were predominantly localised in exon 1. In VHL-disease-related ELSTs, instead, mutations were more evenly distributed in exons 1 and 3 with a mutational hotspot in exon 3 at position R167, which is the most commonly mutated codon in VHL-disease in general (see also VHLdb, database of interactors and mutations; http://vhldb.bio.unipd.it/) [51, 52].

Jester et al. [37] previously reported loss of chromosome 3p in 8/12 ELSTs (66%) and a loss of chromosome 9q in seven of these eight cases (58%). They raised the hypothesis that a combined loss or even derivative chromosome (3q;9p) resulting from an unbalanced translocation was a disease mechanism in ELSTs. In our cohort, we detected loss of chromosome 3p at a slightly higher frequency in 16/21 cases (76%), but only four cases showed an additional loss of chromosome 9p (19%) which renders a combined chromosomal event as a driver less likely. However, chromosome 9q loss was more often observed in sporadic ELSTs in our series (3/4 cases), as well as in the Jester cohort (6/7 cases). Thus, chromosome 9q loss may be more prevalent in sporadic cases.

We additionally identified three cases with loss of 16q, which was only seen in one case of the Jester series, as well as three cases that had gain of chromosome 3q in addition to loss of 3p, which was not described previously and potentially indicates a more complex chromosomal rearrangement involved in the loss of one *VHL* copy. Four VHL-disease-related ELST did not show evidence of chromosome 3p loss (#12, #16, #20, #22) compared to only one sporadic case, which possibly was not detected due to low tumour cell content in this sample (#2). But the frequency of chromosome 3p loss was not significantly different between sporadic and VHL-disease-related ELSTs. Analysis of copy number changes from methylation data does not allow for detection of copy number neutral loss of heterozygosity which might be involved in *VHL* inactivation in several VHL-disease-related cases without chromosome 3p loss.

Although we were not able to identify epigenetic differences between sporadic and VHL-disease-related ELSTs (Figure S2), methylation profiling proved beneficial in delineating ELSTs from morphological mimics of the cerebellopontine angle. The diagnosis of ELST remains challenging due to the rarity of the disease, overlap of immunophenotypes and staining artefacts. Several studies have investigated the usefulness of immunohistochemical markers to differentiate ELST from relevant differential diagnoses [5, 37, 50]. The most relevant differential diagnoses include plexus papillomas, paragangliomas and papillary ependymomas, papillary meningiomas and metastatic carcinomas (in particular lung, thyroid, clear cell renal carcinoma) [3, 5, 37]. Given the high number of ELST patients with VHL syndrome, the exclusion of metastasis from kidney cancer is mandatory, as it occurs in about 27.4% of patients with VHL-disease and tumours larger than three centimetres [53]. ELST and clear cell renal carcinoma both express renal cell markers (e.g., CAIX and pax-8) and occasionally collision tumours of clear cell renal carcinoma and ELST have been reported [50, 54]. DNA methylation profiling is able to increase diagnostic accuracy in ELST despite relatively low tumour cell content and artificial tissue alterations, hindering histological assessment in many cases.

CONCLUSION

Our data suggest that there are no highly distinct differences between sporadic and VHL-disease-related ELST with respect to morphology, epigenetics, copy number alterations or recurrence rate. However, VHL-disease-related ELSTs manifest significantly earlier and show a broader variety of genetic aberrations of the *VHL* gene. Rarely, other, currently unknown, mechanisms may be involved in the pathogenesis of sporadic ELSTs. Moreover, our findings of the epigenetic distinctiveness of ELSTs will contribute to a more accurate identification of these diagnostically challenging tumours. A clear cell phenotype may represent a prognostic marker for both sporadic and VHL-disease-related ELSTs.

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CONFLICT OF INTEREST

DC and AvD have a patent pending: DNA methylation-based method for classifying tumour species (EP 16710700.2).

AUTHOR CONTRIBUTIONS

LS and DC conceived the project and wrote the manuscript. FT, CT and NW contributed to methylation data analysis. LS, FT, AF and PS performed next-generation sequencing analysis. All other authors were involved in the acquisition of data and contributed to the final version of the manuscript.

ETHICS APPROVAL

The study was approved by the ethical committee of the Charité – Universitätsmedizin Berlin (certificate number: EA2/248/18).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/nan.12741.

DATA AVAILABILITY STATEMENT

The methylation data supporting the findings of this study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/, accession number: GSE168808.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1

Figure S2

Table S1

Table S2

Table S3

Table S4

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