

Genotype–phenotype correlation in von Hippel-Lindau disease

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ABSTRACT.

Background/Aims: Retinal haemangioblastomas (RH) remain a major cause of visual impairment in patients with von Hippel-Lindau (VHL) disease. Identification of genotype–phenotype correlation is an important prerequisite for better management, treatment and prognosis.

Methods: Retrospective, single-centre cohort study of 200 VHL patients. Genetic data and date of onset of RH, central nervous system haemangioblastomas (CNSH), pheochromocytoma/paraganglioma (PPGL), clear cell renal cell carcinoma (ccRCC) and pancreatic neuroendocrine neoplasm (PNEN) were collected. The number and locations of RH were recorded.

Results: The first clinical finding occurred at an age of 26 ± 14 years (y) [mean \pm SD]. In $91 \pm 3\%$ (95% CI 88–94) of the patients, at least one RH occur until the age of 60y. A total of 42 different rare *VHL* gene variants in 166 patients were detected. A higher age-related incidence of RH, CNSH, ccRCC and PNEN was detected in patients with a truncating variant (TV) compared to patients with a single amino-acid substitution/deletion (AASD) (all $p < 0.01$), while it is reverse for PPGL ($p < 0.01$). Patients with a TV showed 0.10 ± 0.15 RH per y during their lifetime compared to 0.05 ± 0.07 in patients with AASD ($p < 0.02$). The median enucleation/phthisis-free survival time in patients with a TV was 56y (95% CI 50–62) compared to 78y (95% CI 75–81) in patients with AASD ($p < 0.02$).

Conclusion: Compared to patients with AASD, patients with a TV develop RH, CNSH, ccRCC and PNEN earlier. They experience a higher number of RH and bear a higher risk of enucleation/phthisis. Thus, patients with a TV might be considered for a more intensive ophthalmological monitoring.

Key words: retina – von Hippel-Lindau disease – VHL – haemangioblastoma – genotype –phenotype correlation

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Introduction

Von Hippel-Lindau (VHL) disease is an autosomal dominant inherited phacomatosis with an incidence between 1:27 000 and 1:45 000 (Maher et al. 1991; Maddock et al. 1996; Evans et al. 2010). Von Hippel-Lindau (VHL) is associated with different tumours and cysts in multiple organs like retinal haemangioblastomas (RH), central nervous system haemangioblastomas (CNSH), pheochromocytoma/paraganglioma (PPGL), clear cell renal cell carcinoma (ccRCC) and pancreatic neuroendocrine neoplasm (PNEN). Less frequently, the liver and the reproductive annexes can be affected as well.

A pathogenic germline variant in the *VHL* tumour suppressor gene (3q25.3) can be found in most of the affected patients (Latif et al. 1993). Large/complete deletions, missense, nonsense, frameshift, in-frame and splice variants have been described as pathogenic (Nordstrom-O'Brien et al. 2010). Following a second hit, loss or inactivation of the second, wild-type *VHL* allele, patients develop the disease (Knudson 1971). Inactivation of the *VHL* protein results in accumulation of hypoxia-inducible transcription factor α (HIF α) (Maxwell et al. 1999; Kim & Kaelin 2004), subsequent increased transcription and expression of tumourigenic factors including platelet-derived and vascular endothelial growth factor

(VEGF) (Gnarra et al. 1996; Siemeister et al. 1996) and consequent development of highly vascular tumours (Wizigmann-Voos et al. 1995).

Central nervous system haemangioblastomas (CNSH) and Retinal haemangioblastomas (RH) are the most common tumours in VHL patients (Conway et al. 2001; Lonser et al. 2003; Schmidt & Agostini 2007) and are commonly the onset of disease (Liang et al. 2007). Retinal haemangioblastomas (RH) are benign vascular lesions located in the peripheral retina or juxtapapillary (Singh et al. 2001; Binderup et al. 2018). In the current literature, RH are expected in 50 to 70% of the patients (Webster et al. 1999; Chew 2005; Salama et al. 2019) with increasing probability with age reaching up to 90% in patients over 60 years (Maher et al. 1990; Neumann & Wiestler 1991; Wittebol-Post et al. 1998).

There is significant inter- and intrafamilial phenotypic heterogeneity. Because of the multiorgan involvement, the clinical management, treatment and genetic counselling of VHL patients are complex. A better understanding of genotype–phenotype correlation could, therefore, lead to a more targeted management in the screening examinations and treatment of VHL patients. However, data on the genotype–phenotype correlation of the onset of RH are scarce in the medical literature (Webster et al. 1999). With regard to the ocular involvement of VHL disease, a number of studies describe the genotype–phenotype correlation (Dollfus et al. 2002; Chew 2005; Wong et al. 2007; Wong et al. 2008; Mettu et al. 2010; Hajjaj et al. 2020), some of them with partially contrary results. In the study of Chew (Chew 2005) and Wong et al. (Wong et al. 2007), patients with a complete *VHL* gene deletion developed RH less frequently than those with a single amino-acid substitution. No difference between patients with a truncating variant (variant that shorten the *VHL* protein) and those with a single amino-acid substitution could be found in the study of Wong et al. (2007), whereas Dollfus et al. (2002) described a higher number of RH in patients with a single amino-acid substitution. To investigate these discrepancies, we designed our study to analyse the relationship between

VHL gene variants and the ocular phenotype of the disease.

Methods

Study design and population

This is a single-centre cohort study. In total, 216 patients with clinically expected VHL disease due to positive family history or the presence of VHL typical tumours were routinely examined in our eye centre between January 2019 and January 2020. The complete clinical history of 200 patients with clinically and/or genetically confirmed VHL disease who signed an informed consent could be included. Optical coherence tomography angiography data of the study cohort were published previously (Reich et al. 2020). The study was approved by our institutional Ethics Committee (ID-number 360/19) and adhered to the tenets of the Declaration of Helsinki.

Data collection

Patients underwent a comprehensive ophthalmological routine examination including measurement of best-corrected visual acuity and slit-lamp microscopy with fundus examination. Depending on the tumour and its location, appropriate therapy was initiated if necessary. Demographic, clinical and germline genetic data were collected. The course of disease was retrospectively ascertained through review of medical ophthalmological, nephrological and neurosurgical records. The dates of onset of RH, respectively, CNSH, PPGL, ccRCC and PNEN were collected. The number and location of RH (peripheral or juxtapapillary) were recorded for each eye through review of medical ophthalmological records, fundus photographs and operation reports. Retinal capillary haemangioblastomas which occurred in the area of an already existing, previously treated RH were evaluated as recurrences and were not added to the number of RH per patient.

Genetic subdivision

All patients who fulfilled clinical criteria of VHL (Schmid et al. 2014) were included in the analyses, regardless of the presence of a positive genetic test

result. Molecular genetic testing was conducted by DNA sequencing and gene dosage analysis. Since a molecular genetic testing has often already been performed close to patient's home and since the patients live all over Germany, the molecular genetic data not performed in our internal human genetic laboratory originate from several different external laboratories all over Germany. For molecular genetic testing, DNA sequencing and gene dosage analysis of the *VHL* gene (NCBI/Ensembl gene ID: 7428; reference genome: GRCh37.p13) were conducted. The pathogenicity of the *VHL* gene variant was reviewed via the human gene mutation database (HGMD®). If the genetic test result was known, patients were divided into two groups, patients with a truncating variant or a complete deletion of the *VHL* gene, and patients with a single amino-acid substitution/deletion. If the pathogenicity of detected *VHL* gene variant was uncertain, the patient was not assigned to either of the above groups.

Statistical analysis

SPSS V20.0 was used for statistical analysis. For the descriptive data analysis, mean \pm standard deviation (SD), as well as median, minimal and maximal values (range) were calculated. The number of RH per patient-year was determined by dividing the number of detected RH of a patient by patient's age. For this analysis, only data of patients with completely known ophthalmic clinical history for both eyes were taken into account. To compare between two groups in a non-parametric way, the Mann–Whitney *U*-test was used. To compare between two groups adjusting for potential covariates, a univariate linear model (ANOVA) was used. To analyse the age-dependent penetrance of clinical onset of VHL disease Kaplan–Meier analysis was conducted, including the analysis of mean tumour-free survival time and the corresponding 95% confidence interval (95% CI). To compare between two Kaplan–Meier curves, log-rank test was used, using pairwise comparison if more than two curves were compared. Bonferroni's correction was used due to multiple testing. A probability (*p*) value of $\alpha < 0.05$ was considered statistically significant.

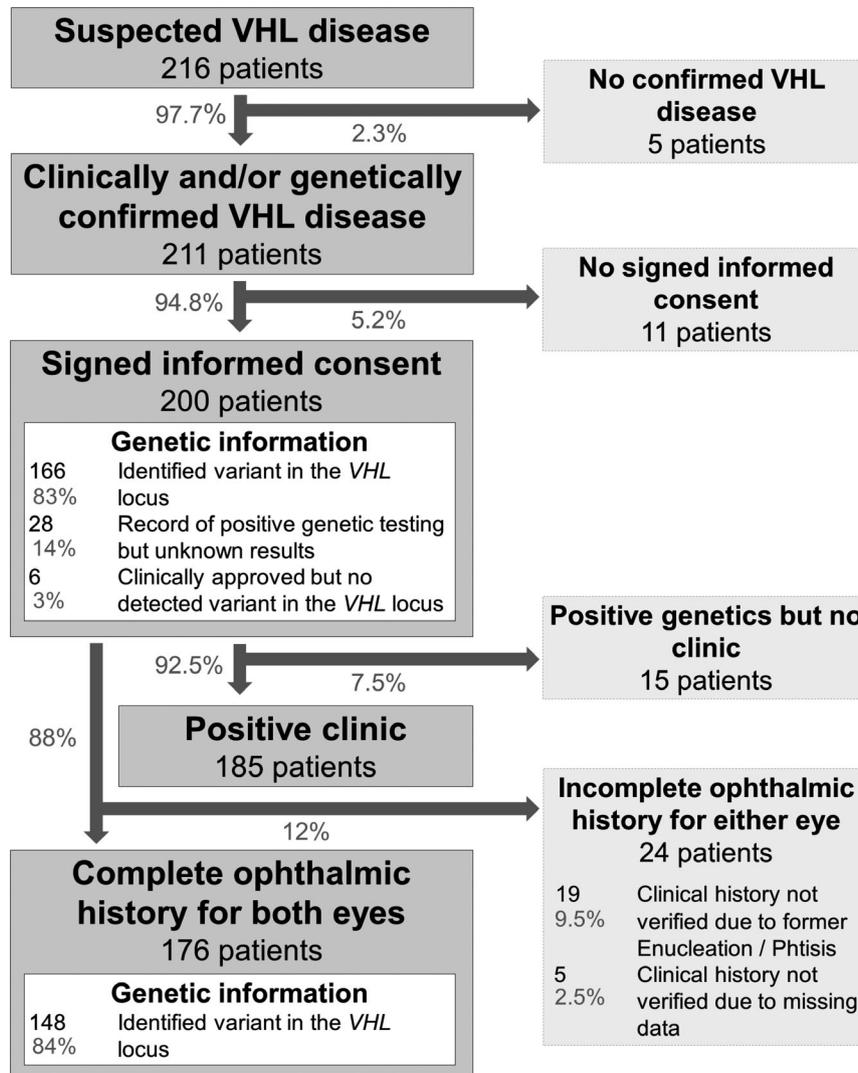


Fig. 1. Flow chart of patients screened and included in the study. VHL = von Hippel-Lindau.

Results

Patient characteristics

Two hundred and sixteen patients with suspected VHL disease were examined between January 2019 and January 2020. Five patients were excluded due to neither clinically nor genetically confirmed VHL disease. Eleven patients were excluded due to missing informed consent. In total, 200 patients (male/female, 79/121, 40/60%), representing 120 families, were included in the study. Details about the data collection are illustrated in Fig. 1. The mean age at the time of the study examination was 39 ± 17 years (y) (median 38, range 7–82). One hundred ninety-two patients (96%) were of European and eight of North African origin (4%).

Table 1. Frequency of clinical findings and age at onset of clinically manifest Hippel-Lindau disease (VHL).

Clinic	Prevalence at onset of clinical VHL (% , n = 185)	Lifetime prevalence (% , n = 200)	Age at onset of clinical VHL [mean ± SD (median, range)]
Retinal haemangioblastoma	79 (42.7)	137 (68.5)	30 ± 15 (28, 6–61)
Haemangioblastoma in central nervous system	77 (41.6)	147 (73.5)	33 ± 13 (31, 9–66)
Pheochromocytoma/paraganglioma	49 (26.5)	72 (36.0)	31 ± 14 (28, 6–59)
Renal			
Clear cell renal cell carcinoma	11 (5.9)	62 (31.0)	36 ± 12 (32, 20–72)
Cysts	19 (10.3)	91 (45.5)	37 ± 13 (36, 13–66)
Pancreatic			
Pancreatic neuroendocrine neoplasm	4 (2.2)	29 (14.5)	36 ± 13 (36, 15–60)
Cysts	22 (11.9)	88 (44.0)	33 ± 13 (30, 12–67)
Liver			
Haemangioblastoma	2 (1.1)	22 (11.0)	39 ± 11 (40, 18–60)
Cysts	3 (1.6)	44 (22.0)	43 ± 11 (45, 20–72)

Onset of disease

The mean age at onset of VHL clinical findings was 26 ± 14 y (median 24, range 6–76). In 15 patients, the diagnosis of *VHL* gene variants was initiated due to positive family history; however, the disease was not clinically apparent (Fig. 1). The mean age of these patients was significantly younger compared to the age of patients having typical VHL findings (20 ± 13 y, median 14, range 7–52 versus 41 ± 16 y, median 40, range 9–82; $p < 0.0001$, Mann–Whitney *U*-test). Table 1 summarizes the frequency of clinical findings at the time of the initiation of VHL disease in the population of 185 patients having typical VHL findings. Furthermore, lifetime frequency of clinical findings and mean age at first onset of these clinical findings with regard to the complete study cohort are shown. At initiation of VHL disease, 54 (29%) patients had multiple VHL typical clinical findings and 131 patients (71%) only one.

Retinal haemangioblastomas (RH) (in 69% of the patients) and CNSH (in 74%) were the most common tumours in our cohort. Figure 2 illustrates the age-dependent probability of RH, CNSH, PPGL, ccRCC and PNEN. Detailed information about the age-dependent probability is listed in Table 2.

Ocular course of disease

One or more RH were seen in one eye in 61 (31%), in both eyes in 75 (38%) patients and 64 patients (32%) did not have any RH. Patients having no RH were younger than patients having RH at least in one eye (33 ± 16 y, median 31, range 7–63 versus 43 ± 16 y, median 44, range 9–82; $p < 0.05$, Mann–Whitney *U*-test). Complete ophthalmic clinical history for both eyes was known in 176 patients (Fig. 1). Five patients were excluded due to missing data and 19 patients due to former enucleation/phthisis (one/both eyes, 18/1). In this subgroup of 176 patients, a total of 414 RH were detected, resulting in a mean number of 2.4 ± 3.5 (median 1, range 0–25) RH per patient. Twenty-two (5%) of these RH were located juxtapapillary with 15 (8%) patients having juxtapapillary RH in one eye and two patients (1%) in both eyes. Three patients (2%) had two

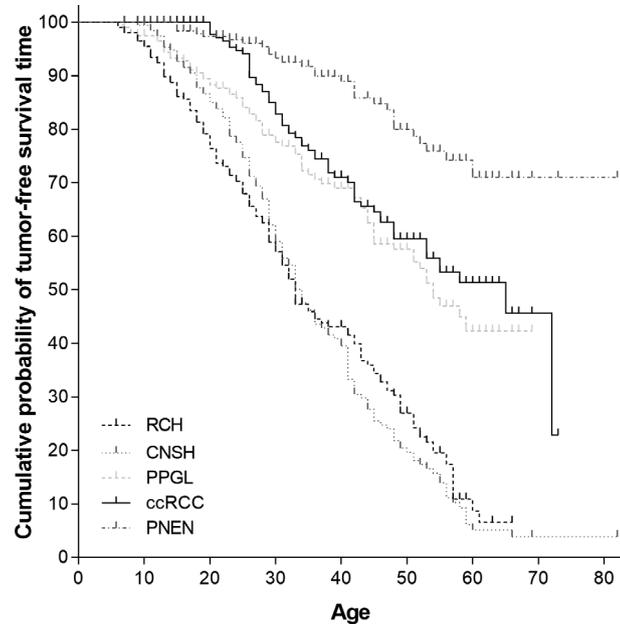


Fig. 2. Age-dependent probability of clinical findings in von Hippel-Lindau disease. Tumour-free survival time of retinal haemangioblastomas (RH, mean 36 years (y), 95% CI 34 to 39y, median 33y, 95%CI 29 to 37y, 137 events, 63 censored), central nervous system haemangioblastomas (CNSH, mean 37y, 95% CI 34 to 39y, median 34y, 95% CI 31 to 37y, 147 events, 53 censored), pheochromocytoma/paraganglioma (PPGL, mean 50.y, 95% CI 47 to 53y, median 54y, 95% CI 48 to 60y, 72 events, 128 censored), clear cell renal cell carcinoma (ccRCC, mean 55y, 95%CI 52 to 58y, median 65y, 95%CI 57 to 73y, 62 events, 138 censored) and pancreatic neuroendocrine neoplasm (mean PNEN, 70y, 95%CI 67 to 74y, median not detectable, 29 events, 171 censored). Log-rank test with pairwise comparison was used for analysis. RH versus PPGL, RH versus ccRCC, RH versus PNEN, CNSH versus PPGL, CNSH versus ccRCC, CNSH versus PNEN, PPGL versus PNEN, ccRCC versus PNEN, all $p < 0.0001$. No difference was detected between RH and CNSH ($p = 1.0$), as well as between PPGL and ccRCC ($p = 0.62$). Bonferroni's correction was used to adjust p-values due to multiple testing. Data of the whole study group ($n = 200$ patients) were used (see Fig. 1).

Table 2. Age-dependent cumulative probability of clinical findings in von Hippel-Lindau disease. More detailed information is given in Fig. 2.

Clinic	Age-dependent cumulative probability (%) [mean (95%CI)]		
	<20 years	<40 years	<60 years
Retinal haemangioblastoma	21 (18–24)	57 (53–61)	91 (88–94)
Haemangioblastoma in central nervous system	15 (12–18)	59 (55–63)	95 (93–97)
Pheochromocytoma/paraganglioma	11 (9–13)	31 (27–35)	58 (53–63)
Clear cell renal cell carcinoma	0 (0–1)	29 (25–33)	49 (44–54)
Pancreatic neuroendocrine neoplasm	3 (2–4)	10 (7–13)	26 (21–31)

juxtapapillary RH in one eye. Mean age and, therefore, mean ocular follow-up of these patients at the time of the data collection was 38 ± 17 y (median 35, range 7–82) resulting in a mean number of 0.07 ± 0.10 RH per patient-year (median 0.03, range 0–0.76).

Genetic influence on the clinical course of disease

The identified variant in the *VHL* locus was known in 166 patients (Fig. 1). In

total, 42 different rare *VHL* gene variants were detected (Table 3). For human gene mutation database (HGMD®) information of the detected mutations see Table S1. A truncating variant was detected in 45 patients (27%), a single amino-acid substitution/deletion in 118 patients (71%) and a synonymous variant of unknown significance (c.93G > A) in a family of three patients (2%). Table 3 summarizes the detected variants in the *VHL* locus in accordance to the number of

Table 3. Detected gene variants in the von Hippel-Lindau disease (*VHL*) locus of the study cohort

<i>VHL</i> gene variant	Protein change	Individuals		RH per patient-year* Mean ± SD (median, range)
		with RH N (%)	without RH N (%)	
TRUNCATING		35 (78) ^{†9}	10 (22)	0.10 ± 0.15 (0.06, 0–0.76)
Deletion		22 (73) ^{†6}	8 (27)	0.09 ± 0.17 (0.03, 0–0.76)
<i>VHL</i> gene deletion		10 (67) ^{†3}	5 (33)	0.09 ± 0.21 (0.02, 0–0.76)
Deletion Exon 1 and 2		0 (0)	1 (100)	0
Deletion Exon 2 and 3		0 (0)	1 (100)	0
Deletion Exon 1		1 (100)	0 (0)	0.10
Deletion Exon 2		3 (100) ^{†1}	0 (0)	0.10 ± 0.02 (0.10, 0.08–0.12)
Deletion Exon 3		8 (89) ^{†2}	1 (11)	0.12 ± 0.14 (0.06, 0–0.40)
Splice		1 (100)	0 (0)	0.12
c.464-2A > G		1 (100)	0 (0)	0.12
Frameshift		3 (75)	1 (25)	0.06 ± 0.01 (0.06, 0.05–0.11)
c.220del	p.Val74Leufs*85	2 (100)	0 (0)	0.06 ± 0.01 (0.06, 0.05–0.08)
c.408del	p.Phe136Leufs*23	0 (0)	1 (100)	0
c.493del	p.Val165Leufs*5	1 (100)	0 (0)	0.11
Nonsense		9 (90) ^{†3}	1 (10)	0.15 ± 0.11 (0.07–0.33)
c.394C > T	p.Gln132X	1 (100)	0 (0)	0.10
c.481C > T	p.Arg161X	2 (100)	0 (0)	0.08 ± 0.01 (0.08, 0.07–0.10)
c.490C > T	p.Gln164X	2 (66.7)	1 (33)	0.15 ± 0.12 (0.15, 0–0.30)
c.548C > A	p.Ser183X	3 (100) ^{†2}	0 (0)	0.33
c.555C > A	p.Tyr185X	1 (100) ^{†1}	0 (0)	/
Single amino-acid substitution/deletion		76 (64) ^{†9}	42 (36)	0.05 ± 0.07 (0.03, 0–0.27)
In frame		3 (100) ^{†2}	0 (0)	0.02
c.227_229del	p.Phe76del	3 (100) ^{†2}	0 (0)	0.02
Missense		73 (64) ^{†7}	42 (36)	0.05 ± 0.07 (0.03, 0–0.27)
c.233A > G	p.Asn78Ser	1 (100)	0 (0)	0.03
c.235C > G	p.Arg79Gly	1 (50)	1 (50)	0.06 ± 0.06 (0.06, 0–0.12)
c.238A > C	p.Ser80Arg	1 (100)	0 (0)	0.09
c.239G > T	p.Ser80Ile	1 (100)	0 (0)	0.27
c.254T > C	p.Leu85Pro	3 (100) ^{†1}	0 (0)	0.12 ± 0.01 (0.12, 0.11–0.13)
c.256C > G	p.Pro86Ala	1 (100)	0 (0)	0.05
c.257C > A	p.Pro86His	2 (50)	2 (50)	0.07 ± 0.07 (0.04, 0–0.18)
c.262T > A	p.Trp88Arg	1 (100) ^{†1}	0 (0)	/
c.266T > C	p.Leu89Pro	2 (67)	1 (33)	0.05 ± 0.04 (0.04, 0–0.10)
c.269A > T	p.Asn90Ile	1 (100)	0 (0)	0.07
c.292T > C	p.Tyr98His	41 (57) ^{†1}	31 (43)	0.05 ± 0.06 (0.03, 0–0.27)
c.319C > G	p.Arg107Gly	3 (75) ^{†1}	1 (25)	0.04 ± 0.04 (0.03, 0–0.10)
c.320G > A	p.Arg107His	4 (80) ^{†1}	1 (20)	0.02 ± 0.01 (0.02, 0–0.03)
c.335A > G	p.Tyr112Cys	1 (100)	0 (0)	0.04
c.386T > C	p.Leu129Pro	1 (100)	0 (0)	0.11
c.388G > A	p.Val130Ile	0 (0)	2 (100)	0
c.395A > C	p.Gln132Pro	0 (0)	1 (100)	0
c.407T > C	p.Phe136Ser	1 (50)	1 (50)	0.07 ± 0.07 (0.07, 0–0.13)
c.461C > T	p.Pro154Leu	1 (100)	0 (0)	0.15
c.463G > C	p.Val155Leu	2 (100)	0 (0)	both 0.22
c.475A > G	p.Lys159Glu	1 (100)	0 (0)	0.02
c.486C > G	p.Cys162Trp	1 (50) ^{†1}	1 (50)	0
c.491A > T	p.Gln164Leu	1 (100)	0 (0)	0.03
c.499C > T	p.Arg167Trp	1 (100) ^{†1}	0 (0)	/
c.562C > G	p.Leu188Val	1 (100)	0 (0)	0.03
Unknown significance				
Synonymous		1 (33)	2 (67)	0.02 ± 0.02 (0, 0–0.05)
c.93G > A	p.Glu31 =	1 (33)	2 (67)	0.02 ± 0.02 (0, 0–0.05)

RH = retinal haemangioblastoma, SD = standard deviation.

[†] N = number of patients with incomplete ophthalmic history for both eyes.

* For analysis only data of patients with complete ophthalmic history for both eyes were used.

detected RH per patient-year for those patients with verified complete ophthalmic clinical history for both eyes (*n* = 148). Mean age and, therefore, mean follow-up of these 148 patients

was 38 ± 17y (median 36, range 7–82). There was no difference in age in the group of patients with a truncating variant compared to the patients with a single amino-acid substitution/deletion

(38 ± 17y, median 38, range 9–73 versus 41 ± 17y, median 40, range 7–82; *p* = 0.33, Mann–Whitney *U*-test). Out of the 18 patients excluded from the analysis due to unverified ophthalmic

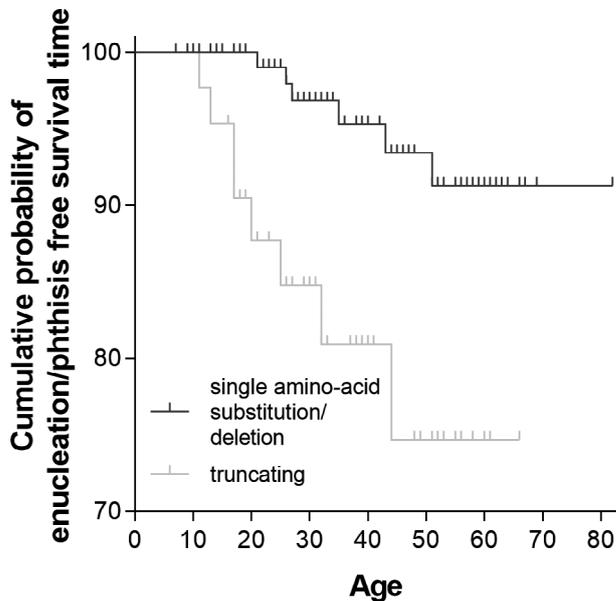


Fig. 3. *VHL* gene variant-dependent risk of enucleation/phthisis of patients with von Hippel-Lindau disease. Mean enucleation/phthisis-free survival time of patients with a single amino-acid substitution/deletion was 78 years (y) (95% CI 75–81y, 6 events, 112 censored), compared to 56y (95% CI 50–62y, 8 events, 37 censored) of patients with a truncating variant ($p < 0.01$). Median enucleation/phthisis-free survival time is not detectable since none of the curves reaches median survival (50%) time. Genetic information was available for 166 patients (see Figure 1). Three patients had a *VHL* gene variant of unknown significance (see Table 3) and, therefore, were excluded from the analyses.

clinical history for both eyes (for genetic information of these patients see Table 2), 14 were excluded due to enucleation/phthisis of at least one eye. Eight of these had a variant assigned to the truncating group (3 patients with *VHL* gene deletion, deletion of exon 3 and nonsense variant c.548C > A each 2 patients, and 1 patient with deletion of exon 2) and six had a single amino-acid substitution/deletion (2 patients with the in-frame deletion c.227_229del, and missense variant c.499C > T, c.319C > G, c.292T > A, c.254T > C each 1 patient). Kaplan–Meier curves are illustrated in Fig. 3. Median enucleation/phthisis-free survival time of patients with a single amino-acid substitution/deletion was 78y (95% CI 75–81y), compared to 56y (95% CI 50–62y) of patients with a truncating variant ($p < 0.01$, Kaplan–Meier analysis, log-rank test). Thus, patients with a truncating variant seem to have a higher risk of enucleation/phthisis.

A higher age-related incidence of RH, CNSH, ccRCC and PNEN was detected in patients with a truncating variant compared to patients with a single amino-acid substitution/deletion (all $p < 0.01$), while it is reverse for PPGL ($p < 0.01$, Kaplan–Meier

analysis, log-rank test, Fig. 4). As patients with a truncating variant have a higher lifetime frequency of RH, they tend to have a higher number of RH during their lifetime ($p < 0.02$, univariate linear model (ANOVA), Fig. 5).

Discussion

Von Hippel-Lindau disease predisposes for different tumours and cysts in multiple organs. Haemangioblastomas of the CNS and in the retina are the most common tumours in patients with *VHL* (Conway et al. 2001; Lonser et al. 2003; Schmidt & Agostini 2007) and are commonly the clinical onset of the disease (Liang et al. 2007). This is also confirmed in our study (Fig. 2, Table 1). Mean age at onset of CNSH and RH in our study was approximately 30 years, which is similar to previous reports (Conway et al. 2001; Lonser et al. 2003; Schmidt & Agostini 2007). However, these tumours can also appear in childhood (Friedrich 2001; Kanno et al. 2013; Findeis-Hosey et al. 2016). Compared to examinations of the CNS, ophthalmic examination can be conducted in young children without the need for complex imaging procedures, which at a young age often

require general anaesthesia. Therefore, the ophthalmologists play an important role in the early diagnosis of the *VHL* disease. The earlier the disease is detected, the earlier therapeutic measures can be initiated, improving the patient's prognosis (Schmidt et al. 2000; Friedrich 2001; Schmidt & Agostini et al. 2007).

Identification of possible genotype–phenotype correlation in *VHL* disease is an important prerequisite for better management, treatment and prognosis (Friedrich 2001). For the genotype–phenotype correlation in our study, we divided our patients into two groups, the ones with truncating variants and the ones with missense variants. The rationale behind this is the simplified assumption truncating variants to lead to a more severe defect, up to a complete loss of the *VHL* protein function, whereas single amino-acid substitutions/deletions may confer minor dysfunctions. However, we are aware that also missense variants in principle could totally abolish protein function and that functional testing would be necessary to rule this possibility out. Despite this caveat, we nonetheless adhered to this simplified classification for comparison as this grouping was used before by several other authors (Dollfus et al. 2002; Wong et al. 2007; Salama et al. 2019). An earlier onset of CNSH in patients with a truncating variant was described, while missense variants predispose for an earlier onset of PPGL (Zbar et al. 1996; Salama et al. 2019). This was also observed in our study (Fig. 4). For RH, such a relationship has not been described in the literature, for example, in the study of Salama et al. (2019), maybe due to a small study cohort of 86 patients. Nevertheless, since there is a close anatomical connection between cells in the brain and in the retina, it is reasonable, that patients with a truncating variant might also show a higher age-dependent penetrance of RH compared to patients with a single amino-acid substitution/deletion. Such a similar relationship between the earlier onset of CNSH and RH in patients with a truncating variant compared to those with a single amino-acid substitution/deletion could be shown in our study (Fig. 4).

With regard to the severity of ocular involvement of *VHL* disease and

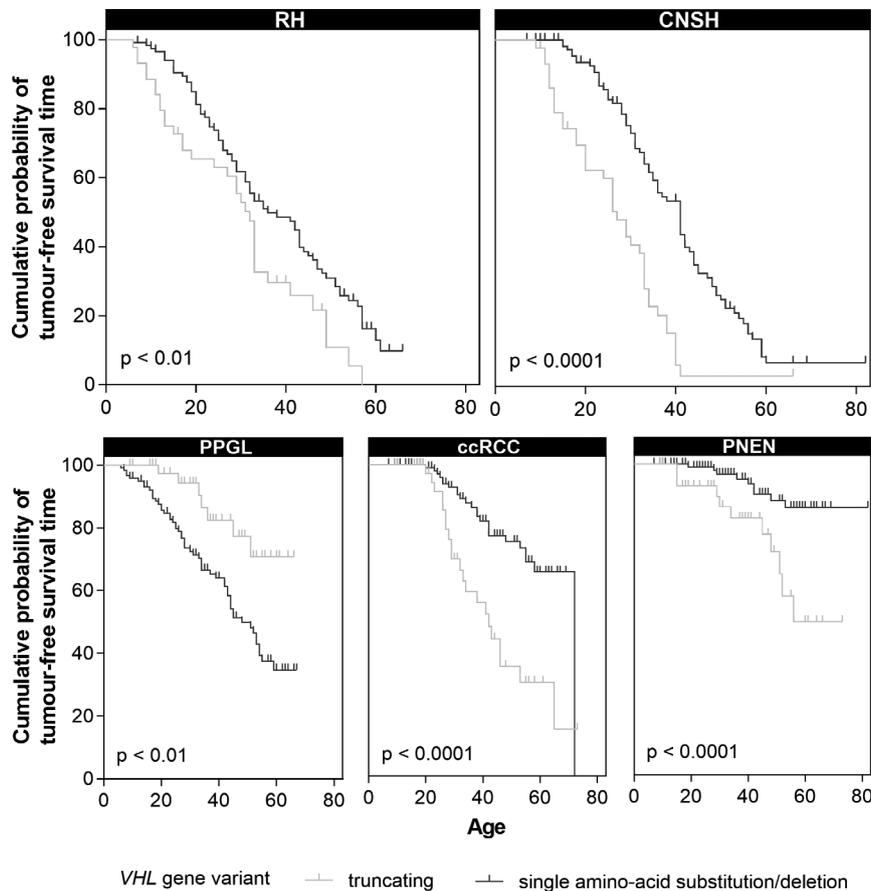


Fig. 4. Age-dependent probability of von Hippel-Lindau disease in patients with a truncating variant compared to patients with a single amino-acid substitution/deletion. Tumour-free survival time was significantly different between patients with a truncating variant (T) and patients with a single amino-acid substitution/deletion (AASD) in all analyses. Retinal haemangioblastomas (RH); T: mean 29 years (y), 95% CI 25–34y, median 32y, 95% CI 29 to 35y, 36 events, 9 censored versus AASD: mean 39y, 95% CI 35 to 42y, median 36y, 95% CI 27–45y, 78 events, 40 censored. Central nerve system haemangioblastoma (CNSH): T: mean 26y, 95% CI 23–30y, median 27y, 95% CI 23–31y, 40 events, 5 censored versus AASD: mean 41y, 95% CI 38–44y, median 41, 95% CI 37–45y, 80 events, 38 censored. Pheochromocytoma/paraganglioma (PPGL): T: mean 58y, 95% CI 53–63y, median not detectable, 7 events, 38 censored versus AASD: mean 46y, 95% CI 42–50y, median 48y 95% CI 41–55y, 54 events, 64 censored. Clear cell renal cell carcinoma (ccRCC): T: mean 45 y, 95% CI 38–51y, median 42y, 95% CI 34–49y, 22 events, 23 censored versus AASD: mean 62y, 95% CI 59–66y, median not detectable, 21 events, 97 censored. Pancreatic neuroendocrine neoplasm (PNEN): T: mean 56y, 95% CI 49–64y, median not detectable, 12 events, 33 censored versus AASD: mean 77 y, 95% CI 73–80y, median not detectable, 8 events, 110 censored. Log-rank test was used for analysis. p-values were not adjusted for multiple testing. Genetic information was available for 166 patients (see Fig. 1). Three patients had a *VHL* gene variant of unknown significance (see Table 3) and, therefore, were excluded from the analyses.

consequential ocular complications, a number of studies describe the genotype–phenotype correlation (Dollfus et al. 2002; Chew 2005; Wong et al. 2007; Wong et al. 2008; Mettu et al. 2010; Hajjaj et al. 2020). For example, the studies of Chew (Chew 2005), and Wong et al. (2007), including 406, and 804 patients respectively, showed a less common ocular involvement and a less severe course of ocular disease in patients with complete deletion of the *VHL* gene. These results could not be confirmed in our study since 67% of our patients with complete *VHL* gene deletion showed ocular involvement. Three out of our 14 patients (21%) with enucleation/phthisis of at least one eye had a complete *VHL* gene

deletion. These observations suggest that this genotype may be associated with more severe disease manifestation and progression. Furthermore, the study of Wong et al. detected no difference regarding the frequency of RH in patients with a truncating variant compared to patients with a single amino-acid substitution, in contrast to the results of our study. This might be explained by the fact that Wong et al. did not adjust their analyses for age, although they detected a significantly younger age of patients with RH and a truncating variant compared to those with a single amino-acid substitution. However, since there is an age-dependent lifetime frequency of RH with more

than 90% of patients affected at an age of 60 years as described in literature (Maher et al. 1990; Neumann & Wiestler 1991) and also in our study (Fig. 2), it is more than likely that patients showing no ocular involvement at the time of the data collection will develop a RH in the future. In our study, patients showing no ocular involvement at the time of the data collection were significantly younger than patients with ocular involvement ($p < 0.0001$). Age is, therefore, a major bias when analysing the number of RH in *VHL* affected patients. Therefore, an age-adjusted data analysis, as conducted in our study, is required when looking for genotype–phenotype correlations (Dollfus et al. 2002).

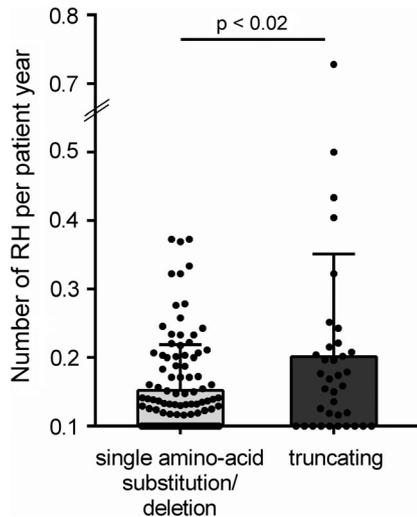


Fig. 5. Comparison of the number of retinal haemangioblastomas per patient-year in patients with a single amino-acid substitution/deletion, compared to patients with a truncating variant. Mean number of retinal haemangioblastomas (RH) per patient-year in 109 patients with an amino-acid substitution/deletion was 0.05 ± 0.07 (median 0.03, range 0–0.27), compared to 0.10 ± 0.15 (median 0.06, range 0–0.76) RH per patient-year in 36 patients with a truncating variant ($p < 0.02$). Age and gender-adjusted univariate linear model (ANOVA) was used for analysis. Both covariates, age and gender, showed a p -value > 0.05 . Only, data of patients with complete ophthalmic history for both eyes and identified variant in the *VHL* locus were used for analyses ($n = 148$, see Fig. 1). Three patients had a *VHL* gene variant of unknown significance (see Table 3) and, therefore, were excluded from the analyses.

In our study, there is a trend for truncating variants to predispose for a higher number of RH during lifetime (Fig. 5, $p < 0.02$). This trend is contrary to the study by Dollfus et al. (Dollfus et al. 2002), who in 196 patients with a mean follow-up of 8.4 years detected a 2.1 higher number of RH in patients who had a substitution variant compared to those with a truncating variant. Like Dollfus et al. (2002), we also used an age-adjusted statistical model for analyses. By reconstructing the complete ophthalmic history of 176 patients, we were able to include an exceptionally long period of individual medical data resulting in a mean follow-up of 38 years. To the best of our knowledge, there is no comparable data set regarding the lifetime frequency of the total number of tumours per patient. It was hypothesized that the risk of developing highly vascular RH might be linked

with the degree of which HIF regulation is compromised. Patients with truncating variants might have forms of the *VHL* protein that are grossly defective with respect to HIF α regulation and consequently increased HIF transcriptional activity of target genes including VEGF (Wizigmann-Voos et al. 1995; Gnarrar et al. 1996; Siemeister et al. 1996). This has been shown for ccRCC (Knauth et al. 2006). Nevertheless, more studies with long-time follow-up and a higher number of patients (possibly by means of multi-centre studies) are needed to further analyse the possible correlation between truncating variants and risk of ocular involvement in *VHL* disease.

Since patients with a truncating variant compared to those with a single amino-acid substitution/deletion tend to have a higher lifetime frequency of the total number of RH, it seems reasonable that these patients also show a higher risk of ocular complications. Nevertheless, the study of Hajjaj et al. (2020) reported higher frequency of progression-related complications in patients with missense variants. However, the study is based on a small cohort of 21 patients. In our study, patients with a truncating variant showed a higher risk of enucleation/phthisis ($p < 0.02$). Detailed information regarding complications during the course of disease was not collected in our study. Therefore, a more precise statement about the relationship between *VHL* gene variant and complication rate based on our data is not possible.

Our study has some limitations such as the relatively disproportionate presence of missense variants. By far, the most common genetic variant of our patients was the missense ‘Black Forest’ variant (c.292T > C), present in 43% of patients with known *VHL* gene variants. This is due to the geographic location of our clinic to an area with endemic *VHL* gene variant. The distribution of different genetic variants among our *VHL* patient cohort is, therefore, shifted in favour to missense variants (69%) compared to cohorts described in other parts of Europe, Japan and North America where missense variants account for 45%–49% (Zbar et al. 1996; Salama et al. 2019). Although number of RH during lifetime was similar in patients with the c.292T > C variant compared to the

group of other missense variants ($p = 0.23$, data not shown), the relatively disproportionate representation of the patient group with missense variants, especially the c.292T > C variant, must, therefore, be taken into account when interpreting our results. The study of Mettu et al. (Mettu et al. 2010), for example, including 412 patients with *VHL* and missense *VHL* gene variant, showed that missense variants in the α -domain of the gene are associated with a higher frequency of RH compared to variants in the β -domain. Such a subgroup analysis in our study was not possible due to a smaller cohort size.

In summary, compared to patients with a single amino-acid substitution/deletion of the *VHL* gene, patients with a truncating variant develop RH, CNSH, ccRCC and PNEN earlier. They additionally tend to experience a higher number of RH during their lifetime and bear a higher risk of enucleation/phthisis. Thus, patients with a truncating variant might be considered for a more intensive ophthalmological screening.

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Ethics Committee approval was obtained (ID number 360/19). Informed written consent was obtained from all VHL patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Human gene mutation database (HGMD[®]) information of the detected mutations in the von Hippel-Lindau disease (*VHL*) locus of the study cohort.