Genotype-Phenotype Correlation in von Hippel-Lindau Disease With Retinal Angiomatosis

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Objectives: To characterize the germline mutations found in a large population of persons having von Hippel-Lindau (VHL) disease mutations with the clinical characteristics of associated retinal capillary hemangioblastomas (RCHs), to measure the prevalence of RCHs among patients with VHL disease generally and specifically for each genotype category, to establish genotype-phenotype correlations between genotype category and phenotypic features of ocular VHL disease, and to establish genotype-phenotype correlations between genotype category and visual function.

Methods: Cross-sectional and molecular genetic study. Of 890 patients with VHL disease, 335 had ocular involvement in the form of RCHs. Statistical analysis was used to correlate the structure of the mutated VHL protein with the ocular phenotype.

Results: Three genotype categories (amino acid substitutions, protein-truncating mutations, and complete deletions of VHL protein) were defined in all patients. The prevalence of RCHs was lowest (14.5%) among patients with complete deletions; the overall prevalence of retinal angiomatosis was 37.2%. Genotype category had no correlation with the unilaterality or bilaterality of ocular disease or with the number or extent of peripheral RCHs. The prevalence of RCHs at the juxtapapillary location was lower among patients with protein-truncating mutations compared with those with amino acid substitutions. Complete deletions were associated with the highest mean visual acuity compared with the other 2 genotype categories.

Conclusion: Patients with complete deletions of VHL protein have the lowest prevalence of ocular disease and the most favorable visual outcome.

Clinical Relevance: The VHL mutation genotype may be used to predict the prevalence and outcome of ocular VHL disease and to guide ophthalmic follow-up.

von Hippel-Lindau (VHL) disease (Online Mendelian Inheritance in Man 193300) is an uncommon (1 in 36,000 live births), multisystem, dominantly inherited cancer syndrome that predisposes affected persons to the development of benign and malignant tumors in the kidney, pancreas, adrenal gland, endolymphatic sac, epididymis and broad ligament, and retina and central nervous system.1 In the retina, VHL disease may result in the formation of benign solitary or multiple retinal capillary hemangioblastomas (RCHs) that have a globular red-pink appearance with prominent dilated feeding and draining vessels.2 Often slow-growing, RCHs in VHL disease (through their exudative or tractional secondary effects) can result in vision loss, structural disruption of the retina and globe, and loss of the eye.3 Treatment, especially at an early stage for a small RCH, may limit vision loss.4,5 Because RCHs may progress or develop de novo over time, annual ophthalmic evaluation is advised for all patients with VHL disease.

von Hippel-Lindau disease results from dominantly inherited germine mutations in a ubiquitously expressed tumor suppressor gene located on chromosome 3p26.6 Patients mostly inherit a mutated copy from an affected parent through the germline and subsequently develop in susceptible tissues a second somatic mutation in another VHL allele. Cells having both a germline and a somatic mutation then become tumorous, according to the 2-hit hypothesis by Knudson.7 The disease is highly penetrant, and almost all patients having a germline VHL mutation develop disease characteristics during the course of their lifetime.8 With the identification of the VHL gene in 1993, germline mutations can be identified in almost all VHL disease pedi-
degrees.9 Encoding a novel protein, the mechanism by which mutations in VHL result in the range of phenotypes seen in VHL disease is incompletely understood.10 There is much variability in the nature of the documented mutations (ranging from single amino acid substitutions to complete deletions of the gene) and in the manifestations of the disease phenotype.11 Genotype-phenotype correlations relating the nature of mutations to the tissue distribution of disease have been performed and have generated some hypotheses about how VHL protein may function.12,13 However, large-scale correlation studies14,15 focusing on ocular manifestations remain few.

Using the largest population of patients with VHL disease collected to date (to our knowledge), our objectives were (1) to characterize the germline mutations found in this large population, (2) to measure the prevalence of RCHs among patients with VHL disease generally and specifically for each genotype category, (3) to establish genotype-phenotype correlations between genotype category and phenotypic features of ocular VHL disease, and (4) to establish genotype-phenotype correlations between genotype category and visual function. These findings may help us to better understand how VHL mutations affect the occurrence and progression of RCHs in the eye and to assist clinicians in using genotype information for the prognosis and monitoring of ocular disease in patients with VHL disease.

METHODS

PATIENT SELECTION AND ASCERTAINMENT OF VHL DISEASE

All patients referred to the study were enrolled in a study protocol at the National Cancer Institute from October 1988 to August 2005. Patients were systematically evaluated at this single center by history and physical examination, laboratory evaluation, and radiographic studies (computed tomography or magnetic resonance imaging) of the abdomen, pelvis, brain, and spine. Patients were also evaluated with a complete ophthalmologic workup with slitlamp examination, indirect funduscopy, and, when clinically indicated, fluorescein angiography.

Eight hundred ninety patients met the clinical diagnostic criteria of VHL disease based on genetic testing, physical examination, imaging studies, or pathologic findings and were admitted to the study. Of these, 335 patients were confirmed to have a history of ocular involvement from VHL, either by history and physical examination, laboratory evaluation, or present evidence of retinal angiomatosis in at least 1 eye. Patients were followed up longitudinally, and treatments, if necessary, were delivered. Owing to the cross-sectional nature of this study, each patient's most recent visit was designated as the study visit.

GENOTYPE ANALYSIS

Analysis for VHL mutations was carried out on peripheral blood samples from at least 1 member of each kindred as previously described.9 Mutations were classified into 1 of the following groups: splice, missense, nonsense, partial deletion, complete deletion, and amino acid deletion (microdeletions in multiples of 3). These were further classified into 3 main genotype categories according to their expected effect on protein structure: missense mutations were classified as single amino acid substitutions; frameshift, nonsense, and partial deletion mutations as protein-truncating mutations; and complete deletions of the coding sequence as complete deletions. Amino acid mutations and splice mutations were not assigned to any subgroup, as their effect on the structure of VHL protein is uncertain.

ASSESSMENT OF RETINAL PHENOTYPE

Patients with retinal angiomatosis were interviewed about whether they had a history of ocular involvement from VHL, the date of onset of eye findings, and any prior therapy. Ocular examination included best-corrected visual acuity, intraocular pressure measurements, slitlamp examination, and indirect ophthalmoscopic funduscopy. Both eyes in each patient were examined, including healthy eyes with no VHL ocular lesions, eyes that had been enucleated because of complications of retinal angiomas, and phthisical or prephthisical eyes for which a view of the posterior pole was often unavailable. For eyes that could be evaluated by funduscopy, the number of RCHs was counted, the location of RCHs was noted (juxtapapillary, macular, or peripheral), and the extent of retinal involvement was recorded (extending to ≥1 quadrant of the retina). Tumors were described as optic nerve tumors if they were located on or were touching the optic nerve, as macular tumors if they were located within the vascular arcades, and as peripheral tumors if they were located peripheral to the arcades and twice the fovea-to-disc distance from the optic nerve.

STATISTICAL ANALYSIS

Age-adjusted logistic regression analysis and analysis of variance were used to assess the relationship between the germline mutations and age, visual function, and the clinical phenotypes of the patients included in the analysis. Because outcomes occurred in familial clusters of varying sizes, multiple outputation10 was applied for all comparisons to account for intrafamily correlation and family size. For the outputation technique, it was determined that 2000 resamplings within a family were optimal for drawing inferences from these data. Analysis of variance was performed to evaluate the differences in age and the differences in mean visual acuity among the 3 genotype categories. The clinical phenotypes that were analyzed using logistic regression included presence or absence of RCH, laterality of angiomas, presence of severe ocular involvement, location of retinal angiomas, number of peripheral RCHs, and extent of peripheral involvement. Given that the mean ages were significantly different among the 3 mutation classes, all analyses were adjusted for age. Prevalence rates were calculated by dividing the number of patients with a phenotype by the total number of patients.

GENOTYPE ANALYSIS

Germline VHL mutations were characterized in 873 (98.1%) of 890 patients (with and without retinal angiomas) included in the study. In the remaining patients, germline VHL mutations were not found. The distribution of patients in each mutation category according to DNA sequence and in each genotype category according to effect on protein structure is given in Table 1. Among the entire group of patients, 834 (93.7%) had mutations that have known effects on VHL protein and could be placed in 1 of 3 genotype categories of amino acid substitutions, protein-truncating mutations, or complete deletions. For the remainder of the study, genotype-
phenotype correlations were performed among these 3 genotype categories and the patients’ demographic, ocular, and visual function phenotypes.

GENOTYPE CATEGORY AND THE PREVALENCE OF RETINAL ANGIOMATOSIS

Among 834 patients in the 3 major genotype categories, approximately one third of patients (37.2% [310/834]) had a history or clinical evidence of retinal angiomatosis in at least 1 eye. This compares similarly with the overall prevalence of retinal angiomatosis (37.6% [335/890]) when all patients in the study regardless of genotype were considered. The prevalences of retinal angiomatosis in the 3 genotype categories were as follows: amino acid substitutions, 38.0% (159/418); protein-truncating mutations, 40.1% (142/354); and complete deletions, 14.5% (9/62). These analyses were adjusted for age, sex, and familial relationships.

We examined the question of whether the prevalence of retinal angiomatosis was affected by genotype category. The results summarized in Table 2 and Figure 1 demonstrate that the prevalence of RCHs is significantly lower among patients with complete deletions of VHL protein compared with those with amino acid substitutions and protein-truncating mutations. The odds ratio of having RCHs vs not having RCHs was about 5 to 6 times greater among patients with amino acid substitutions and protein-truncating mutations compared with those with complete deletions, indicating a clear effect of mutation genotype on RCH prevalence.

GENOTYPE CATEGORY AND DEMOGRAPHICS OF PATIENTS WITH RETINAL ANGIOMATOSIS

The demographic features of 310 patients in the 3 genotype categories are as follows: mean ± SD age, 37.1 ± 13.9 years (age range, 8.6-84.3 years); sex, 142 male and 168 female (male-female ratio, 1:1.18); and self-reported race/ethnicity, 284 white, 15 Hispanic, 6 Asian, and 5 black. We examined the possible correlation between genotype category and the demographic categories of age, sex, and race/ethnicity in this group of patients. For sex, there was no evidence to reject the null hypothesis that all genotypes had a similar prevalence (P = .50 for all pairwise comparisons). For age, the cross-sectional ages (with 95% confidence limits) for the 3 major genotype categories are shown in Figure 2. The difference in the mean ages of patients with amino acid substitutions (39.2 years) and protein-truncating mutations (34.6 years) was statistically significant (P < .001). The mean age of patients with complete deletions (38.3 years) was not significantly different from the mean ages in the other categories (P = .34 when compared with protein-truncating mutations; and

Table 2. Genotype Analysis of 890 Patients With von Hippel-Lindau Disease (With and Without Retinal Angiomatosis)

<table>
<thead>
<tr>
<th>Germline Mutation</th>
<th>No. (%) of Patients</th>
<th>Genotype Category</th>
<th>No. (%) of Patients</th>
<th>No. (%) of Pedigrees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense</td>
<td>418 (47.0)</td>
<td>Amino acid substitution</td>
<td>418 (47.0)</td>
<td>138 (42.2)</td>
</tr>
<tr>
<td>Partial deletion</td>
<td>218 (24.5)</td>
<td>Protein-truncating mutation</td>
<td>354 (39.8)</td>
<td>128 (39.1)</td>
</tr>
<tr>
<td>Non-sense</td>
<td>70 (7.9)</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Frameshift</td>
<td>66 (7.4)</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Complete deletion</td>
<td>62 (7.0)</td>
<td>Complete deletion of VHL protein</td>
<td>62 (7.0)</td>
<td>23 (7.0)</td>
</tr>
<tr>
<td>Splice</td>
<td>21 (2.4)</td>
<td>Undefined effect</td>
<td>38 (4.3)</td>
<td>22 (6.7)</td>
</tr>
<tr>
<td>Amino acid deletion</td>
<td>17 (1.9)</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Unknown</td>
<td>18 (2.0)</td>
<td>Unknown</td>
<td>18 (2.0)</td>
<td>16 (4.9)</td>
</tr>
<tr>
<td>Total*</td>
<td>890 (100.1)</td>
<td>Total*</td>
<td>890 (100.1)</td>
<td>327 (99.9)</td>
</tr>
</tbody>
</table>

*Due to rounding percentages do not total 100%.

Table 2. Comparison of the Prevalence of Retinal Angiomatosis by Genotype Category

| Comparison | Odds Ratio (95% Confidence Limits) for the Presence of Retinal Capillary Hemangioblastomas | P  
|------------|-----------------------------------------------------------------------------------------------|---
| Amino acid substitution vs protein-truncating mutation | 1.01 (0.67-1.54) | .95 |
| Amino acid substitution vs complete deletion of VHL protein | 5.85 (2.51-13.65) | <.001 |
| Protein-truncating mutation vs complete deletion of VHL protein | 5.77 (2.45-13.59) | <.001 |

Abbreviation: VHL, von Hippel-Lindau disease.

Figure 1. Prevalence of retinal angiomatosis (in ≥1 eye) among patients with von Hippel-Lindau disease in the 3 genotype categories. * Indicates a significantly lower prevalence among patients with complete deletions compared with patients with amino acid substitutions and protein-truncating mutations (P < .001).
GENOTYPE CATEGORY AND OCULAR PHENOTYPE IN EYES WITH RETINAL ANGIOMATOSIS

The ocular phenotypes of 310 patients were documented and categorized according to phenotypic features as summarized in Table 3. These phenotypic features illustrate the nature, extent, and severity of retinal angiomatosis in affected eyes.

Approximately 60% of the patients in this subset had retinal angiomatosis in both eyes; the remainder had only 1 eye affected. The prevalences of bilateral involvement among the 3 genotype categories were similar (Table 4), and the odds of having bilateral involvement vs unilateral involvement did not differ statistically among the categories (P > .60 for all pairwise comparisons). Therefore, genotype category had no correlation with whether 1 or both eyes had angiomatosis.

Both eyes in each patient were examined. Some eyes had been enucleated secondary to complications of severe retinal angiomatosis. Other eyes had undergone structural disruption in the form of total retinal detachment, massive subretinal exudation, and phthisical changes that prevented visualization of the posterior pole. These eyes were collectively referred to as having severe ocular involvement to distinguish them from eyes in which the posterior pole was intact and individual RCHs were visualized and counted. These eyes had poor (<20/160) or no visual acuity. About 1 in 5 patients had 1 or both eyes that were severely affected. Genotype correlation analysis did not produce evidence to reject the null hypothesis that all genotypes have a similar rate of severe involvement in at least 1 eye (P = .60 for amino acid substitutions vs protein-truncating mutations). However, none of the 9 patients in the complete deletions category had severely affected eyes (Table 4). Owing to the few patients in this genotype category, the statistical significance of this rate could not be established.

Retinal capillary hemangioblastomas were located in 2 fundus locations, the peripheral retina and the juxtapapillary area (referred to as optic nerve involvement). The predilection of RCHs to occur in these locations and to avoid the macular retina is not understood. The location of RCHs is of clinical and functional significance as, unlike peripheral RCHs, juxtapapillary RCHs often cannot be safely treated using commonly available treatment modalities of laser photocoagulation and cryotherapy. The growth and exudative behavior of juxtapapillary lesions, left unchecked, may in some cases lead to vision loss and structural disruption to the posterior pole more frequently than peripheral lesions. Approximately 1 in 5 patients having at least 1 eye affected by retinal angiomatosis have a juxtapapillary tumor in at least 1 eye. On the level of the individual eye, about 1 in 6 eyes affected by retinal angiomatosis have a juxtapapillary tumor (W.T.W., unpublished data, August 2006). We examined the effect of genotype category on the location of RCHs. The frequency of having a juxtapapillary tumor was lowest among patients with protein-truncating mutations (14.1%), and this was statistically lower than the frequency among patients with amino acid substitutions (24.5%; P = .007) but was not distinct from the frequency among patients with complete deletions (22.2%; P = .19) (Figure 3).

Retinal capillary hemangioblastomas located in the peripheral retina were found in most patients in at least 1 eye (n = 269). In these eyes, individual hemangioblastomas were counted, and the extent of peripheral retinal involvement was scored. We examined whether genotype category affected the number of peripheral RCHs or the extent of peripheral involvement in this group of patients. We correlated genotype category with the proportions of patients having at least 3 RCHs and having at least 5 RCHs in either eye (Table 4). There were no statistically significant differences among the genotype categories for the number or extent of peripheral lesions (P > .30 for all comparisons).
Visual acuities (in letters on the Early Treatment of Diabetic Retinopathy Study chart) in both eyes were separately evaluated for patients in the 3 genotype categories (eyes that had been enucleated were given a score of 0). The mean visual acuities in the better- and worse-seeing eyes of 310 patients were correlated separately with genotype category (Figure 4) and were adjusted for age and familial relationships. In the better-seeing eye, the mean visual acuities in all 3 genotype categories were similar and did not vary statistically from each other ($P_{H11022}$ for all comparisons). In the worse-seeing eye, patients with complete deletions had a statistically higher visual acuity score (84.7 letters) than patients with either amino acid substitutions (54.9 letters; $P_{H11022}$=.01) or complete deletions (51.7 letters; $P_{H11022}$=.01).

The findings from the present study are drawn from the largest group of patients with VHL disease thus far assembled in the literature, to our knowledge. These patients had been referred to the study as a result of systemic findings of VHL disease rather than ocular findings or complaints. They had also been evaluated systemically in a single center, and germline genotype information was obtained in almost all patients. The nature of ascertainment of the patients, the systemic clinical diagnosis of VHL disease, and the comprehensive genotype analysis of patients provide a comprehensive evaluation of retinal angiogenesis in VHL disease.

The prevalence of retinal angiogenesis among our study population was 37.2%, slightly lower than the previously reported prevalences of 49% to 68% for ocular involvement.14,19,20 Our study focused only on hemangioblastomas in the retina and excluded tumors in the retrobulbar optic nerve and nonangiomatic retinal lesions that appear as retinal neovascularization.21 Our method of ascertainment did not draw primarily from patients referred from eye centers with ocular findings or who had complaints but rather from patients with systemic VHL disease. As such, the lower prevalence of retinal angiogenesis in this study may reflect more accurately the occurrence of RCHs among all patients with VHL disease as a whole.

We chose to analyze genotype mutations in VHL according to their effect on protein structure. The 3 genotype categories of amino acid substitutions, protein-truncating mutations, and complete deletions of VHL protein provide a comparison of how ocular phenotypes may differ when a single residue on the protein is altered or when only part or none of the protein is present. These genotype categories were previously used for genotype-
phenotype correlations for ocular disease\textsuperscript{14,15} and for other systemic associations.\textsuperscript{22} Because members in the same pedigree share not only the same genotype category but also many other genes that may affect the ocular phenotype, our statistical analyses of genotype-phenotype correlations consider familial relationships (Corbin, MD, unpublished data, October 2005). The objectives for defining the correlations in this study were to disclose possible mechanisms by which VHL mutations may result in retinal disease and to provide ways in which clinicians may use genotype information for the prognosis and monitoring of eye disease.

Our analyses indicate that the prevalence of RCHs among patients with VHL disease is significantly lower among those with complete deletions compared with those with the other genotype categories. The reason the complete absence of VHL protein from an allele results in less frequent eye disease than a focally mutated or a truncated protein is not clear. It is possible that adjacent sequences flanking VHL that are also lost in large deletions are important for the production of RCHs. Alternatively, the complete loss of a VHL allele in all cells may produce compensatory mechanisms in the retina that partially protect it from the formation of RCHs.

Genotype category correlated slightly but significantly with age. Amino acid substitutions were found among a slightly older distribution of patients compared with those with protein-truncating mutations. Because the overall prevalence of RCH is not different among the 2 genotype categories, protein-truncating mutations (perhaps resulting in lower levels of residual VHL function) may cause the RCH phenotype to emerge at a slightly younger age.

On the whole, genotype category had little correlation with the overall phenotype of RCHs. Previous studies\textsuperscript{14,15} found few differences between genotype and various aspects of ocular phenotype. In our study, genotype category did not significantly affect whether 1 or both eyes were affected by RCHs, how many peripheral RCHs were found, and how much area of the retina was occupied. However, the presence of protein-truncating mutations was associated with a lower prevalence of juxtapapillary lesions. The mechanism underlying this trend and the functional significance of this finding are not clear.

Genotype category did not correlate with the visual acuity of the better-seeing eye of affected patients. Many better-seeing eyes of affected patients lack any RCH involvement, and most had good visual acuity (range, 20/20 to 20/40). However, all of the worse-seeing eyes of affected patients had RCHs, and some had significantly compromised vision. In these eyes, amino acid substitutions and protein-truncating mutations were associated with a significantly lower level of visual acuity compared with complete deletions. Although these eyes did not differ significantly in RCH number or area involved by the genotype, the natural history of RCHs in patients with complete deletions may be more favorable (ie, have less exudative behavior or result in fewer tractional complications) compared with the other genotype categories, resulting in better preservation of visual function.

As a whole, patients in our study with complete deletions developed RCHs less frequently (and with lower overall visual morbidity) than patients with the other genotype categories. The genetic and cellular mechanisms underlying this difference may arise from additional deleterious gain-of-function properties that are absent in complete deletions but are present in the other mutations. Alternatively, the complete absence of protein or flanking genetic regions may produce compensatory mechanisms that ameliorate the ocular phenotype.

In the present age of genetic diagnosis and counseling, ophthalmologists are often consulted when a patient is found to have a germline VHL mutation of a certain genotype category.\textsuperscript{23} This study provides data for helping clinicians counsel about the risk of developing retinal angiomatosis and about the resulting visual morbidity for each genotype category. Although multiple screening protocols exist for the surveillance of patients
with or at risk for VHL disease. Future protocols may be formulated that take into account the prognostic information provided by genotype analysis.

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