

# An Analysis of Phenotypic Variation in the Familial Cancer Syndrome von Hippel–Lindau Disease: Evidence for Modifier Effects

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## Summary

von Hippel–Lindau disease (VHL) is a dominantly inherited familial cancer syndrome predisposing to ocular and CNS hemangioblastomas, renal-cell carcinoma (RCC), and pheochromocytoma. Both interfamilial and intrafamilial variability in expression is well recognized. Interfamilial differences in pheochromocytoma susceptibility have been attributed to allelic heterogeneity such that specific missense germ-line mutations confer a high risk for this complication. However, in most cases, tumor susceptibility does not appear to be influenced by the type of underlying *VHL* mutation. To probe the causes of phenotypic variation, we examined 183 individuals with germ-line *VHL* gene mutations, for the presence and number of ocular tumors. The prevalence of ocular angiomas did not increase with age, and the distribution of these tumors in gene carriers was significantly different than the expected stochastic distributions. Individuals with ocular hemangioblastomas had a significantly increased incidence of cerebellar hemangioblastoma and RCC (hazard ratios 2.3 and 4.0, respectively). The number of ocular tumors was significantly correlated in individuals of  $\frac{1}{2}$  degree relatedness but not in more distantly related individuals. These findings suggest that the development of VHL ocular tumors is determined at an early age and is influenced by genetic and/or environmental modifier effects that act at multiple sites. Functional polymorphisms in the glutathione-S-transferase M1 gene (*GSTM1*) or the cytochrome P450 2D6 gene (*CYP2D6*) did not show a significant association with the severity of ocular or renal involvement.

## Introduction

Familial cancer syndromes, although rare, offer a unique opportunity to identify the causal factors in human tumorigenesis. Most familial cancer syndromes result from germ-line mutations in tumor-suppressor genes, and the identification of these genes has provided novel insights into human tumorigenesis. Variability in clinical expression is characteristic of these syndromes. In many cases, phenotypic variation has been correlated with allelic heterogeneity. In addition, genetic or environmental modifier effects have been implicated in some disorders. In a mouse model of familial polyposis coli, a modifying locus has been identified (*Mom-1*) (Dietrich et al. 1993; Gould et al. 1996), and the risk of ovarian cancer in human subjects with *BRCA1* mutations appears to be modified by allelic variation at the *H-RAS* locus (Phelan et al. 1996). In a large study of affected relative pairs with neurofibromatosis type 1, there was significant evidence for modifier effects on the number of café au lait spots and neurofibromas (Easton et al. 1993).

von Hippel–Lindau (VHL) disease (OMIM 193300) is a rare familial cancer syndrome causing susceptibility to benign vascular tumors of the eye and CNS (angioma or hemangioblastoma), renal-cell carcinoma (RCC), and pheochromocytoma (Maher et al. 1990; Maher and Kaelin 1997). The gene responsible for VHL has been characterized (Latif et al. 1993) and has been shown to encode a protein of 213 amino acids that is ubiquitously expressed in fetal and adult tissues (Richards et al. 1996). Germ-line mutations have been detected in ~500 VHL kindred and include deletions of part or whole of the gene, as well as intragenic point mutations and microdeletions/insertions (Crossey et al. 1994b; Whaley et al. 1994; Chen et al. 1995; Zbar et al. 1996). Somatic inactivating mutations have been identified in tumors (e.g., cerebellar hemangioblastoma) from VHL-affected individuals (Prowse et al. 1997) and in sporadic clear-cell RCC and cerebellar hemangioblastoma (Foster et al. 1994; Gnarr et al. 1994; Kanno et al. 1994; Oberstraß et al. 1996). Loss of the wild-type VHL allele is frequent in tumors (including RCC, cerebellar hemangioblas-

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toma, and pheochromocytoma) from VHL patients, and VHL methylation occurs both in VHL tumors (RCC and cerebellar hemangioblastoma) and in sporadic RCC (Crossey et al. 1994a; Herman et al. 1994; Prowse et al. 1997; Clifford et al., in press). Interfamilial differences in predisposition to pheochromocytoma in VHL disease correlate with the type of germ-line mutation. Missense VHL gene mutations confer a high susceptibility to pheochromocytoma, and large deletions or protein-truncating mutations confer a low risk (Crossey et al. 1994b; Chen et al. 1995; Maher et al. 1996). However, allelic heterogeneity does not appear to explain the majority of the tumor-susceptibility variation between individuals affected by VHL disease (Maher et al. 1996; A. R. Webster, F. M. Richards, A. T. Moore, and E. R. Maher, unpublished data).

Unlike other organs affected by VHL disease, the eye is readily accessible to examination by specialists and can be scrutinized with high magnification to detect lesions as small as 100  $\mu$ m in diameter. Furthermore, because these tumors are benign, the presence and position of previously treated lesions can also be documented. This allows a unique opportunity for in vivo assessment of the presence and severity of one manifestation of a familial cancer syndrome. Using this method, we have (i) examined the influence of sex and age on VHL ocular angiomas; (ii) determined whether the presence of ocular angiomas in an affected individual affects the risk of tumors at other sites; (iii) compared the distributions of the number of angiomas versus theoretical distributions derived from the stochastic nature of somatic mutation and previously predicted for retinoblastoma (Knudson 1971); (iv) compared pairs of individuals with differing degrees of relatedness, to determine the presence of shared modifier effects; and (v) tested our cohort directly for the influence of two genotypes shown formerly to influence tumor susceptibility.

## Subjects and Methods

### Ascertainment of Cases

Families and individuals affected by VHL were ascertained via departments of clinical genetics, ophthalmology, and neurosurgery from throughout the United Kingdom. For each family ascertained, all affected patients and at-risk relatives were invited to participate so that as many as possible, if not all, at-risk and affected individuals from each family were included. In this way a complete ascertainment was made of most of the VHL families, without a bias toward the recruiting of either particularly severely affected individuals or patients presenting to specific specialists. Fully informed consent was given by each individual after a full explanation of the intended research and the nature of the interview and

examination. Ethical approval was provided by the Cambridge District Ethics Committee.

### Patient Interview and Examination

Individuals were examined and interviewed by one or both of two of the authors (A.R.W. and A.T.M.), and all were examined by one of the authors (A.R.W.). Interview and case-note review included the full documentation of the individual's past ocular history and family history, with particular reference to the presentation, diagnosis, and treatment of the ocular and other complications of VHL. Examination in each case included indirect ophthalmoscopy and slit-lamp biomicroscopic funduscopy. An angioma count of lesions in each eye was made on the basis of findings on examination and with reference to case notes. The ocular history of eyes with no fundal view was determined from case notes, as far as possible, although an accurate angioma count in these eyes was not possible. A 10-ml sample of venous blood from each patient was extracted into EDTA for DNA analysis. The age-related complications were determined for 16 living affected relatives and 80 deceased affected relatives for whom accurate case notes and/or family records were available. These data, being less accurate than data obtained from examination and interview, were used only to confirm that the differences in renal and cerebellar susceptibility remained when all known affecteds were included and to overcome any ascertainment bias incurred by the exclusion of deceased relatives.

### Molecular-Genetic Analysis

*VHL mutation detection.*—DNA was extracted by standard methods. The gene-carrier status of patients was confirmed by the presence of the family mutation if the latter had been identified; otherwise, family members were typed for the informative microsatellite polymorphism D3S1038, as described elsewhere (Jones et al. 1992), which has a rate of <1% recombination with the VHL gene. Specific VHL mutations were determined, as described (Crossey et al. 1994b; Richards et al. 1994; Maher et al. 1996). Southern analysis was first performed on blood DNA, to detect/exclude a large deletion in the VHL gene. A 5- $\mu$ g sample of genomic DNA was digested with 1 U of *Eco*RI (Boehringer Mannheim) at 37°C, as recommended by the manufacturers, and then was run overnight on a 0.8% agarose gel. The DNA was transferred to a Hybond nitrocellulose filter by standard methods and hybridized with the VHL cDNA. A single band of 22 kb is normally detected after autoradiography, and a second larger or smaller band identifies a deletion (Richards et al. 1994). PCR was used to amplify the VHL coding and promoter region in six fragments from genomic DNA, and SSCP analysis was

performed to detect intragenic mutations, as described elsewhere (Crossey et al. 1994b). Fragments producing an aberrant SSCP band were purified by use of a Promega Wizard purification column and were sequenced on an ABI 373 automated sequencer by dye-terminator chemistry (AmpliTag FS), as recommended by the manufacturers. Direct sequencing of the whole coding region was performed in a similar fashion in those pedigrees in whom the mutation remained unidentified. All base changes so found were absent from  $\geq 200$  normal chromosomes. This analysis is expected to identify mutations in  $\geq 80\%$  of VHL kindreds (Maher et al. 1996). The germ-line mutations identified have been described in detail elsewhere (Crossey et al. 1994b; Richards et al. 1994; Zbar et al. 1996).

*Analysis of modifier-gene polymorphisms.*—Functional polymorphisms in the glutathione S-transferase M1 gene (*GSTM1*) and the cytochrome P450 2D6 gene (*CYP2D6*) were analyzed by standard methods. VHL patients were screened for the *GSTM1*-null allele polymorphism, by a PCR-based assay with three primers, P1–P3 (Zhong et al. 1993). In this assay, a 230-bp P1–P3 fragment reveals the presence of one or two copies of the wild-type allele. Absence of the 230-bp fragment identifies a homozygous-null individual. A control 157-bp fragment indicates a successful P1–P2 PCR amplification from either *GSTM1* or the related *GSTM4*, in all individuals. VHL patients were screened, by PCR and restriction digestion, for the functional polymorphisms in the *CYP2D6* locus (Smith et al. 1992). A high annealing temperature (60°C) was used, in order to avoid having the primers anneal to the homologous genes *CYP2D7* and *CYP2D8* (Smith et al. 1992). In the normal, extensively metabolizing (EM) allele, a *Bst*NI restriction site (CCAGG) at the intron 3/exon 4 boundary allows digestion of the 334-bp PCR product into fragments of 230 bp and 105 bp. However, in the mutated, poorly metabolizing (PM) allele *CYP2D6*\*4, a G→A transition causes the loss of this *Bst*NI restriction site (i.e., the sequence becomes CCAAG). The biological effect of this mutation is to shift the splice-acceptor sequence (AG) at the end of intron 3 so that it is 1 bp downstream. This leads to the loss of the first G residue in exon 4 of the processed transcript, with the consequent frameshift mutation causing a premature-termination codon at nucleotide 554 of the mRNA, hence preventing translation into a normal *CYP2D6* enzyme. By means of this *Bst*NI assay, patients could be divided into those with normal *CYP2D6* alleles (i.e., EM phenotype), heterozygotes, and homozygotes for mutated *CYP2D6*\*4 alleles (i.e., PM phenotype).

#### Statistical Methods

*Age-related prevalence.*—Prevalence of ocular angiomas was calculated both for the whole cohort and

separately for five age groups of gene carrier (ages 0–19 years, 20–29 years, 30–39 years, 40–49 years, and  $\geq 50$  years). Confidence intervals were calculated for the prevalence of each group, and a point-age-related prevalence was determined by use of the mean age in each group. The observed prevalence data were tested against two theoretical models:

1. A “constant risk” model assuming a constant risk per unit time, throughout life, that a gene carrier will convert from having bilateral healthy retinas to having one or more ocular angiomas. Under such a condition the relationship between prevalence ( $y$ ) and age ( $x$ ) would be of the form  $y = 1 - e^{-m(x+\mu)}$ , where  $m$  and  $\mu$  are constants with dimensions  $\text{time}^{-1}$  and  $\text{time}^1$ , respectively. The value of  $\mu$  was fixed at 0.75 years, to fix a value of 0 prevalence at conception. The constant  $m$  was varied between the limits 0.055 and 0.130  $\text{years}^{-1}$ , to generate a group of curves to fit within the 95% confidence interval (95% CI) of the actual prevalence of the youngest age group (0–19 years;  $n = 34$ ; prevalence .70, 95% CI = .55–.85),

2. A fixed prevalence model assuming that ocular lesions are congenital and that prevalence is fixed throughout life.

One likely cause for the lower than expected prevalence in surviving older gene carriers compared with younger ones is that patients with ocular angiomas have generally more severe disease and therefore die earlier from systemic complications. We therefore examined whether our data were consistent with the null hypothesis that each model was operating and that, by a certain age, all deceased gene carriers would have had ocular angiomas. To do this, we calculated a theoretical proportion of survivors who would be seen to have ocular angiomas ( $a_t$ ) under this hypothesis, as follows:  $a_t = (p_t + s_t - 1)/s_t$ , where  $s_t$  is the survival proportion at age  $t$  (by survival analysis of 279 gene carriers from the 81 pedigrees included in this study; data not shown) and  $p_t$  is the proportion of all individuals (dead and alive) who either are or would have been affected by ocular angiomas at age  $t$  (calculated on the basis of the models described above). The calculated prevalences under this hypothesis were compared with our data for each of the age groups, and the null hypothesis was rejected if the calculated theoretical prevalence was outside the 95% CIs of the actual data.

*Distributions of number of angiomas and affected eyes in gene carriers.*—Molecular-genetic studies of VHL tumors have shown loss of heterozygosity and methylation of the wild-type allele. If the development of VHL tumors is similar to that in retinoblastoma (a two-hit model), the development of a tumor from any one founding retinal cell depends on a somatic *VHL* mutation in that cell. Even if there were no external influences on tumorigenesis, variation would occur purely because of

the stochastic nature of these mutational events. In the absence of other influences, the distribution of the number of individuals having each discrete number of ocular tumors in their eyes, when the mean number of ocular tumors per individual in the population is, say,  $m$ , would follow a Poisson distribution with parameter  $m - \text{Po}(m)$ . Similarly, the distribution of the number of individuals having each number of eyes suffering from one or more ocular angioma (none, one, or two), in a sample of  $n$  people with a total of  $x$  eyes affected, would follow a binomial distribution with parameters  $\text{Bin}(2, x/2n)$ . These two theoretical distributions were calculated for the cohort of individuals in this study and were compared with the actual observed distributions, by the  $\chi^2$  test for goodness of fit. To compensate for any age-related effects that may affect the independence of observations (e.g., premature death of angioma-positive cases and differing age-related prevalence), similar analysis was also performed on restricted age groups.

*Cumulative prevalence of cerebellar and renal disease in angioma-positive and -null individuals.*—Since the diagnosis of the complications that occur in VHL is age dependent, in previous studies we had used survival analysis to compare the age-related cumulative prevalence for a specific complication in two or more groups of individuals (Maher et al. 1990, 1996). Both to analyze the association between ocular angiomatosis and cerebellar and renal complications and to compensate for patient age, we have used a similar analysis in the present study. Patients were divided into groups on the basis of their ocular status at the time of the study. For each group and for each year of analysis ( $y$ ), the cumulative probability of having incurred a cerebellar or renal complication was calculated as follows: cumulative probability =  $1 - [p_{y-1} * (r_y - f_y)/r_y]$ , where  $p_{y-1}$  is the probability that an individual will survive, without the complication, to year  $y - 1$ ,  $r_y$  is the number of individuals still at risk at year  $y$ , and  $f_y$  is the number of individuals incurring the complication at year  $y$ . The log-rank test and the log-rank test for trend were used to assess the significance of differences between groups.

*Relative-pair analysis.*—To determine whether the degree of ocular angiomatosis was influenced by factors shared by related individuals, we generated pairs of affected individuals of differing degrees of relatedness. We also generated pairs of unrelated individuals who were known to share exactly the same underlying germ-line VHL mutation. Pairwise correlation coefficients were calculated for the number of ocular angiomas. Because the distribution of the number of ocular angiomas in affecteds is highly skewed, the data were transformed by use of the function  $\log(1 + x)$ . This is similar to the analysis used by previous studies in the analysis of the neurofibromatosis 1 phenotype (Easton et al. 1993). To test the significance of specific pairwise correlations, the

following statistic was calculated:  $t = r\sqrt{n-2}/\sqrt{1-r^2}$ , where  $r$  is the Pearson correlation coefficient and  $n$  is the number of pairs.  $t$  is distributed as a  $t$  distribution with  $n - 2$  df, under the assumption that there is no correlation. We used a null hypothesis of no positive correlation, thus using a one-tailed test of significance for each group of pairs.

*Effect of GSTM1 and CYP2D6 on ocular angiomatosis.*—The proportion of patients with angioma who were in the different genotype groups (GSTM1 null vs. other; CYP2D6 EM vs. other) were compared, by the  $\chi^2$  test for independence, for the null hypothesis that angioma status is independent of genotype. Furthermore, groups of patients with increasing numbers of ocular angiomas in these same groups were examined by  $\chi^2$  for trend. To determine whether one or more specific combination of genotypes at these two loci (six genotypes in all) might have a significant effect through their interaction, the six genotypes were tested against the null hypothesis that all were similar with regard to their effect on the number of angiomas, by nonparametric analysis of variance (Kruskall-Wallis).

The cumulative probability of a solid renal lesion was calculated by survival methods (see above), for the comparisons GSTM1 null versus other and CYP2D6 EM versus other. To control for the potential confounding effect of ocular angiomatosis, each group was stratified for the presence or absence of ocular angiomatosis, and the stratified log-rank test was used to compare groups.

## Results

### Patient Details

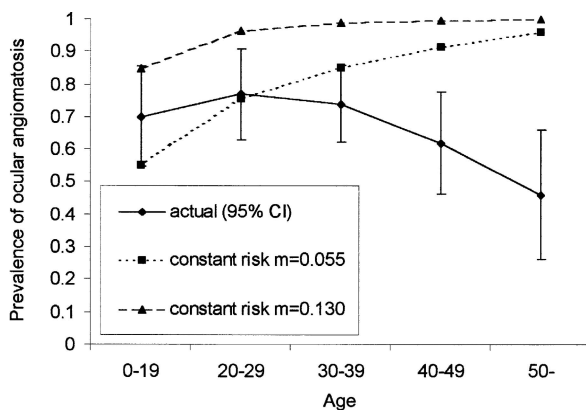
A total of 183 gene carriers were identified through clinical and molecular genetic criteria and were interviewed and examined as described above (mean age 33.8 years, range 7–74 years; 101 males [ $P = .15$ ]). Families originated from all over the United Kingdom, including Scotland and Northern Ireland. Patients were derived from 81 unrelated pedigrees (mean number of affected examined members per pedigree 2.3, range 1–10). Clinical details were collected from case notes and relatives' interviews, for 80 deceased affecteds and 16 living affecteds who were unavailable for interview (56 males and 40 females [ $P = .08$ ]). By means of survival analysis for all affected individuals in these 81 pedigrees, the median survival was calculated as 57 years.

### Sex- and Age-Related Prevalence of Ocular Angiomatosis in VHL

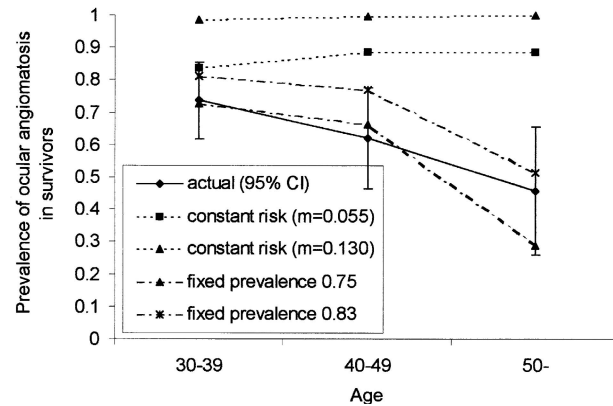
The overall prevalence of ocular angiomatosis in 183 examined gene carriers was 68%. The prevalence of ocular angiomatosis was similar in males and females (65% in males and 71% in females  $\chi^2_1 = 0.36$  [not sig-

nificant]). The mean number of ocular angiomas in males and females in whom an accurate bilateral count was possible did not significantly differ (mean 1.78 in 88 males and mean 1.94 in 68 females [not significant]). The mean age of patients with ocular angiomas (32.2 years in 124 patients) was lower than the mean age of patients without (37.0 years in 59 patients), and this was statistically significant ( $T_{181} = 2.14$ ;  $P < .013$ , two-tailed). The prevalence of ocular angiomas in different age groups is shown in figure 1. Curves for a constant-risk model were constructed (see Statistical Methods subsection) and were plotted together with the actual data (the age used is the mean age of the group). Figure 1 also shows two curves ( $m = 0.055$  and  $m = 0.130$ ) that fit within the 95% CIs of the prevalence of the youngest age group. Neither of these curves lies within the 95% CIs of the observed prevalence of the older age groups.

One likely explanation for the reduction in prevalence of ocular angiomas in the older gene carriers is the selective death of angioma-positive patients because they generally have a more severe type of the disease (see below). A theoretical prevalence of ocular angiomas in survivors for the three oldest age groups was calculated (see Statistical Methods subsection) by use of the following models: (i) a constant-risk model with  $m = 0.055$  and  $m = 0.130$  and (ii) models using a fixed prevalence of .75 and .83, as shown with the actual data in figure 2. The calculated values for the constant-risk models fall well outside the actual 95% CIs. Therefore, even when a correction is made for the selective early death of angioma-positive patients, the age-related prevalence of angiomas does not concord with the hypothesis that the probability of angiomas development is a



**Figure 1** The prevalence of ocular angiomas (95% CI) in the cohort of 183 affected VHL patients for different age groups. The two superimposed curves are derived from the expected prevalence that would occur if the chance of converting to having ocular angiomas remained constant throughout life.

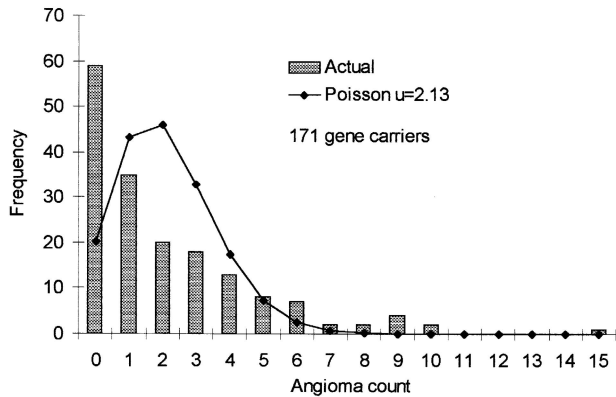


**Figure 2** The actual prevalence of ocular angiomas (95% CI) in over 30 year old affected VHL patients. Against these are four calculated values of the prevalence of ocular angiomas in survivors assuming that all deceased VHL patients had ocular angiomas and (i) a constant risk of developing ocular angiomas is present throughout life and (ii) the prevalence of ocular angiomas is fixed from birth.

constant throughout life. Alternatively, the theoretical prevalence in survivors that is generated under the assumption of a fixed-prevalence model does fit within the 95% CIs of the actual data. Hence, the data are compatible with the ocular lesions being present at an early age and having a fixed prevalence thereafter.

*Distribution of Number of Ocular Angiomas in VHL Carriers*

If the probability of an angioma originating from a susceptible retinal cell in a VHL carrier is a rare, independent event, then the number of angiomas occurring in gene carriers should conform to a Poisson distribution (as has been reported for retinoblastoma) (Knudsen et al. 1971). Factors that would cause deviation from such a distribution include an influence of age, type of underlying germ-line mutation, or other genetic risk- or environmental risk-modifying factors. For the 183 gene carriers, an accurate angioma count was possible in 155. The mean number of angiomas occurring per affected individual was 1.83. The observed distribution was significantly different than a Poisson distribution with the same mean ( $\chi^2_4 = 82.1$ ;  $P \ll .0001$ ). Of the remaining 28 patients, 16 of 22 unilaterally blind individuals had accurate case records concerning the number of angiomas occurring in the blind eye. The distribution of the total number of angiomas in these 171 gene carriers is shown in figure 3. Superimposed is the theoretical Poisson distribution with the same mean (Po[2.1]; mean no. of angiomas 2.1). Clearly, the distribution of angiomas does not conform to a Poisson distribution, the difference being significant ( $\chi^2_4 = 117.5$ ;  $P \ll .0001$ ). If the



**Figure 3** The actual distribution of the number of ocular angiomas in affected patients drawn against the theoretical Poisson distribution with the same mean.

outliers with  $\geq 10$  angiomas are omitted from the analysis, the mean number of angiomas is reduced to 1.79, but the data remain significantly different from a Poisson distribution with this mean. These most severely affected patients were unrelated and did not share the same VHL germ-line mutation. The distribution remains non-Poisson when the data are restricted to a narrow age range (e.g., 20–39 years) (mean 2.79 in 85 individuals;  $\chi^2_5 = 66.92$ ;  $P \ll .0001$ ). Also, the inclusion, within such a cohort of surviving patients, of the expected number of patients who would have suffered VHL-related deaths—and the assignment to each such patient of one or more angiomas—would not compensate for the number of individuals with no angiomas, to allow the distribution to fit a Poisson curve (data not shown). It can be concluded, therefore, that the variability in the number of angiomas in the eyes of VHL patients is not due to stochastic factors alone.

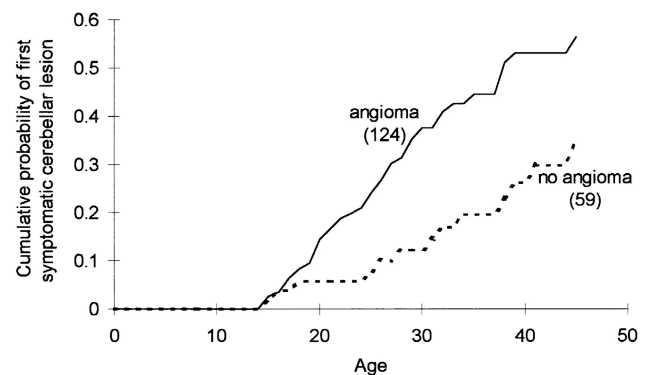
#### Distribution of Number of Affected Eyes in Gene Carriers

In a similar way, if the event of a gene carrier's eye becoming angiomatous is independent with regard to the fate of the fellow eye and has a probability that is shared among all gene carriers, then the number of eyes (zero, one, or two) affected by angiomatosis should fit the binomial distribution  $\text{Bin}(2, p)$ , where  $p$  is the mean probability of angiomatosis occurring in an eye of a gene carrier. Of the 183 patients, angiomatosis occurred in 199 eyes. The estimated mean probability that an eye in a gene carrier will suffer angiomatosis is, therefore,  $199 / (183 * 2) = .54$ . The observed distribution for 183 affected patients was as follows: angiomatosis in neither eye, 59 patients; angiomatosis in one eye, 49 patients; and angiomatosis in both eyes, 75 patients. The theo-

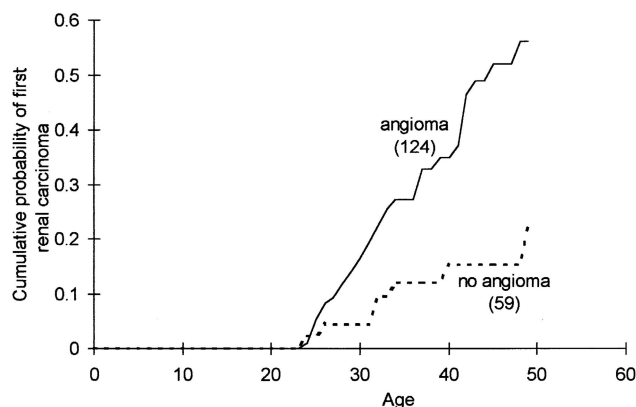
retical binomial distribution for the number of eyes affected in patients is  $\text{Bin}(2, .54)$  and would give a theoretical distribution of (angiomatosis in neither eye, 39 individuals; angiomatosis in one eye, 91 individuals; and angiomatosis in both eyes, 53 individuals); this is significantly different from the observed data ( $\chi^2_1 = 39.62$ ;  $P < .005$ ). There is also significant difference when only patients restricted to a narrow age group are included (e.g., 20–39 years [theoretical  $\text{Bin}(2, .62)$ ;  $\chi^2_1 = 20.17$ ;  $P < .005$ ]). These findings suggest that the probability of an eye being affected is not independent of the state of the fellow eye, and the excess of pairs of eyes that are both either affected or unaffected suggests that other factors in addition to the stochastic nature of tumorigenesis influence the variation in tumor susceptibility.

#### Effect of Status of Ocular Angioma on Susceptibility to Other Tumors

Survival curves for the cumulative risk of development of a first symptomatic cerebellar lesion were constructed for individuals with and without ocular angiomatosis (fig. 4). The occurrence of ocular angioma significantly increased the susceptibility to cerebellar hemangioblastoma (log-rank test  $\chi^2_1 = 7.48$ ;  $P < .006$ ), the hazard ratio being 2.3. The occurrence of ocular angioma also significantly increased the susceptibility to a first solid renal lesion (fig. 5) (log-rank test  $\chi^2_1 = 11.15$ ;  $P < .0008$ ), the hazard ratio being 4.0. These differences were still significant when retrospective data from 96 unexamined individuals (80 of whom were deceased) from the same pedigrees were included (data not shown). Furthermore, there was a significant increase in the susceptibility to a first symptomatic cerebellar lesion when individuals were divided into three groups on the basis



**Figure 4** The cumulative probability of a first symptomatic cerebellar lesion occurring in the cohort of VHL patients divided into two groups on the basis of the presence of ocular angiomatosis



**Figure 5** The cumulative probability of a first solid renal lesion occurring in the cohort of VHL patients divided into two groups on the basis of the presence of ocular angiomas

of the degree of ocular angiomas (i.e., no angiomas, one or two angiomas, and more than two angiomas) and were examined for trend (fig. 6) (log-rank test for trend  $\chi^2_1 = 9.09$ ;  $P < .003$ ). With regard to renal lesions, the majority of individuals were screened for this complication, according to a standard protocol (Maher et al. 1990). The small proportions not screened were similar in the angioma-positive and -null groups and so did not confound this analysis.

A similar analysis, comparing the cumulative probability of symptomatic cerebellar lesions in those with a previous solid renal lesion (38 individuals, 75% probability at 60 years of age) and in those without it (145 individuals, 66% probability at 60 years of age), showed a greater susceptibility in those who had had a renal lesion diagnosed, although this did not reach statistical significance (log-rank test  $P > .05$ ).

#### Modifier Effects in Ocular Angiomas

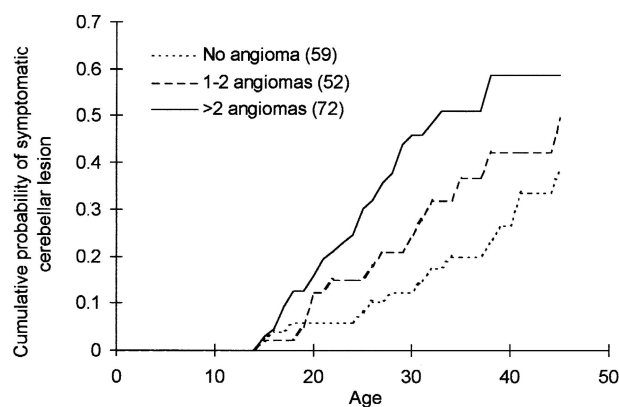
Table 1 shows the results of pairwise correlation coefficients for the number of ocular angiomas occurring in pairs of relatives of different degrees of relatedness. It can be seen that the correlation was significantly positive, at the 5% level, only in pairs of relatives who would be expected to share half their genome, both in parent-child pairs and in sib pairs. No significant positive correlation was seen in other relative pairs, despite a similar sample size (and, hence, similar statistical power). Furthermore, pairs of individuals not knowingly related who had the same underlying mutation did not show significant positive correlation. Mutations were deemed identical only if the same-size abnormal fragments were detected on Southern analysis or, alternatively, if the same nucleotides were involved in intragenic

mutation. We have reported elsewhere that, in the United Kingdom, most families with identical mutations do not share a similar haplotype, suggesting that there is not a common ancestry (Richards et al. 1995).

#### *GSTM1 and CYP2D6 Genotype and Ocular Angiomatosis*

A total of 163 VHL patients were typed successfully for *GSTM1*-allele status. Of these, 75 (46%) were shown to have a 230-bp fragment as well as the control 157-bp fragment and therefore possessed a functional *GSTM1* allele. The remaining 88 (54%) demonstrated only the control, 157-bp fragment and therefore were identified as homozygous nulls. The proportion of patients affected by ocular angiomatosis was not significantly different in the two groups—*GSTM1*-positive group, 54/75 (72%); *GSTM1*-null group, 57/88 (65%) ( $\chi^2_1 = 0.67$ ;  $P = .41$ ). Of the 163 VHL patients typed for *GSTM1*-allele status, an accurate bilateral angioma count was available in 151. The mean number of angiomas in patients from each group was not significantly different (*GSTM1* positive, 2.30; *GSTM1* null, 2.04 [ $T_{138} = 0.59$ , not significant]). Furthermore, when the data were examined for trend, there was no influence of the *GSTM1* genotype on the number of angiomas in patients ( $\chi^2_1 = 0.12$ , not significant). These results are shown in table 2.

A total of 161 VHL patients were typed successfully for the *CYP2D6* genotype; 99 (61.5%) of them were homozygous for the PM allele of *CYP2D6*, 51 (31.7%) of them were identified as heterozygotes, and 11 (6.8%) of them were PM. The proportion of patients affected by ocular angiomatosis was not significantly different in patients homozygous for the EM allele of *CYP2D6*



**Figure 6** The cumulative probability of a first symptomatic cerebellar lesion occurring in the cohort of VHL patients divided into three groups on the basis of the presence of 0, 1-2, >2 ocular tumors.

**Table 1**

**Correlation Coefficients for Number of Ocular Angiomas in Pairs of Affected Patients of Differing Degrees of Relatedness**

Relative Pair (No.)	Degree of Relatedness	Correlation	P <sup>a</sup>
Pairs of patients' eyes (155)	1	+.56	<.0001
Parent-child pair (45)	1/2	+.27	.04
Siblings (53)	1/2	+.32	.01
1 in 4 (48)	1/4	+.14	.16
≤1 in 8 (45)	<1/8	-.08	NS
Unrelated (49)		-.21	NS

<sup>a</sup> NS = not significant.

(64%), compared with either the heterozygotes or the PM individuals (74%) ( $\chi^2 = 1.19$ ;  $P = .27$ ). Of the 161 VHL patients typed for *CYP2D6*-allele status, an accurate bilateral angioma count was available in 149. The mean number of angiomas in patients from each group did not significantly differ (EM individuals, 2.13; heterozygotes or PM individuals, 2.22 [ $T_{140} = 0.21$ , not significant]). Furthermore, when the data were examined for trend, there was no influence of the *CYP2D6* genotype on the number of angiomas in patients ( $\chi^2 = 1.23$ ;  $P = .26$ ). These results are shown in table 2.

To determine whether a specific combination of *GSTM1* and *CYP2D6* genotypes either was protective or caused particular susceptibility with regard to ocular angiomatosis, we divided the 149 patients into the six genotype groups (null/EM [42 individuals], null/heterozygous [27 individuals], null/PM [3 individuals], positive/EM [42 individuals], positive/H heterozygous [20 individuals], and positive/PM [8 individuals]). We then performed nonparametric analysis of variance (Kruskal-Wallis) on all the data. The data did not significantly differ from those expected under the null hypothesis that none of the six individual groups were significantly protective or caused more susceptibility to ocular angiomatosis than the cohort as a whole ( $\chi^2 = 2.27$ ;  $P = .13$ ).

#### *GSTM1* and *CYP2D6* Genotypes and RCC

To determine whether *GSTM1* or *CYP2D6* status had an effect on the diagnosis of a first solid renal lesion, survival analysis was used. To overcome the potential confounding effect of ocular angioma, each group was stratified with regard to the presence or absence of ocular angiomatosis. When the stratified log-rank test was used, there was no significant difference, in the development of a first solid renal lesion, either between patients with a *GSTM1*-null genotype (88 individuals) and patients with a *GSTM1*-positive genotype (75 individuals) ( $\chi^2 = 1.78$ ;  $P > .1$ ) or between patients homozygous for the EM allele of *CYP2D6* (99 individuals) and patients either heterozygous or homozygous for the PM allele (62 individuals) ( $\chi^2 = 0.039$ , not significant).

## Discussion

Variable expression is a feature of many genetic disorders and may result from either allelic heterogeneity or the influence of environmental and modifying genetic factors. In inherited cancer syndromes caused by germline mutations in tumor-suppressor genes such as *RB1*, tumors occur through the chance event of one or more somatic mutations in a susceptible cell, and variability will also occur solely because of this stochastic mechanism. Molecular studies of VHL tumors (e.g., cerebellar hemangioblastoma and RCC) suggest a tumorigenesis model similar to that for retinoblastoma (Crossey et al. 1994a; Prowse et al. 1997). However, for retinal hemangioblastomas in VHL disease, we have shown that there is a significant deviation from the theoretical stochastic distributions, in both the number of individual tumors and the number of affected eyes, in individuals with VHL disease. It is of interest that, in Knudson's (1971) original work on retinoblastoma, Poisson and binomial distributions were assumed in the analysis of, respectively, the number of retinoblastoma tumors and the number of affected eyes from data on familial retinoblastoma cases. As far as we are aware, this is the first time that such assumptions have been tested empirically, and we find that, for ocular angiomatosis in VHL disease, these assumptions are incorrect. Thus the stochastic nature of tumorigenesis cannot fully account for the variability of ocular involvement in VHL disease, and other factors therefore must be important (or a different model of tumorigenesis is operating).

If the risk of development of ocular angioma in VHL disease were lifelong, then the mean age of individuals affected by this manifestation would be expected to be higher than that of those not affected. In the present study, however, VHL gene carriers with ocular angiomatosis were significantly younger than those without it. In a cross-sectional study such as this, individuals of different ages are sampled simultaneously. One explanation for this observation, therefore, is that, with regard to their exposure to environmental mutagens, younger

**Table 2**

**Numbers of Affected Patients with Ocular Angiomas Counts, by *GSTM1* and *CYP2D6* Genotype**

NO. OF ANGIOMAS	NO. OF PATIENTS					
	<i>GSTM1</i>			<i>CYP2D6</i>		
	Null	Positive	Total	EM	Heterozygous/PM	Total
0	31	21	52	35	16	51
1	13	17	30	18	12	30
2	10	9	19	10	9	19
3-4	12	14	26	14	11	25
≥5	14	10	24	14	10	24
Total	80	71	151	91	58	149



subjects differ from those born earlier. We propose that an alternative explanation is that patients with retinal involvement are more likely to suffer the other major complications of the disease. In the present study, we found that the major complications of VHL disease are not independent of each other. Individuals with ocular angiomas were 2.3 and 4 times more susceptible to development of cerebellar and renal lesions, respectively. Since these complications are a cause of mortality in VHL, older surviving individuals are more likely to be generally less severely affected and, therefore, more likely to be free of ocular angiomas. Although a similar analysis showed that subjects who had renal lesions were more likely to have suffered cerebellar lesions, this was not statistically significant. This may be because, unlike our analysis of ocular involvement, this comparison relies wholly on retrospective data and therefore is likely to be less accurate.

The age-related prevalence observed in this cohort of gene carriers was not consistent with a theoretical model of a constant risk, throughout life, of conversion from the presence of normal eyes to eyes with one or more ocular tumors. This inconsistency remained after a correction based on the assumption that all deceased VHL individuals had been affected by ocular angiomas. Hence, our data suggest that the risk of development of ocular angiomas is not lifelong. This would be consistent with the hypothesis that an ocular angioma will develop only if the important tumorigenic somatic mutations occur during a specific time interval. This situation would be similar to that of familial and sporadic retinoblastoma, as well as to that of other childhood-related tumors, but is apparently different from that of most familial and sporadic adult cancers. The data would also be compatible with the ocular lesions being congenital hamartomas, a view supported also by the fact that, histologically, the tumors have three distinct cell types.

The relationship between visual acuity and the presence of ocular angiomas is not straightforward, and the reasons why some, but not all, lesions cause ocular complications leading to loss of vision are unknown. The present study did not address this issue. However, the data presented here suggest that adult gene carriers who are free of angiomas are likely to be at low risk of development of further retinal angiomas.

Previous studies of genotype-phenotype correlations in VHL have shown an association between deletions/protein-truncating mutations and a low risk of pheochromocytoma (Crossey et al. 1994b; Chen et al. 1995; Maher et al. 1996). Also, a specific missense mutation has been associated with a low risk of RCC (Brauch et al. 1995), although this mutation did not occur in the pedigrees included in this study. Otherwise, ocular, CNS, and renal involvement are not influenced by the type of

underlying germ-line mutation (Maher et al. 1996). With regard to ocular angioma, this result is supported by the data in this study, in the lack of correlation between pairs of distantly related individuals despite the fact that the latter have identical mutations. Closely related individuals, however, did show a significant correlation with regard to the number of ocular angiomas. This was evident only in relative pairs who share half the genome, being apparent in both sib pairs and parent-child pairs. This suggests that important modifying factors might be shared between closely related individuals. Furthermore, since the risks of cerebellar hemangioblastoma and RCC were increased in patients with retinal angiomas, these modifying factors appear to influence susceptibility to each of the three major complications of VHL disease.

The putative modifiers could be genetic and/or environmental. However, genetic factors are likely to be involved, since, because of differences in age, parent-sib pairs would not preferentially share environmental factors. Candidate modifiers in VHL disease would include factors that determine the clearance of environmental mutagens from the body and the rate at which tumorigenic metabolites are generated from inactive procarcinogens. Although specific environmental mutagens that predispose to ocular hemangioblastomas have not been identified, functional polymorphisms in *GSTM1* and *CYP2D6* have been implicated in cancer susceptibility (for reviews, see Wolf et al. 1994; Raunio et al. 1995). Foreign compounds are metabolized in two stages (phase I and phase II metabolism). Phase I is usually an oxidation step giving rise to an electrophilic intermediate that may be mutagenic. Phase II enzymes catalyze the conjugation of specific chemical groups (e.g., glutathione) to previously created electrophilic intermediates, to produce a water-soluble metabolite that is excreted. Individuals with efficient phase I metabolism and inefficient phase II enzymes would be predicted to be predisposed to cancer when they are exposed to appropriate carcinogens. Approximately 50% of the population are homozygous null for the phase II enzyme *GSTM1*, and the null phenotype has been associated with susceptibility to colorectal, bladder, and squamous-cell lung cancer (Zhong et al. 1991, 1993; Daly et al. 1993). Similarly, polymorphic variations in the cytochrome P450 2D6 (*CYP2D6*) gene (a phase I enzyme) may produce EM individuals or PM individuals. Approximately 66% of the Caucasian population are EM, 30% are heterozygous, and 4% are PM, with 80%–90% of PM alleles being the *CYP2D6*\*4 (G1934A) frameshift mutation detected by *Bst*NI digestion (Smith et al. 1992; Sachse et al. 1997). The EM genotype may predispose to lung and liver cancer (Hirvonen et al. 1993; Agundez et al. 1995, 1996). However, we found no evidence to suggest that, in patients affected by VHL disease, allelic variation at either *GSTM1* or *CYP2D6* modifies either

the number of retinal angiomas or the age at development of a first solid renal lesion. Nevertheless, the identification of such modifying factors would extend our understanding of the mechanisms of tumorigenesis and would improve the accuracy of tumor-risk predictions in patients with VHL disease.

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## Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim> (for VHL [MIM 193300])

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