EMANGIOBLASTOMAS of the CNS are rare, benign lesions (World Health Organization Grade 1) that account for 1 to 2% of all intracranial tumors.4,31 They occur predominantly in the cerebellum, and typically appear on neuroimaging studies as contrast-enhancing nodules with associated cysts.4 Symptoms often arise from an increase in intracranial pressure caused by impaired cerebrospinal fluid flow. The standard treatment is surgical removal; radiosurgery is an alternative for deeply located, small HBs.7,33

Hemangioblastomas occur in sporadic form (75%) or as a manifestation of von Hippel–Lindau (VHL) disease. In the majority of VHL-related HBs, inactivation of the VHL tumor suppressor gene (TSG), which is located on chromosome 3p25-26, is found. The VHL gene is assumed to be involved also in the development of sporadic HBs. In a previous study of chromosomal aberrations of sporadic HBs, multiple chromosomal imbalances were found in the majority of tumors. The aim of this study was to analyze further both sporadic HBs and VHL-related HBs to determine if these histopathologically identical tumors have a different genetic background.

Methods. Sixteen sporadic HBs and seven VHL-related HBs were identified by clinical criteria and analyzed. Comparative genomic hybridization was used to screen for chromosomal imbalances throughout the entire HB genome. Additionally, mutation analysis of the VHL gene was performed using direct sequencing.

Loss of chromosome 3 and multiple other chromosomal imbalances were found in the sporadic HBs, although only one imbalance, a loss of chromosome 3, was detected in the seven VHL-related HBs. Somatic VHL gene mutations were found in one third of sporadic HBs, whereas a mutation of the VHL gene was detected in all VHL-related HBs.

Conclusions. These results indicate that the molecular mechanisms underlying sporadic HBs and VHL-related HBs are different. Inactivation of the VHL gene is probably not the most important event in the tumorigenesis of sporadic HBs. Other mechanisms of inhibition of VHL protein function, or inactivation of other TSGs, on chromosome 3p or on other chromosomes, might be important in the development of sporadic HBs.

Key Words • hemangioblastoma • von Hippel–Lindau disease • gene mutation • comparative genomic hybridization

Abbreviations used in this paper: CGH = comparative genomic hybridization; CNS = central nervous system; dUTP = deoxyuridine triphosphate; HB = hemangioblastoma; HIF = hypoxia-inducible transcription factor; LOH = loss of heterozygosity; TSG = tumor suppressor gene; VHL = von Hippel–Lindau.
to 100% of families in which VHL disease has been observed.13,16,40 Up to 50% of sporadically occurring counterparts of the tumors observed in VHL disease.15,37 Conflict- ing results of the importance of VHL hypermethylation in these histopathologically identical tumors.9

The VHL TSG maps to chromosome 3p25-26,25 and a broad spectrum of germline mutations has been reported in 63 to 100% of families in which VHL disease has been identified.15,28,43,48 In concordance with Knudson’s ‘two-hit’ hypothesis,23 the majority of VHL-related tumors show the germline mutation and LOH on 3p, small intragenic mutations, or hypermethylation of the other allele.15,37 Conflicting results of the importance of VHL hypermethylation have been reported in HBs in the CNS.15,37,44

Inactivation of the VHL gene has also been reported to play a role in the sporadically occurring counterparts of the tumors observed in VHL disease.13,16,48 Up to 50% of sporadic HBs showed LOH on 3p, whereas somatic mutations of the VHL gene have been found in, on average, in 35%.15,21,38,34 Bi-allelic inactivation, however, was found in only one of 13 sporadic HBs investigated by Gläsker, et al.,15 and they suggested that inactivation of other TSGs on chromosome 3p might be crucial for the development of sporadic HBs. Other chromosomes might be involved in the tumorigenesis of sporadic HBs, as indicated by our previous study,41 in which we used CGH to screen for chromosomal imbalances throughout the entire tumor genome of 10 sporadic HBs. In the present study, we analyzed chromosomal imbalances by using CGH, and aberrations of the VHL gene by mutation analysis in sporadic HBs and VHL-related HBs to elucidate further the molecular genetic differences in these histopathologically identical tumors.

### Materials and Methods

Twenty-three tumor samples of HBs in the CNS were obtained in 20 patients who were treated at the University Medical Center Nijmegen, The Netherlands (Tumors N240–N244, N298, N341, N342, N379, and N380), the University Hospital Rotterdam, The Netherlands (Tumors N364–N368 and N370), or the University Hospital, Leuven, Belgium (Tumors N245–N249, N373, and N378). One sporadic HB was a recurrence (Tumors N298 and N243), and two patients with VHL had two different VHL-related HBs (Tumors N379 and N380 and Tumors N364 and N368). Samples of 21 HBs were collected during surgery, snap-frozen, and stored at −80°C. In two cases (Tumors N341 and N342) no frozen tissue was available and, therefore, formalin-fixed and paraffin-embedded tissue was analyzed. Clinical criteria provided by the Dutch National VHL Working Group, based on international guidelines, were used to establish VHL disease.14,27 Criteria for VHL disease include a single HB and a positive family history of VHL disease, at least two HBs (including retinal or spinal), or a single HB in association with other typical VHL-related tumors such as clear-cell renal cell carcinoma or pheo-

<table>
<thead>
<tr>
<th>Tumor No.</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Location</th>
<th>CGH</th>
<th>Chromosomal Imbalances</th>
<th>Mutation of VHL Gene in Tumor</th>
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<tr>
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<td>34, F</td>
<td>cerebellar</td>
<td>+</td>
<td>-</td>
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<td>spinal</td>
<td>+</td>
<td>-</td>
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<td>N245</td>
<td>31, M</td>
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<td>+</td>
<td>-</td>
<td></td>
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</tr>
<tr>
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<td>+4, +5, +7, +16, +17, -22</td>
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<tr>
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<td></td>
<td>-</td>
<td>-</td>
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<td>42, M</td>
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<tr>
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</tbody>
</table>

* † = reported in previous study; — = the deletion could not be confirmed by Southern blotting because of insufficient DNA.
† Recurrence of N243.
‡ Different HB, same patient.
§ Different HB, same patient.

**TABLE 1**

Patient and tumor characteristics and results of CGH and mutation analysis*
Mutation analysis in sporadic and hereditary hemangioblastomas

The CGH was performed as previously described by Jeukens, et al. Briefly, DNA was extracted by a salting-out procedure. Control and tumor DNA were labeled by nick translation with digoxigenin-dUTP and biotin-dUTP, respectively. The DNA isolated from paraffin-embedded tissue (Tumors N341 and N342) was labeled with a rhodamine universal linkage system as described by the manufacturer, with minor modifications. Labeled DNA was precipitated in the presence of human COT-1 DNA and herring sperm DNA. Target metaphase spreads were prepared using standard procedures; the probe and the metaphase slides were denatured simultaneously. After hybridization and posthybridization washes, biotin and digoxigenin were detected using streptavidin–fluorescein isothiocyanate and sheep antidigoxigenin–tetramethyl rhodamine isothiocyanate. The chromosomes were counterstained with 4,6-diamino-2-phenylindole-dihydrochloride and were then mounted in Fluoroguard.

Negative and positive control experiments were included in each series of CGHs to monitor CGH quality. For CGH analysis, commercially available software was used and detection thresholds for losses and gains were set at 0.8 and 1.2, respectively.

Analysis of VHL gene mutations was performed by direct sequencing, as described by Bodmer, et al. In summary, five primer sets were used to amplify exons 1 (three overlapping primer sets), 2, and 3 of the VHL gene from tumor tissue DNA. The polymerase chain reaction products were purified and subsequently sequenced, after which the sequence products were analyzed.

Sources of Supplies and Equipment

The digoxigenin-dUTP and the biotin-dUTP used in the nick translations were purchased from Roche Molecular Biochemicals, Almere, The Netherlands, as were the streptavidin–fluorescein isothiocyanate and sheep antidigoxigenin–tetramethyl rhodamine isothiocyanate. The rhodamine universal linkage system (model ULS/Ulys) was acquired from Kreatech Biotechnology, Amsterdam, The Netherlands. The human COT-1 DNA was supplied by Gibco–BRL Life Technologies, Inc., Gaithersburg, MD. The 4,6-diamino-2-phenylindole-dihydrochloride and the Fluoroguard were obtained from Biorad, Almere, The Netherlands, as were the streptavidin–fluorescein isothiocyanate and sheep antidigoxigenin were detected using streptavidin–fluorescein isothiocyanate and sheep antidigoxigenin–tetramethyl rhodamine isothiocyanate. The chromosomes were counterstained with 4,6-diamino-2-phenylindole-dihydrochloride and were then mounted in Fluoroguard.

The Quips CGH software was purchased from Applied Imaging, Newcastle upon Tyne, UK. The polymerase chain reaction purification kit (QIAquick) was supplied by Qiagen, Westburg, The Netherlands. The ABI PRISM BigDye Terminator Cycle sequencing kit was acquired from Applied Biosystems, Foster City, CA, as was the ABI PRISM DNA analyzer (model 3700).

Results

In Table 1 we have summarized the clinical characteristics of the patients and tumors, chromosomal imbalances detected by CGH in the tumors, and VHL mutations detected by direct sequencing in the tumors. Clinical evaluation identified 16 sporadic HBs and seven VHL-related HBs. The 16 sporadic HBs (obtained in 11 male and four female patients; mean age at surgery 50 years, range 14–69 years) included one recurrence and one spinal HB. All patients with VHL disease (two men and three women; mean age at surgery 33 years, range 21–47 years) harbored multiple cerebellar and/or spinal HBs, in two patients there were also multiple pancreatic and epididymal (Tumors N379 and N380) or renal (Tumor N378) cysts, in one patient there was a retinal HB (Tumor N370), and two patients there was a positive family history for VHL disease (Tumors N373 and N378).

The CGH results are depicted in Fig. 1. The chromosomal imbalances most frequently detected in 16 sporadic HBs were loss of chromosome 3 (designated −3) in 11 tumors (69%); −6 in seven (44%); −18(q) in five (31%); and −9 in four (25%); and gain of chromosome 19 (designated +19) in four sporadic HBs (25%). In three sporadic HBs (19%) no abnormalities were detected using CGH. In the seven VHL-related HBs only one aberration (−3) was found on CGH (14%). This patient (Tumor N370) had multiple cerebellar and spinal HBs and a retinal HB.

Sequencing of the VHL gene identified three somatic frameshift mutations and two missense mutations in the sporadic HBs (five [31%] of 16; Table 1). These mutations, to our knowledge, have not been previously described.2,14,15,21,26,35,37,44 Mutations were identified in six (86%) of seven VHL-related HBs (Table 1). These mutations are not germline mutations per se; blood was available for only two VHL-related HBs (Tumors N379 and N380) in which the germline mutation T152P was confirmed. To our knowledge, the mutation T152P has not been reported previously.2,14,15 All were missense mutations, of which S80R, L158V, and N78S were previously described as germline mutations.2,48 In one VHL-related HB a homozygous dele-
of exon 2 was found (Table 1). We could not confirm this deletion by Southern blotting due to the small amount of DNA available. Germline homozygous deletions have been reported in VHL disease.²

Discussion

Loss of Chromosome 3 and Mutations of the VHL Gene

A complete loss of chromosome 3 (designated −3) was found in 11 (69%) of 16 sporadic HBs, whereas −3 was detected in only one (14%) of the seven VHL-related HBs, and multiple other chromosomal imbalances were detected in most sporadic HBs, but not in VHL-related HBs. Furthermore, mutations of the VHL gene were identified in only five (31%) of the 16 sporadic HBs, but in all seven of the VHL-related HBs (Table 1). Thus, these results show genetic differences in sporadic compared with VHL-related HBs.

Inactivation of the VHL TSG at 3p25-26 is considered to be a common oncogenic mechanism in VHL-associated tumors.¹³,³⁷ and germline mutations of this gene are reported to be present in up to 100% of VHL-related HBs.¹⁵,²⁸,⁴³,⁴⁸ Until recently, it was generally assumed that the VHL TSG was also involved in the tumorigenesis of the sporadic counterparts of the VHL tumors.²¹,²⁶,³⁴,⁴⁴,⁴⁵ as was shown in clear-cell renal cell carcinomas.¹³,¹⁶,⁴⁰ Somatic mutations of the VHL gene have been reported, however, in only 25% of sporadic HBs (17 of 69; range 10–44%),¹⁵,²¹,²⁶,³⁴,⁴⁴ whereas LOH on 3p has been found in 52% of tumors (16 of 31 overall; range 20–53%).¹⁵,²⁶,⁴⁴ Furthermore, in a recent study inactivation of both alleles of the VHL gene was found in only one (8%) of 13 sporadic HBs, compared with 13 (62%) of 21 VHL-related HBs, suggesting that biallelic inactivation of the VHL gene plays a minor role in tumorigenesis of sporadic HBs.¹⁷ These latter findings corroborate our suggestion that the VHL gene might be involved in only a subset of sporadic HBs, and that other TSGs on chromosome 3p might be important in the development of sporadic tumors.

Although according to Knudson,²³ inactivation of both alleles of a TSG is required to cause tumor development, TSGs may also cause tumors by giving rise to dominant negative mutations or haploinsufficiency.²⁴,⁴¹ Dominant negative mutations inhibit the function of the wild-type allele and the production of wild-type protein. Haploinsufficiency is caused by a relative shortage of the wild-type protein as a direct result of loss of one of the alleles. Such mechanisms might be involved in the tumorigenesis of sporadic HBs. Because the detection limit of CGH is 2 to 5 Mb, smaller losses and gains may be present in these tumors.

Putative TSGs on Chromosome 3

The frequent loss of chromosome 3 in sporadic HBs suggests a prominent role for genes located on this chromosome in the development of sporadic HBs. In a review of DNA copy number losses in various human neoplasms it was reported that a complete or partial loss of chromosome 3p is often found by LOH and CGH analysis, whereas losses of chromosome 3q are relatively rare.³ Putative TSGs have been suggested⁶,¹³,⁴²,⁴⁹ at several regions on 3p: at 3p12-14 containing, among others, fragile histidine triad gene;³⁵ at 3p21.3-p22 containing, for instance, lung cancer TSG region 1; and in the VHL gene region 3p25-26 containing, among others, the DNA repair gene Xeroderma pigmentosum C.⁴⁴ An important role for TSGs at 3p12-14 and 3p21.3 in the tumorigenesis of sporadic and VHL-associated renal cell carcinomas has already been suggested,¹⁵,²⁶,³⁴ but the involvement of these putative TSGs in sporadic HBs or VHL-related HBs has not yet been reported.

In three of the sporadic HB samples (Tumors N247, N249, and N367), no chromosomal aberrations were detected with CGH. Because the detection limit of CGH is 2 to 5 Mb, these HBs may contain small aberrations not detectable using this method. It is also possible that chromosomal aberrations were not detected because the percentages of normal DNA were too high. Nevertheless, because the VHL-related HBs and sporadic HBs are phenotypically identical, there is no reason to assume that the percentage of normal DNA in the sporadic HBs and the VHL-related HBs is different.

Involvement of Other Chromosomes in Sporadic HBs

Genes located on other chromosomes may also play a role in the genesis of sporadic HBs. As reported in our previous study³¹ and confirmed by analyzing this larger group of sporadic HBs, the frequency of the aberrations and their occurrence in individual sporadic HBs might indicate a pathway of sequential events in sporadic HBs (see Table 1): −3 may be a primary event, followed by −6, −18, and −9, and/or +19. Interestingly, a loss of chromosome 8 was detected in three sporadic HBs, two of them with a normal constitution of chromosome 6. The third sporadic HB containing −8 was a tumor with multiple imbalances but with a normal chromosome 3. Therefore, eight of 10 sporadic HBs with multiple chromosomal aberrations had two of the following imbalances: −3, −6, or −8.

In human tumors, (putative) TSGs and oncogenes have been reported on chromosomes 6, 8, 9, 18, and 19.²⁴ Wheth-
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found in only one VHL-related HB. Additionally, losses of chromosomes 6, 9, and 18, and gain of chromosome 19 are often present in sporadic HBs; no other imbalances were found in VHL-related HBs. Mutations of the VHL gene were detected in 31% of sporadic HBs and in all VHL-related HBs. These findings indicate that inactivation of the VHL gene is probably not the most important molecular event in sporadic HBs, and that these tumors and VHL-related HBs have a different genetic background. The pathogenetic pathway of sporadic HBs remains to be elucidated. Other than the VHL gene, TSGs on chromosome 3 and TSGs on other chromosomes might be instrumental in the development of these tumors.

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References


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