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Partial Deletions of the Long and Short Arm of Chromosome 3 Point to Two Tumor Suppressor Genes in Uveal Melanoma[1]

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ABSTRACT

Uveal melanoma is the most common form of primary eye cancer. Monosomy 3, which is an unusual finding in tumors but is present in ~50% of uveal melanomas, is significantly correlated with metastatic disease. To obtain positional information on putative tumor suppressor genes on this chromosome, we have investigated tumors from 333 patients by comparative genomic hybridization, microsatellite analysis, or conventional karyotype analysis. A partial deletion of the long arm was found in eight tumors, and the smallest region of deletion overlap (SRO) spans 3q24–q26. We found six tumors with a partial deletion of the short arm and were able to define a second SRO of about 2.5 Mb in 3p25. This SRO does not overlap with the *VHL* gene. Our finding suggests a role for two tumor suppressor genes in metastasizing uveal melanoma and may explain the loss of an entire chromosome 3 in these tumors.

INTRODUCTION

Uveal melanoma is the most common primary intraocular tumor with an incidence of six per one million Caucasians per year. Cytogenetic investigations have revealed recurrent primary aberrations including the loss of an entire chromosome 3 in ~50% of tumors (1–3). Loss of chromosome 3 is frequently accompanied by gain of 8q material, whereas most tumors with disomy 3 show 6p alterations (4). Intriguingly, monosomy 3 is significantly correlated with metastatic disease (5–7). Long-term studies have shown that 4 years after diagnosis ~70% of patients showing monosomy 3 in the primary tumor have died of metastases (5). On the other hand, tumors with disomy 3 rarely give rise to metastatic disease. Therefore, monosomy 3 is a specific and sensitive prognostic indicator of poor survival in uveal melanoma patients.

In many tumors, recurrent loss of genetic material is part of a two-step inactivation of TSGs.[4] The location of several of these genes was narrowed down by detailed mapping of regions showing LOH in tumor DNA (8). In individual tumors, LOH most often affects only one arm of a chromosome or segments thereof. Loss of an entire chromosome 3, although typical for uveal melanomas, is rarely detected in other tumor entities. To reconcile this finding with a two-step mutation model, it was hypothesized that monosomy 3 in uveal melanoma targets two or more distinct TSGs located on the short and long arms of chromosome 3 (5, 9). Inactivation of the respective second alleles may be caused by local mutations such as point mutations, gene deletions, or epigenetic silencing. So far, no alterations on the remaining homologue have been detected by karyotyping or CGH. However, alterations at these putative loci may be below the detection limit of these methods. Several TSGs have been identified on the short arm of chromosome 3, but there is no evidence that any of these genes is involved in tumorigenesis of uveal melanoma. In one study, homozygous loss of the thyroid hormone receptor β gene (*THRB*), which is located in 3p24, was detected in 3 of 19 uveal melanomas using Southern blot hybridization (10). However, in another study, the finding could not be confirmed in 13 uveal melanomas informative at the *THRB* locus (9). In another 46 tumors, comparative PCR including a marker located in the *THRB* region, also did not show any homozygous deletion.[5] There are only very few findings that might help to narrow down the location of putative TSGs on chromosome 3. In two uveal melanomas, a translocation with breakpoint at 3q23 and 3p13 was reported as the only clonal aberration (11, 12). However, no other translocations or deletions involving these regions have been reported so far. Other partial deletions of chromosome 3 have been reported in only two uveal melanomas and in both, large segments of the long arm were lost (9). To obtain positional information on putative TSGs in uveal melanoma, we have searched for partial deletions in the tumors from 333 patients. The tumors were examined by conventional karyotype analysis, CGH, or MSA. Here we describe 13 tumors with a partial deletion of chromosome 3. With this new information, it was possible to define two distinct SRO. One is on the long arm of chromosome 3, spanning 3q24–q26; the other is located on the short arm at 3p25. Genotyping of microsatellite markers shows that the latter is close to the *VHL* gene but does not overlap with this locus.

MATERIALS AND METHODS

Patients. Diagnosis of uveal melanoma was established following current ophthalmological and histological criteria. Vital tumor samples were obtained from patients treated by primary enucleation without prior radiation or chemotherapy. Peripheral blood and tumor material were obtained at the time of operation and stored at −20 and −80°C, respectively.

Cytogenetic Analyses, CGH, and Genotyping of Polymorphic Microsatellites. Cytogenetic analyses and CGH were performed as described previously (5, 13, 14). DNA extractions from blood and tumor samples and PCR-based diagnosis of chromosome 3 loss were carried out as described previously (15). In brief, fluorescently labeled primers (Research Genetics, Huntsville, AL) for amplification of microsatellites *D3S3050, D3S2406, D3S3045, D3S1744, D3S2421, D3S1311,* and *D3S1272* from DNA of tumors and corresponding blood samples were used in individual reactions. In addition, *D3S1481* was investigated in cases M13789 and M16397. Reaction

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[4] The abbreviations used are: TSG, tumor suppressor gene; CGH, comparative genomic hybridization; MSA, microsatellite analysis; SRO, smallest region(s) of deletion overlap, LOH, loss of heterozygosity; NCBI, National Center for Biotechnology Information.

[5] Unpublished data.

Table 1 *Uveal melanomas as primarily analyzed by CGH, MSA, and karyotype analysis*

Laboratory	Method	Total no. of tumors	Chromosome 3 status		
			Disomy	Monosomy	Partial monosomy
Essen	Karyotyping	27	15	12	0
	CGH	126	42	78	6 (5%)
	MSA	95	59	34	2 (2%)
Vancouver	Karyotyping	85	39	41	5 (6%)
Total		333	155	165	13 (4%)

Table 2 *Clinical data of uveal melanomas with partial monosomy 3*

Tumor	Follow-up (yr)	Metastases	Tumor thickness	Ciliary body involvement	Cell type
M6167	4	No	Unknown	Yes	Epithelioid
VGH1	3.5	Yes	17 mm	Yes	Epithelioid
VGH2	No		12 mm	No	Mixed
VGH3	No		60 mm	Yes	Mixed
VGH4	7	No	33 mm	No	Mixed
M16800	1	No	13 mm	Yes	Spindle
M6159	5	No	Unknown	Yes	Unknown
M13789	No		15 mm	No	Spindle
VGH5	No		20 mm	Yes	Mixed
M4352	4	Yes	Unknown	Yes	Mixed
M2358	6	No	7 mm	No	Spindle
G1350	7	No	12 mm	No	Spindle
M16397	2	No	16 mm	Unknown	Spindle

products were analyzed using Abi 310 and 3100 automated capillary genetic analyzers and GeneScan and Genotyper software (Applied Biosystems, Foster City, CA).

Primers for analysis of all other markers as listed in Fig. 1 were custom synthesized without fluorescent label according to sequences available from the University of Texas Health Science Center at San Antonio, TX.[6] Amplification was carried out by adding 4 pmol of each primer to a 20-μl reaction mixture, containing 1× GeneAmp PCR buffer (Applied Biosystems), 50 ng of template DNA, 0.2 mM each dNTP, and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems). To enable automatic analysis, 12.5 pmol of dUTP fluorescently labeled with dye R110 (Applied Biosystems) were added to each reaction. Amplification was performed in a Gene Amp PCR system 9700 thermocycler (Applied Biosystems). PCR conditions were as follows: 94°C for 2 min initial denaturation, 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, with a final extension for 7 min at 72°C. To test for PCR efficiency, 5 μl of the products were loaded on 2% agarose gels. Usually, the PCR products were diluted 1:8 in water and 1 μl was added to 12.5 μl of deionized formamide and 1 μl of TAMRA fluorescently labeled GeneScan-500 size standard (Applied Biosystems). Analyses were performed using the automated analysis systems as described above.

RESULTS

The data presented here are based on the analysis of all of the 333 uveal melanomas that have been successfully examined at the Vancouver General Hospital or at the Universitätsklinikum Essen since 1989 and 1987, respectively. Tumors were analyzed by karyotype analysis, CGH, or genotyping of polymorphic microsatellites on chromosome 3. Loss of an entire chromosome 3 was identified in 165 (49%) of 333 tumors (Table 1). Alterations involving parts of chromosome 3 were detected in 13 tumors (Table 2; Fig. 1). The results obtained in two tumors (VGH1 and VGH4), both with a large deletions involving 3q, were included in a previous report (9). In two tumors (VGH3 and M16800), partial loss of chromosome 3 was the only detectable aberration. Conventional karyotyping or CGH detected a partial deletion of chromosome 3 in 11 tumors (Fig. 1). In two other tumors (M16397 and M13789), an alteration of chromosome 3 was at first detected by MSA using eight polymorphic chromosome 3 markers, a test that was established to replace CGH for diagnosis of monosomy ("Materials and Methods"). To distinguish between a deletion and isodisomy, both of the tumors were also analyzed by CGH. The average ratio profile of tumor M16397, which showed LOH of *D3S3050* located at 3p25, did not cross the lower threshold line set for losses of material at any point along chromosome 3. However, because the ratio profile comes very close to the threshold at the telomere (Fig. 2), the extent of material lost in this region may be below the detection limit of CGH. Tumor M13789 showed two discontinuous interstitial deletions on the short and long arms of chromosome 3 involving 3p14–25 and 3q11–26, respectively (Fig. 2).

Eight tumors showed loss of genetic material on the long arm of chromosome 3. Comparing the affected segments, we were able to

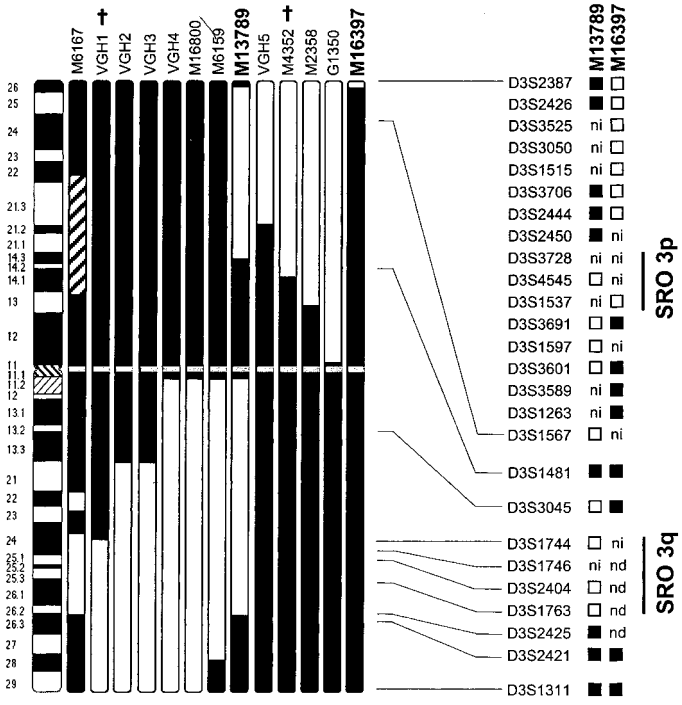

Fig. 1. *Left*, losses and gains of chromosome 3 in tumors with partial monosomy as assessed by CGH and karyotype analysis. Tumors are the same as listed in Table 2. *VGH*, tumors analyzed in Vancouver. *Right*, microsatellite analysis of tumors M13789 and M16397 defining the 3p SRO. *Vertical bars*, SROs. Approximate location of microsatellite markers is indicated; *ni*, not informative; *nd*, not done. ■/filled chromosome, retention of both alleles/no copy number change; *hatched region*, gain of material; □/open chromosome, loss of material/heterozygosity; †, dead of disease.

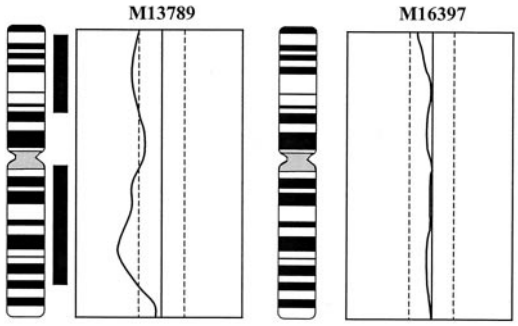

Fig. 2. Results of CGH analysis of chromosome 3 (tumors *M13789* and *M16397*). *Next to each ideogram*, average ratio profiles along the chromosome. *Stippled vertical lines left and right of the solid line in the middle*, upper and lower thresholds of the normal range as described previously (14).

[6] Human Genome Project: http://apollo.uthscsa.edu.

define a SRO at 3q24–q26 (Fig. 1). The centromeric boundary of this SRO is defined by LOH at *D3S196* and retention of heterozygosity of the dinucleotide marker at the *RHO* locus in tumor VGH1 (9). Tumor M13789 localizes the telomeric border of the SRO between *D3S1763* and *D3S2425*, which show LOH and retention of heterozygosity, respectively.

Six tumors had lost genetic material on the short arm of chromosome 3, and the affected segments indicate a second SRO at 3p25 (Fig. 1). To delimit the commonly deleted region on 3p, we analyzed 17 polymorphic markers available from this region. Tumor M16397 showed LOH at all of the informative markers from *D3S1537* at 3p25 to *D3S2387* in the telomeric region. In tumor M13789, LOH was found in a region spanning *D3S1567* to *D3S4545* (Fig. 1). The SRO on 3p was flanked by markers *D3S2450* and *D3S3691*.

We were interested to know whether the *VHL* gene, a well-established TSG located in 3p25–26, is contained in the uveal melanoma SRO on 3p. Integrated physical and genetic map information (Ref. 16; NCBI[7]), indicates that *D3S3691* and *D3S2450* are located at a distance of 11.491–10.601 and 8.092 Mb from the telomere, respectively. According to this data, the SRO on 3p has an extent of ~2.5 Mb and does not overlap with the *VHL* gene, which is located at a distance of 13.252–13.320 Mb from the telomere. Follow-up data for more than 3 years were available for 7 of 13 patients with partial monosomy 3 (Table 2). Two patients died of metastatic disease: one whose tumor (VGH1) showed a deletion of a segment of the long arm (3q24–29) and another whose tumor (M4342) had lost a substantial part of the short arm of chromosome 3 (3p14–26).

DISCUSSION

Metastases of uveal melanoma are found almost exclusively in patients whose primary tumors have lost an entire chromosome 3 (5, 9). This indicates that loss of genes on chromosome 3 is, either by itself or in addition to other genetic alterations, a prerequisite for metastasizing disease. Loss of one chromosome 3 may be one step towards inactivation of one or more TSGs on chromosome 3. For the localization of these putative loci, the rare uveal melanomas with a partial deletion of chromosome 3 are pivotal. Among a consecutive series of 333 uveal melanomas referred to us for cytogenetic or molecular analysis, we identified hemizygous deletions restricted to the long or short arm of this chromosome in seven and five tumors, respectively. In addition, one tumor showed an interstitial deletion on both chromosome arms. Intriguingly, although most of the 13 tumors had lost different parts, it was possible to identify a SRO on each arm of chromosome 3.

The SRO on the long arm is located at bands 3q24–q26. Deletions involving this region are rarely seen in other tumor types. Recurrent losses of 3q have been reported in osteosarcomas (17), abdominal paragangliomas, and pheochromocytomas (18). Interestingly, the minimal region of LOH in osteosarcomas was bounded by *D3S1212* and *D3S1246*, which are located at the telomeric border of the SRO identified here (19). A TSG that might be the target of deletions involving 3q has not been reported yet. The second SRO in uveal melanoma is defined by LOH at *D3S4545* and *D3S1537*, which map to a region in 3p25. Losses involving various regions of 3p are a frequent finding in many tumors (8). The *VHL* gene, which is frequently altered in many tumors, is located in 3p25–26. However, available data indicate that the SRO on 3p identified here does not contain the *VHL* gene. In fact, data on allele loss in other tumors have also indicated the presence of a TSG telomeric of *VHL* (20). According to the Human Genome Resources available at the NCBI only about 30 expressed sequences are localized between *D3S2450* and *D3S3691*, which bind the SRO on 3p reported here. Of the few sequences that represent genes with known function, none is a likely candidate for a putative TSG in uveal melanoma.

Long-term follow-up data (>3 years) were available for seven of the patients whose tumor had a partial deletion. Two of them died of metastasizing disease. The tumor of one of these patients has a deletion on the short arm, whereas in the other, a segment of the long arm is lost. Thus, the presence of a single SRO related to metastatic disease is most unlikely.

In most human tumors, the chromosomal mutations that may contribute to biallelic inactivation of a TSG are accompanied by allele loss of only subchromosomal regions. Loss of an entire chromosome is very uncommon (21). To account for monosomy 3 in uveal melanoma, it was hypothesized that, in this tumor, two TSGs, one each on the long and the short arms of chromosome 3 are inactivated (5, 19). If partial deletions in uveal melanoma target the same genes that are subject to biallelic inactivation in monosomic tumors, the two SROs identified here represent the first molecular evidence for this hypothesis. In view of the tight association between the metastasizing potential and monosomy 3 in uveal melanomas, identification of the genes that are the possible targets of inactivation will bring insights into the mechanisms underlying fatal outcome of patients with uveal melanoma.

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