Expression of \textit{APITD1} Is Not Related to Copy Number Changes of Chromosomal Region 1p36 or the Prognosis of Uveal Melanoma

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**PURPOSE.** Concurrent loss of chromosome arm 1p, region 36, and chromosome 3 leads to decreased disease-free survival in patients with uveal melanoma. A candidate tumor-suppressor gene \textit{APITD1} is located on the critical region on chromosome arm 1p, and it was therefore hypothesized that lower expression levels of this gene could lead to decreased survival in patients, with concurrent loss of a region on chromosome arm 1p and chromosome 3. Using neuroblastoma cells, which, like uveal melanoma, originate from neural crest cells, a former study showed that \textit{APITD1} has cell growth and/or cell death properties. In this study, \textit{APITD1} expression was analyzed to determine whether it corresponds with the DNA copy number and is related to survival in uveal melanoma.

**METHODS.** To detect whether loss in the copy number of \textit{APITD1} results in lowered mRNA expression of the gene, FISH analysis was combined with real-time PCR. In addition, the effect of \textit{APITD1} expression on survival was studied by using Kaplan-Meier survival analysis.

**RESULTS.** Expression of \textit{APITD1} mRNA was not related to DNA copy number \((P = 0.956)\) or chromosome 3 status \((P = 0.958)\). Kaplan-Meier survival analysis showed very similar survival curves for tumors with high and low \textit{APITD1} expression, with a log-rank significance value of \(P = 0.9682\).

**CONCLUSIONS.** These results indicate that \textit{APITD1} is not the tumor suppressor gene on 1p36 responsible for the negative prognostic effect in uveal melanoma with concurrent loss of chromosome arm 1p, region 36, and chromosome 3. (Invest Ophthalmol Vis Sci. 2007;48:4919 - 4923) DOI:10.1167/iovs.07-0061

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Disclosure: W. van Gils, None; H.W. Mensink, None; E. Kilic, None; J. Vaarwater, None; M.M. Verbiest, None; D. Paridaens, None; G.P. Luyten, None; A. de Klein, None; H.T. Bruggenwirth, None.

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Uveal melanoma (UM) is the most common primary malignant intraocular tumor in the Western world, with a yearly incidence of six per million.\textsuperscript{1} Cytogenetic and molecular genetic studies revealed that over 80% of the UM from sporadic cases have a nearly diploid character with simple nonrandom chromosomal aberrations, of which amplification of chromosome arms 8q and 6p and the loss of chromosome 3, the chromosome arm 6q, and the distal part of chromosome arm 1p are the most frequent.\textsuperscript{2,3} Loss of chromosome arm 1p, region 36, is also frequently observed in various other tumor types, including neuroblastoma and pheochromocytoma, which also originate from neural crest-derived cells. In neuroblastoma, loss of chromosome arm 1p is known to be a predictor of unfavorable clinical outcome.\textsuperscript{4,5} In UM, loss of the tip of 1p, as was identified with FISH-probe RP11-48E9 located on 1p36, has been detected in metastasizing tumors.\textsuperscript{3} Furthermore, concurrent loss of this region and chromosome 3 is associated with decreased survival of patients with UM.\textsuperscript{2} This suggests that a tumor suppressor gene involved in UM is located on the distal region of 1p. In our own tumor set, we could not identify losses of the telomeric part of 1p that were smaller than 1p34-pter, and Hughes et al.\textsuperscript{6} identified the smallest region of overlap (SRO) ranging from 1p34-pter using array-CGH. This region is still considerably large and is very gene dense, which makes it hard to identify candidate genes. However, in neuroblastoma, a 500-kb region on 1p36.2-1p36.3 was reported,\textsuperscript{7} which includes the promising candidate tumor suppressor gene \textit{APITD1} (apoptosis-inducing, TAF9-like domain 1) positioned at 1p36.22. The protein, encoded by this gene contains a domain that is similar to the human TATA box binding protein-associated factor, TAFII31 (locus name TAF9). TAFII31 has been identified as a critical protein in p53-mediated transcription activation.\textsuperscript{8} As p53 is associated with apoptotic cell death and growth arrest, \textit{APITD1} may have a role in tumor suppression. Krona et al.\textsuperscript{9} showed that addition of \textit{APITD1} mRNA to neuroblastoma cells results in a reduction of cell growth (up to 90%) compared with nontreated cells, suggesting that \textit{APITD1} indeed has a role in the cell death pathway of neuroblastoma. Loss of function or downregulation of \textit{APITD1} can thus be a way for tumor cells to overcome the cell growth-regulating properties of the p53 pathway. In UM, the p53 pathway is not affected through alterations in p53 protein levels.\textsuperscript{10} Therefore, decreased expression of \textit{APITD1} could be involved in UM by interfering with the p53 pathway. We have analyzed whether loss of 1p36 leads to decreased expression of \textit{APITD1} in UM. Furthermore, we evaluated whether lower expression levels of \textit{APITD1} were associated with a decreased patient survival. A relation between 1p36 loss and decreased expression would indicate \textit{APITD1} as a possible candidate tumor-suppressor gene responsible for poor prognosis in UM with concurrent loss of region 1p36 and chromosome 3.
METHODS

Patient Samples and Cell Lines

Fresh tumor tissue was obtained from patients without prior radiation or chemotherapy within 1 hour after primary enucleation. Informed consent was obtained before enucleation, and the study was performed according to the tenets of the Declaration of Helsinki. Tumors were processed for fluorescence in situ hybridization (FISH) and cytogenetic analysis as described previously. Part of the tumor was snap frozen and stored in liquid nitrogen. In addition, 11 UM-derived cell lines were used. Mcl270, Mel 202, EOM 3, OCM1, and 92.1 are cell lines derived from primary tumors. OMM1, -2, and -3 were established from metastases from different patients with UM and OMM2.2, -2.3, and -2.6 are all cell lines derived from different metastases of the same patient from whom Mel 270 was also derived. Also, two cell lines obtained from normal eye melanocytes, EMC1 and -4, were included as a control for expression in normal melanocytes.

Fluorescence In Situ Hybridization

Dual color FISH on uncultured tumor material, using centromeric and locus-specific cosmid P1 or YAC probes for chromosomes 1 (short arm), 3, 6, and 8, was performed as described previously. Twenty tumors were selected from our UM database based on the FISH scores on chromosome arm 1p, 10 with loss of 1p36.33, and 10 with normal copy numbers of this region. All tumors were further analyzed with BAC probe RP1-1-199O1 mapping to the APITD1 sequence at 1p36.22, combined with BAC probe RP11-48E9, mapping to 1p36.33, as a reference probe. Both probes were selected from the human genome browsers of the University of California at Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgGateway/ provided in the public domain by UCSC Genome Bioinformatics, University of California at Santa Cruz, Santa Cruz, CA) and the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi). The probes were validated on normal peripheral blood cell metaphase spreads and interphase nuclei. For each probe, 10 metaphases and 100 interphases were analyzed. Abnormal copy numbers were detected in less than 3% of the scored interphase nuclei. In the tumors, signals were counted in 200 interphase nuclei according to the criteria of Hopman et al. Cutoff limits for deletion (15% of the nuclei with one signal) or amplification (>10% of the nuclei with three or more signals) were adapted from the available literature.

RNA Purification, cDNA Synthesis, and PCR Assay

Five to eight sections with a thickness of 50 μm were made from fresh-frozen tumor tissue, depending on the size of the tumor. RNA was isolated from the sections (RNA-Bee; TelTest Inc., Friendswood, TX), and cell line RNA was isolated (RNeasy Mini Kit; Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol. RNA quantity was measured by spectrophotometer (model ND-1000; NanoDrop Technologies, Wilmington, DE), and the quality was assayed (Bioanalyzer 2100; Agilent, Palo Alto, CA). From 1 μg of total RNA cDNA was synthesized using 2 μL of a random hexamer primer (0.5 μg/μL) and 10 U of reverse transcriptase (HT Biotechnology, Ltd., Cambridge, UK), according to the manufacturer’s instructions. Solutions were diluted to 10 ng/μL for cDNA synthesis.

For the PCR, master mix (TaqMan Universal Master Mix; Applied Biosystems, Inc. [ABI], Foster City, CA) was used. Gene expression assays were selected for APITD1 and three reference genes: β2-microglobulin (B2M; 15q21-q22), β-glucuronidase (GUSB; 7q21.11), and hypoxanthine phosphoribosyltransferase (HPRT1; Xq26.1). Each of these assays consists of two unlabeled primers and a probe labeled with the 5’ reporter dye FAM and the 3’ quencher TAMRA (ordered from the ABI Assays-on-Demand Platform). Every reaction contained 3.575 μL H2O, 12.5 μL master mix (2X) (without AmpErase UNG; ABI), 2.5 μL of gene expression assay reagent, and 1 μL of cDNA. The reactions were run on a sequence-detection system (Prism 7700; ABI).

The solution was subjected to a protocol of 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute.

The efficiency of the PCR assay was determined by assaying a control pool of human RNA extracted from three lymphoblastic cell lines in dilutions of 10, 20, 100, 200, and 1000 times. With the ΔRn (threshold) set at 0.1, a standard curve of mean Ct for three replicates at each dilution versus log10 amount of cDNA was determined. The efficiency of the reaction was calculated from the slope of this standard curve using the formula $e^{\text{slope}} = 10^{-1/\text{slope}}$. These efficiencies were 1.9991, 1.9996, and 1.9996 for the reference gene assays B2M, GUSB, and HPRT1, respectively. APITD1 had an efficiency of 1.9855. Of the three endogenous control genes tested, GUSB and HPRT1 showed the least intratumor variation, and GUSB expression levels came closest to APITD1 expression (data not shown). Therefore, GUSB was chosen as the endogenous control in APITD1 relative expression measurements. To estimate the relative expression of APITD1 the difference in Ct of APITD1 and the chosen endogenous control gene, ΔCt, was determined for each sample. This ΔCt was transformed by $2^{-\Delta C_{\text{t}}/1000}$ to correct for the logarithmic nature of the Ct. Differences in amplification efficiency between the assays, approximately 2 in all assays, were insignificant and therefore not taken into account in the calculations.

Statistical Analysis

Relative expression of APITD1 mRNA in a group of 10 tumors with loss of one copy of the APITD1 region was compared with relative expression in a group of 10 tumors without loss of this region in a two-sample t-test. The relative expression of APITD1 was also compared between groups of tumors with and without monosomy of chromosome 3, consisting of 10 and 9 samples, respectively (the chromosome 3 status of one patient was uncertain). The tumors were divided into a group with high and low expression to analyze the influence of APITD1 expression levels on disease-free survival, with 12 (the geometric mean of all samples) chosen as the highest value for $2^{-\Delta C_{\text{t}}/1000}$ in the group of low expression, thereby dividing the patients into two groups of 10 each. Kaplan-Meier survival analysis and the log rank test were performed to determine the influence of APITD1 expression on survival. Disease-free survival is the time from enucleation to the development of metastatic disease or disease-related death. All tests were two-sided. Statistical analyses were performed with commercial software (SPSS, ver. 11; SPSS, Chicago, IL).

RESULTS

Fluorescence In Situ Hybridization

Twenty tumors were selected from our UM database on the basis of routine FISH scores on chromosome arm 1p, region 36. Ten showed loss of one copy of 1p36.33 and 10 had normal copy numbers of this region. All tumors were analyzed with FISH with BAC probes RP11-199O1 (1p36.22) and RP11-48E9 (1p36.33). Results are presented in Table 1. The results found with the diagnostic probe RP11-48E9 were not different from the results obtained with the APITD1 probe RP11-199O1, indicating that the region of loss detected in routine FISH encompassed at least the APITD1 gene in all cases.

Real-Time PCR

APITD1 expression was analyzed by quantitative real-time PCR. APITD1 was not differentially expressed in melanocytic and UM derived cell lines. Independent-sample t-tests showed a 4.1 times higher expression in cell lines compared with the expression in the primary tumors ($P < 0.001$; Fig. 1). There was no significant difference in APITD1 expression levels between tumors with and without loss of 1p36, irrespective of
chromosome 3 status \((P = 0.956)\). Irrespective of 1p36 status \(APITD1\) expression levels were also not significantly different between tumors, with and without loss of chromosome 3 \((P = 0.958)\), and between tumors with concurrent loss of 1p36 and chromosome 3 and tumors without loss of chromosome arm 1p and chromosome 3 \((P = 0.764)\). To test for a significant difference between \(APITD1\) expression and the survival of patients, the samples were separated into groups of high and low \(APITD1\) expression. Kaplan-Meier survival analysis showed very similar patterns of survival in both groups, with a resulting log rank probability close to 1 \((P = 0.9682; \text{Fig. 2})\).

**DISCUSSION**

A frequent characteristic of UM is deletion of the distal part of chromosome arm 1p. In combination with the loss of chromo-

### Table 1. Relative Expression of \(APITD1\) Compared with \(GUSB\) with DNA Copy Number and Follow-up Data

<table>
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<tr>
<th>Tumor</th>
<th>Relative Expression of (APITD1) ((2^{-\Delta\text{Ct} \times 1000}}))</th>
<th>DNA Copy Number*</th>
<th>Survival (y)</th>
<th>Event†</th>
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</table>

* The reported copy numbers were scored in 72% of the counted nuclei in one case and over 80% in all other cases. NA, data not available.
† 0, no event has occurred; 1, event (melanoma-related death).

**Figure 1.** \(APITD1\) expression in UM and melanocyte cell lines and primary UM. Results of the primary UM were divided into two groups, based on the number of \(APITD1\) DNA copies. The \(y\)-axis indicates the relative expression of \(APITD1\) compared with the \(GUSB\) housekeeping gene, calculated with \(2^{-\Delta\text{Ct} \times 1000}}\). The identity of each sample is indicated along the \(x\)-axis.
some 3, loss of 1p36 leads to decreased disease-free survival. 2 This implicates 1p36 as a location of a UM prognosis-related suppressor gene. In neuroblastoma, also originating from neural crest-derived cells, a small cluster of genes in a 500-kb SRO was reported. The genes in this cluster—APITD1, UBE4B/UFD2, KIF3B, PGD, DFFA, and PEX14—are all downregulated in high-stage neuroblastomas and are all candidate tumor-suppressor genes. For APITD1, UBE4B/UFD2, and DFFA, a relation with high-stage neuroblastoma was reported. Besides a lower expression in high-stage neuroblastomas, a splice site mutation was detected in UBE4B/UFD2 in a high-stage neuroblastoma with a fatal outcome, and there were also some coding mutations found in the DFFA gene in neuroblastoma. The DFFA gene has essential functions in the final stage of apoptosis. Altogether, this evidence suggests a role for these genes at least in high-stage neuroblastoma. 7,9,19–22 In UM, the p53 pathway is not affected through alterations in p53 protein levels. 10 Therefore, interference of the p53 pathway could be caused by another mechanism. Kilic et al. suggested that p73, a p53 homologue located on 1p36 is a possible prognosis-related suppressor gene (unpublished data, March 2006). Another candidate gene located on 1p36 is CHD5, which encodes a protein that functions in the p53 pathway, and was recently shown to function as a tumor suppressor in vivo. 23 The APITD1 gene in the reported neuroblastoma gene cluster is associated with p53 activity and has been shown to inhibit cell growth. 7,9 Because expression of APITD1 is almost absent in a variety of tumors 2 and because of its relation with p53 activity, downregulation of APITD1 could provide an alternative way to interfere with the p53-mediated pathway in tumors without alterations in p53 protein levels. Therefore, we characterized expression and copy number of the APITD1 gene in UM.

We combined FISH analysis with real-time PCR to assess whether a reduced APITD1 copy number results in lowered expression of the gene. A Kaplan-Meier survival analysis was performed with APITD1 expression as a discriminator to study the effect of APITD1 expression on survival. We analyzed APITD1 expression in 10 tumors with and 10 tumors without loss of 1p36, using a FISH probe mapping to 1p36.33. In all cases, loss of this region concurred with the loss of one copy of the APITD1 region, whereas retention of two copies of 1p36.33 was always combined with two copies of the APITD1 region. There was no difference found in APITD1 expression between tumors with and without loss of 1p36. Similar results were obtained for tumors with and without loss of chromosome 3 and for tumors with and without concurrent loss of 1p36 and chromosome 3. In addition, in Kaplan-Meier survival analysis based on high and low expression of APITD1, the two groups showed very similar survival curves with a log-rank significance of 0.9682 (Fig. 2). This result indicates that down-regulation of APITD1 is probably not the mechanism for the immortality of those cell lines. We showed that expression of the APITD1 transcript is generally elevated in both melanocyte and UM-derived cell lines, compared to primary UMAs. This finding is in concordance with the earlier report on APITD1 expression in neuroblastoma in which APITD1 expression was also considerably lower in primary tumors compared to neuroblastoma-derived cell lines. 9

The negative effect on prognosis of loss of 1p36 in tumors with monosomy of chromosome 3 is most probably caused by decreased expression of a tumor suppressor gene located on this region as a result of the chromosomal loss. After the data in Table 1, we could verify for this group of cases that concurrent loss of 1p36 and chromosome 3 has an adverse effect on patient outcome (log rank \( P = 0.0259 \)), which shows that that this cohort has the correct size and composition to evaluate this adverse survival effect. In the present study, APITD1 mRNA levels alone are not associated with survival. Furthermore, we could not find differential expression for APITD1 in tumors, with and without loss of 1p36. From these results, we conclude that APITD1 is not the suppressor gene on 1p36 responsible for the poor prognosis in UMAs with concurrent loss of 1p36 and chromosome 3.

**Acknowledgments**

The authors thank Eline van Meel for excellent technical assistance and Ruchi Saxena for critically reviewing the manuscript and helpful comments.

**References**


