Purpose. Although studies on uveal melanoma (UM) revealed prognostic significance of chromosomal aberrations, they resulted in classification errors in survival prediction. A robust prognostic classifier with strong predictive value and further insight in genes responsible for poor prognosis were obtained by performing a gene-expression profile in tumors of UM patients for which extensive clinical, histopathologic, cytogenetic, and follow-up data were available. Furthermore, the UM microarray expression data were compared with cytogenetic data.

Methods. Gene-expression profiles of 46 UMs were obtained with microchip assays. Data were analyzed with cluster-analysis and predictive analysis of microarrays (PAM) software and validated with real-time PCR. The prognostic significance of UMs with specific molecular signatures was determined. Furthermore, LAP analysis resulted in the identification of differentially expressed chromosomal regions.

Results. The primary UMs were classified in two distinct molecular classes with a strong prognostic value ($P < 0.001$; hazard ratio 7.7). Classifier gene sets for microarray class and disease-free survival were validated with real-time PCR, and the predictive value of the UM class marker set was validated with gene-expression profiles of tumors provided by other institutions, showing a sensitivity of 0.93 and specificity of 1.00 for class II tumors. A locally adaptive statistical procedure identified two regions on the short arm of chromosome 3 with decreased gene-expression in tumors with shorter disease-free survival.

Conclusions. Microarray classification outperforms known prognostic indicators for UM, such as clinical, histopathologic, and cytogenetic parameters. In addition, the identified regions with lower expressed genes on 3p could harbor genes that are responsible for the poor prognosis of patients with UM. (Invest Ophthalmol Vis Sci. 2008;49:4254 – 4262) DOI:10.1167/iovs.08-2033
Expression Profiling in UM: Two Regions on 3p

PAM Analysis

Supervised class-prediction analyses were performed by applying PAM (predictive analysis of microarrays) software (Excel, PAM version 2.1 Excel plugin; Microsoft, Redmond, WA). This program uses the method of the nearest shrunken centroids to identify a subgroup of genes that best characterizes a predefined class. The gene contents of the different classifier sets are shown in Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/49/10/4254/DC1. The prediction error was calculated by 10-fold cross-validation within a training set (two thirds of the patients) followed by analysis of a test set (one third of the patients).

Validation by Real-Time PCR

The eight most differentially expressed known classifying genes for UM class and the 4-year disease-free survival (DFS) classifier were tested in real-time PCR, together with a candidate tumor suppressor and oncogenes that were present in the microarray class and other genes that have been described by others to be associated with prognosis in other gene-expression profiling studies on UM. The cutoff of 4 years for DFS was chosen since death in the first 48 months is probably mainly due to micrometastatic spreading before initiation of treatment. For methods and results of this validation see Supplementary Data and Supplementary Table S2, online at http://www.iovs.org/cgi/content/full/49/10/4254/DC1.

Identification of Differentially Expressed Chromosomal Regions

Differentially expressed chromosomal regions were identified with a computational tool called the locally adaptive statistical procedure (LAP), which combines transcriptional data with structural information and estimates the differential expression of chromosomal regions accounting for variations in the distance between genes and gene density. LAP analysis was performed in R, version 2.2.1. Functions in R for implementing the LAP method were obtained from the Website http://www.dpc.i.unipd.it/Bioeng/Publications/LAP.htm at the University of Padua, Italy.

Statistical Analysis

Statistical analyses were performed with commercial programs (SPSS software, ver. 11.0; SPSS, Chicago, IL). Odds ratios with corresponding probabilities were calculated to identify associations between the different parameters. Actuarial probabilities of DFS (with an event defined as development of metastatic disease or death by disease) were estimated according to the Kaplan-Meier method. To examine the possibility that other clinical, histopathological or chromosomal variations affected the prognosis, we performed Cox proportional hazards analysis for each confounding variable. An effect was considered significant at $P \leq 0.05$.

RESULTS

Selection

Patients ($n = 49$) were selected from the tumor database such that numerical abnormalities of chromosomes 1, 3, 6, and 8 were all represented in at least 10 patients, as well as UM with no numerical chromosome anomalies.

Unsupervised Clustering and Visualization

After applying a hierarchical clustering algorithm on the 528 probe sets with the highest differential expression, the UMs

Tumor Selection

Patients ($n = 49$) were selected from our extensive database with information on follow-up and clinical, cytogenetic, and histopathologic parameters. The selection was made such that numerical abnormalities of chromosomes 1, 3, 6, and 8 were all represented in at least 10 patients, as well as UM with no numerical chromosome anomalies.

Preparation of RNA and Hybridization

From fresh-frozen tumor tissue, a 5-μm section was made for H&E staining and depending on the size of the tumor, five to eight sections of 50 μm were used for RNA isolation (RNA-Bee; TelTest, Friendswood, TX) according to the manufacturers’ instructions. RNA quantity (20–80 μg in 20 μL) was measured with a spectrophotometer (model ND-1000; NanoDrop Technologies, Wilmington, DE), and quality was assayed on a bioanalyzer (model 2100; Agilent, Palo Alto, CA). A commercial cRNA extraction kit (GeneChip One-Cycle Target Labeling and Control Reagents package; Affymetrix, Santa Clara, CA) was used to create biotinylated cRNA out of 5 ng per slide for RNA isolation (RNA-Bee; TelTest, Friendswood, TX) according to the manufacturers’ instructions. RNA quantity (20–80 μg in 20 μL) was measured with a spectrophotometer (model ND-1000; NanoDrop Technologies, Wilmington, DE), and quality was assayed on a bioanalyzer (model 2100; Agilent, Palo Alto, CA). A commercial cRNA extraction kit (GeneChip One-Cycle Target Labeling and Control Reagents package; Affymetrix, Santa Clara, CA) was used to create biotinylated cRNA out of 5 μg of total RNA. The cRNA was used for hybridization on microchips (Hu133 2.0 Plus GeneChips with Fluidics 450 station; Affymetrix), and the arrays were scanned (GeneChip Scanner 3000; Affymetrix), according to the manufacturer’s instructions.

Normalization

Only gene chips with at least 30% present calls and no signs of degradation were analyzed. For normalization, variance-stabilizing normalization (VSN) was used (aiffy, vsn and BioConductor packages in the open-source statistical language R, version 2.2.1). The MisMatch (MM) intensities were ignored, and by doing so only the perfect match (PM) intensities from the created CEL file were taken for normalization. This strategy circumvents systematic overestimation of intensities by implementation of a heuristic when PM intensities are smaller than MM probe intensities. For each probe set, the geometric mean of the hybridization intensities of all tumor samples was calculated. To reflect the differential expression, the level of expression of each probe set in every sample was determined relative to its geometric mean and was logarithmically transformed (on a base-2 scale) to ascribe equal weight to gene-expression levels with relative distances similar to the geometric mean.

Unsupervised Clustering and Visualization

The 528 annotations that had a standard deviation of at least 1.25 were selected for unsupervised hierarchical clustering with a K-means algorithm. Pearson’s correlation was used for unsupervised heat-mapping analysis. Probe sets that were differentially expressed in at least one patient were selected for further analysis. Hierarchical cluster analysis, heatmap cluster analysis, and visualization were all performed with commercial software (OmniViz, Inc., Maynard, MA).

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clustered into two discrete molecular classes (UM class 1: \( n = 23 \); UM class 2: \( n = 23 \)). This unsupervised clustering is shown in Figure 1A. With Pearson’s correlation analysis, the same two blocks of related UMs were found (Fig. 1B). Next to this correlation plot, cytogenetic and clinical prognostic markers of the status of 1, 3, 6p, 6q, 8p, and 8q; the presence of epithelioid cells and closed vascular patterns; largest tumor diameter (LTD); ciliary body involvement; and sex are shown (Fig. 1C). Odds ratios of UM class with the different parameters were calculated, and the corresponding probabilities are displayed in Table 1. The parameters 4-year DFS, monosomy of chromosome 3, gain of 6p and closed vascular patterns correlated with UM class with the highest significance. To assess the prognostic significance of UM class and the other parameters, we performed a multivariate analysis. The parameter UM class remained significant after correction for possible confounders and nearing significance when corrected for 8q gain (\( P = 0.053 \)). Also, chromosome 3 loss, 6p gain, 8p loss, 8q gain, ciliary body involvement, and closed vascular patterns were significant when corrected for almost all confounders (Table 2).

**Gene Expression Signatures**

To create classifier sets, we applied PAM analysis for the categorical variables 4-year DFS and UM class (Supplementary Table S1). The 61-gene annotation classifier set for 4-year DFS has a sensitivity of 0.89 and a specificity of 0.6. For UM class, we selected 69 gene annotations that classified all samples correctly in training and cross validation. This 69-classifier set predicts with a maximum score of 1 for both sensitivity and specificity. Both classifier sets were confirmed in real-time PCR (Supplementary Data).

**Survival Analysis**

We evaluated the predictive value of the classifiers for patient survival using Kaplan-Meier analysis and the log rank test (data not shown). Survival analysis of all 46 patients showed that all patients in UM class 2 developed metastatic disease within 7 years of follow-up whereas 86% of the patients in class 1 did not have metastatic disease during this period (\( P = 0.0004 \)). Survival analysis of the groups predicted with the 4-year DFS classifier also showed no 7-year DFS survival in the shorter survival class versus 80% DFS of the patients in the longer survival class (\( P = 0.04 \)). Kaplan-Meier survival analysis was also performed for the parameters chromosome 3 loss, 6p gain, 8p loss, 8q gain, closed vascular patterns and ciliary body involvement, all significant after correcting for possible confounders in Cox proportional hazards analysis. UM class outperformed all other cytogenetic, clinical, and pathologic prognostic factors (data not shown).

**Analysis of Other UM Expression Data Sets**

To evaluate the predictive value of the UM classifier, we used the expression profiles, kindly provided by Tschentscher et al. and Onken et al. All annotations in our classifier were present on the gene chips U133A and B chips (Affymetrix) that were used by Onken et al., but were on the gene chip U95Av2 (Affymetrix) that was used by Tschentscher et al. only 32 gene annotations of our classifier could be identified. For this reason, we have analyzed the complete data set of the three different research groups with an adjusted UM classifier containing the 32 annotated genes, present in all datasets. As a training set, we have chosen the same set of tumor samples that was used earlier in the UM array class prediction, the remaining samples of our dataset were analyzed together with the other datasets. Results are visualized in Figure 2. All 62 tumors in the test set were classified according to the results presented by Tschentscher et al. and Onken et al. with the exception of two samples (MM27 and M18672).

**LAP Analysis**

The locally adaptive statistic procedure (LAP analysis) was used to identify differentially expressed chromosomal regions for chromosome 3 status, UM class and 4-year DFS. LAP analysis, with monosomy 3 as the discriminator (Fig. 3A), confirmed a lower expression of genes over the entire chromosome 3 in tumors with monosomy 3. These monosomy 3 tumors appear to have a decreased expression of genes in the telomeric 2 Mb of 1p36 but on 8q, large regions of higher expression. Other regions of higher expression in tumors with chromosome 3 monosomy were detected on 1q, and on chromosomes 7, 9, 14, 15, 17, 20 and 21. In tumors with two copies of chromosome 3, a 6p region (15 Mb from 6p24.3 to 6p22.2) showed significantly higher expression.

LAP analysis on UM class (Fig. 3B) revealed lower expression of almost the entire short arm of chromosome 3 in class 2 tumors, whereas no significant differential expression of chromosome 3q was found between the two tumor classes. In addition, a part of chromosome 6p (6 Mb from 6p24.3 to 6p23) showed significantly higher expression in class 1, and chromosome 8q had large regions of higher expression in class 2. Also higher expression of 1q in the poor prognosis class and other smaller regions of differential expression were found.

Less differentially expressed chromosomal regions were found when comparing tumors from patients \( \geq 4 \)-year DFS with tumors from patients <4-year DFS (Fig. 3C). Remarkably, on the short arm of chromosome 3, two smaller regions with lower expression in the short survival group were found: one region of 8 Mb ranging from the end of 3p23 to 3p25.3 and one region of 9 Mb ranging from 3p12 to 3p14.1. A small region of 0.5 Mb on 6p (6p23 to 6p24.1) showed significantly higher expression in tumors with a longer DFS. On chromosome 8, a large fraction of the short arm showed significantly lower expression in the short DFS group whereas large regions on the long arm were upregulated in this group. In addition, regions of significantly higher expression in the short survival group are located on chromosomes 10, 12, and 15 and in regions of chromosome 15.

**Discussion**

We report the results of gene-expression profiling analysis of primary tumors from 46 selected patients with UM of all age groups (26–84 years; median, 59) and tumor sizes (7–19 mm; median, 13). Unsupervised clustering of the gene-expression profiles grouped the tumors into two distinct classes that are strongly related with prognosis and 4-year DFS. Other parameters that have a strong correlation with this microarray-based classification are chromosome 3 loss, chromosome 6 abnormalities, closed vascular patterns, and sex. Chromosome 3 loss and the presence of closed vascular patterns, both occurring more often in class 2, are known predictors of poor survival. The higher frequency of gains in 6p in class 1 corresponds with the earlier observed positive relation of prognosis with abnormalities of chromosome 6, resulting in a relative increase of 6p material compared with the long arm. We were able to confirm these findings in our analyzed cohort by using Kaplan-Meier survival analysis. Array class, chromosome 3 loss, 6p gain, 8p loss, 8q gain, closed vascular patterns, and ciliary body involvement were strongly related with prognosis in multivariate analysis with Cox proportional hazards analysis. Because of the tumor selection, the results differ from previous results obtained by our group on a larger cohort of UMs. In this latter study, chromosome 3 loss and 8q gain were also significant after correcting for most confounding parameters, but LTD was also significant. In contrast, 6p gain, 8p loss, presence of closed vascular patterns, and ciliary body involvement were
FIGURE 1. Correlation view of specimens from 46 UM patients involving 528 probes combined with data of follow-up, clinical, pathologic, and cytogenetic parameters. (A) Results of unsupervised hierarchical clustering of probes and tumors with a K-means algorithm are shown. (B) The results of heat-map cluster analysis with Pearson’s correlation algorithm. Two clusters were identified on the basis of the correlation view, separated by a black line. (C) Data of follow-up clinical, pathologic, and cytogenetic parameters are shown.
not significant after correction for confounders in that study. The relatively small size of our currently analyzed cohort and an apparent overrepresentation of tumors with structural abnormalities on chromosomes 1, 3, 6, and 8 could explain this discrepancy.

Because of the very strong relation of microarray-based UM class with survival, that outperforms all known prognostic clinical, histopathologic, and cytogenetic parameters in the studied patients, we used PAM analysis to obtain a gene signature that accurately predicts for UM microarray class. The created 69-gene classifier flawlessly predicted for UM class. We also developed a gene-expression signature for 4-year DFS. The cutoff of 4 years was chosen, since death in the first 48 months is probably mainly due to micrometastatic spreading of tumor cells before initiation of treatment, and preoperative spreading is most likely responsible for a significant part of the melanoma-related deaths after 4 years; these deaths could be prevented by irradiation before enucleation. Therefore, this 4-year time-point serves as a valuable prognostic time-point.\textsuperscript{13,19} This 61-gene 4-year DFS classifier set had a sensitivity of 0.89 and a specificity of 0.60. The low-specificity value could be a result of the low number of patients in the poor survival group in the test set (only four patients), thereby increasing the influence of one misclassified patient. However, when the tumors were classified with this signature, it resulted in two groups of patients, one of which had a survival rate of 80% after 120 months of follow-up, whereas all patients in the other group developed metastatic disease during this period (\(P = 0.014\)). This classification was also outperformed by UM-based tumor classification, and corroborate results reported by Onken et al.,\textsuperscript{7} Worley et al.,\textsuperscript{20} and Petrausch et al.\textsuperscript{21}

LAP analysis was used to determine whether the differential expression was associated with specific genomic regions. We used chromosome 3 status, UM class, and 4-year DFS as discriminatory factors in the LAP analysis. As expected, tumors with monosomy 3 showed lower expression of all the chromosome 3 genes. The results of LAP analysis on UM class showed that only chromosome-arm 3p and not 3q is significantly downregulated in the poor survival group. In both of these LAP analyses, a large part of the genes on 8q were upregulated and downregulation of genes in a region on 6p was observed.

TABLE 1. Correlation between Prognostic Parameters for Uveal Melanoma in the Analyzed Cohort\textsuperscript{4,5}†

<table>
<thead>
<tr>
<th>Array class</th>
<th>4-Year Survival</th>
<th>1p Loss</th>
<th>3p Loss</th>
<th>6p Gain</th>
<th>6q Loss</th>
<th>8q Gain</th>
<th>Age</th>
<th>Epithelioid Present</th>
<th>Closed Vascular Patterns</th>
<th>LTD</th>
<th>Ciliary Body Involvement</th>
<th>nMIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Year Survival</td>
<td>0.003</td>
<td>NS</td>
<td>0.000</td>
<td>0.006</td>
<td>NS</td>
<td>0.026</td>
<td>NS</td>
<td>0.018</td>
<td>NS</td>
<td>0.003</td>
<td>NS</td>
<td>0.018</td>
</tr>
<tr>
<td>1p Loss</td>
<td>NS</td>
<td>NS</td>
<td>0.016</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>3p Loss</td>
<td>0.000</td>
<td>0.016</td>
<td>NS</td>
<td>0.003</td>
<td>NS</td>
<td>0.016</td>
<td>NS</td>
<td>0.025</td>
<td>0.002</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>6p Gain</td>
<td>0.000</td>
<td>0.036</td>
<td>NS</td>
<td>0.003</td>
<td>0.003</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>6q Loss</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>8q Gain</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.016</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.046</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
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<td>Epithelioid Present</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>0.025</td>
<td>0.038</td>
<td>NS</td>
<td>NS</td>
<td>0.046</td>
<td>0.025</td>
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<td>0.003</td>
<td>0.001</td>
<td>NS</td>
<td>0.002</td>
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<td>NS</td>
<td>NS</td>
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<td>0.025</td>
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<td>LTD</td>
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<td>NS</td>
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<td>Ciliary Body Involvement</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>0.030</td>
</tr>
<tr>
<td>nMIF</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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</tr>
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</table>

* The odds ratios of the parameters were calculated with \(\chi^2\) analysis based on the available data of the 46 uveal melanomas; correlation is expressed as probability.
† Dark gray boxes/NS: not significant (\(P > 0.05\)); light gray boxes: 0.01 < \(P \leq 0.05\); white boxes: \(P \leq 0.01\).
MITF acts in the development of various cell types, including neural-crest–derived melanocytes and optic-cup–derived retinal pigment epithelial cells. It transactivates the tyrosinase gene, a key enzyme for melanogenesis known to be critically involved in melanocyte differentiation. However, no correlation between MITF positivity and the parameters cell type, largest tumor diameter, sclera invasion, and mitotic figures was observed in UM as reported by Mouriaux et al. In melanoma cell lines in which the gene was repressed, induced expression of MITF-M showed growth-inhibitory effects and led to a change from epithelioid toward a spindle-cell type in vivo. This suggests that decreased expres-

Table 2. Prognostic Significance of Histopathologic, Clinical, and Chromosomal Aberrations in the Uveal Melanoma Cohort*†

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Array Class</th>
<th>3-Year DFS Class</th>
<th>6p Loss</th>
<th>6p Loss</th>
<th>6q Loss</th>
<th>8p Gain</th>
<th>8q Gain</th>
<th>Age</th>
<th>Epithelioid Present</th>
<th>Closed Vascular Patterns</th>
<th>Ciliary Body Involvement</th>
<th>M/F</th>
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<td><strong>P†</strong></td>
<td>0.000</td>
<td>0.014</td>
<td>0.513</td>
<td>0.008</td>
<td>0.363</td>
<td>0.006</td>
<td>0.397</td>
<td>0.003</td>
<td>0.067</td>
<td>0.057</td>
<td>0.000</td>
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<td>Array class</td>
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<td>0.593</td>
<td>0.287</td>
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<td>0.369</td>
<td>0.255</td>
<td>0.461</td>
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<td>0.535</td>
<td>0.160</td>
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<td>0.013</td>
<td>0.016</td>
<td>0.297</td>
<td>0.017</td>
<td>0.402</td>
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<td>0.059</td>
<td>0.043</td>
<td>0.000</td>
<td>0.149</td>
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<td>1p Loss</td>
<td>0.037</td>
<td>0.477</td>
<td>0.339</td>
<td>0.073</td>
<td>0.871</td>
<td>0.280</td>
<td>0.378</td>
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<td>0.726</td>
<td>0.233</td>
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<td>0.008</td>
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<td>0.341</td>
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<td>0.491</td>
<td>0.025</td>
<td>0.137</td>
<td>0.004</td>
<td>0.328</td>
</tr>
<tr>
<td>Epithelioid present</td>
<td>0.004</td>
<td>0.123</td>
<td>0.204</td>
<td>0.036</td>
<td>0.046</td>
<td>0.781</td>
<td>0.046</td>
<td>0.430</td>
<td>0.012</td>
<td>0.180</td>
<td>0.008</td>
<td>0.076</td>
</tr>
<tr>
<td>Closed vascular patterns</td>
<td>0.017</td>
<td>0.096</td>
<td>0.061</td>
<td>0.048</td>
<td>0.203</td>
<td>0.594</td>
<td>0.013</td>
<td>0.295</td>
<td>0.023</td>
<td>0.292</td>
<td>0.389</td>
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<td>LTD</td>
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<td>0.911</td>
<td>0.598</td>
<td>0.023</td>
<td>0.025</td>
<td>0.730</td>
<td>0.019</td>
<td>0.398</td>
<td>0.014</td>
<td>0.147</td>
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<tr>
<td>Ciliary body involvement</td>
<td>0.006</td>
<td>0.062</td>
<td>0.342</td>
<td>0.037</td>
<td>0.041</td>
<td>0.542</td>
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<td>0.192</td>
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<tr>
<td>M/F</td>
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<td>0.079</td>
<td>0.523</td>
<td>0.022</td>
<td>0.028</td>
<td>0.477</td>
<td>0.017</td>
<td>0.355</td>
<td>0.015</td>
<td>0.116</td>
<td>0.093</td>
<td>0.002</td>
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</table>

* Log rank test.† Multivariate analysis with the Cox proportional hazards analysis.

Figure 2. Test probabilities (threshold = 2) in multiplex PAM analysis of UMs with the 32 UM class gene classifier. Tumors belonging to class 1, according to the literature, are placed on the left side, the tumors belonging to class 2 on the right side. The class 1 prediction probability of a sample with our 32-gene classifier is indicated with open diamonds, the class 2 probability with filled boxes. The samples were classified according to the highest prediction probability. Tumors: M1 numbers from data set Onken et al.; MM numbers from Tschentscher et al.; EOM numbers from our own data set. Class 1: sensitivity 1 and specificity 0.99; class 2: sensitivity 0.93 and specificity 1.
sion of MITF would lead to a more epithelioid phenotype, which is related with poor prognosis in UM. It is interesting that, although MITF expression was also significantly lower in the shorter DFS group, it was not significantly lower in tumors with an epithelioid cell type (data not shown). Our data point to association of MITF expression and survival and it would be interesting to corroborate this in a larger UM cohort. Other candidate tumor suppressor genes on the proximal 3p region are the TATA element modulatory factor 1 (TMF1) and EGF receptor antagonist leucine-rich repeats and immunoglobulin-
like domain 1 (LRIG1). TIMF is a transcription factor that probably regulates the expression of genes via the TATA element.\(^{28}\) Downregulation of LRIG1 increases cell-surface EGF receptor levels, enhances activation of downstream pathways, and stimulates epithelial cells proliferation.\(^{29}\) The other region, ranging from 3p23 to 3p25.3, harbors multiple candidate TSGs, of which XPC, \(\text{WT}^7\text{A}, \text{PPARG}, \text{and TIMP}\text{4}\) are the most promising genes. XPC is a well-described DNA repair gene that functions via nucleotide excision repair (NER) and is linked to type C xeroderma pigmentosum that concurs with a high incidence of malignant melanoma.\(^{30}\) \(\text{WT}^7\text{A}\) belongs to the \(\text{Wt}\) gene family, which genes are implicated in oncogenesis and several developmental processes.\(^{31,32}\) Peroxidase proliferator-activated receptor \(g\) (PPARG) is a nuclear hormone receptor that acts in differentiation of adipocytes in particular, although this gene is also expressed in other tissue types. Activation of PPARG in different cancer cell types induces cell growth inhibition and differentiation.\(^{33}\) This inhibitory effect would be impaired by decreased expression of this gene. TIMP4 belongs to the tissue inhibitors of metalloproteinases (TIMPs), which inhibit matrix metalloproteinases (MMPs), which are involved in degradation of the extracellular matrix. Overexpression of recombinant TIMP4 in breast cancer cells inhibited the invasion potential of the cells in vitro.\(^{34}\) Decreased expression of TIMP4 may therefore lead to an increased invasive potential.

The selective decreased expression of genes in the two regions on 3p in poor-prognosis UMs could, besides loss of chromosomal DNA, also be caused by epigenetic mechanisms, such as methylation of gene promoter regions. Promoter methylation has been identified as the mechanism responsible for lower expression of the classifier gene TIMP3 in UM.\(^{35}\) In addition, methylation of RASSF1A has been shown to be a sensitive prognostic marker for UM.\(^{36}\) Lower expression of blocks of genes in those regions on 3p in the absence of deletions could indicate epigenetics as an alternative mechanism of regulation of gene expression. It is therefore interesting to look at the methylation status of classifier genes in tumors of patients in the poor-prognosis UM class without chromosome 3 aberrations. This could explain the presence of two UMs with normal copy numbers of chromosome 3 in our UM class 2 group and the misclassification of the MM27 and M1B672 from the Tschentscher et al.\(^{3}\) and Onken et al.\(^{3}\) cohorts, respectively.

In summary, we have created a very robust gene-expression signature for microarrays that classifies correctly tumors analyzed on different microarray platforms. Survival analysis with our extensive follow-up data revealed a very strong relation between this classification and DFS that is superior to all other known UM prognostic classifiers. Recently, the superiority of this classification was confirmed by Worley et al.\(^{30}\) and Petrusch et al.\(^{3}\) This offers great perspectives for predictive screening, and prospective studies with UM biopsies as a source of RNA are under way.\(^{37}\)

Furthermore, the genes in the microarray classifier set that are located at the plasma membrane are especially potential markers for prognostic screening. There are nine such genes in the classifier, of which four (ENPP2, ENTPD1, IL12RB2, and TBC1D8) have lower and five (HTR2B, PM, PTGER4, SDC2, and TNFRSF19) have higher expression levels in class 2 tumors. Immunohistochemical studies may reveal their value for prognostic screening and their potential use in treatment strategies.

Using expression profiling, we have identified two small regions on chromosome 5, the lower expression of which correlates with poor survival. If indeed epigenetic mechanisms such as methylation are the cause of this decreased expression, it offers an excellent starting point for a better predictive, noninvasive test for the early detection of metastatic disease.

**References**


