Gene Expression Profiling–Based Identification of Molecular Subtypes in Stage IV Melanomas with Different Clinical Outcome

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Abstract

Purpose: The incidence of malignant melanoma is increasing worldwide in fair-skinned populations. Melanomas respond poorly to systemic therapy, and metastatic melanomas inevitably become fatal. Although spontaneous regression, likely due to immune defense activation, rarely occurs, we lack a biological rationale and predictive markers in selecting patients for immune therapy.

Experimental Design: We performed unsupervised hierarchical clustering of global gene expression data from stage IV melanomas in 57 patients. For further characterization, we used immunohistochemistry of selected markers, genome-wide DNA copy number analysis, genetic and epigenetic analysis of the CDKN2A locus, and NRAS/BRAF mutation screening.

Results: The analysis revealed four distinct subtypes with gene signatures characterized by expression of immune response, pigmentation differentiation, proliferation, or stromal composition genes. Although all subtypes harbored NRAS and BRAF mutations, there was a significant difference between subtypes (P < 0.01), with no BRAF/NRAS wild-type samples in the proliferative subtype. Additionally, the proliferative subtype was characterized by a high frequency of CDKN2A homozygous deletions (P < 0.01). We observed a different prognosis between the subtypes (P = 0.01), with a particularly poor survival for patients harboring tumors of the proliferative subtype compared with the others (P = 0.003). Importantly, the clinical relevance of the subtypes was validated in an independent cohort of 44 stage III and IV melanomas. Moreover, low expression of an a priori defined gene set associated with immune response signaling was significantly associated with poor outcome (P = 0.001).

Conclusions: Our data reveal a biologically based taxonomy of malignant melanomas with prognostic effect and support an influence of the antitumoral immune response on outcome.

Malignant melanoma is an aggressive form of skin cancer with a rapidly increasing incidence in the western world (1). Approximately 15% of patients diagnosed with primary melanoma develop distant metastases (2), and current treatment regimens for metastatic melanoma have little effect on long-term survival. Single agent dacarbazine (DTIC) has been standard treatment for many years with response rates of 7% to 13%; however, long-lasting responses are few (3). Importantly, several novel treatment approaches, systemic and targeted, are emerging (4). The concept and arguments for immunotherapy in melanoma include reports on spontaneous remissions and lymphocytic infiltration in tumors (5). However, a rationale for selecting patients eligible for immunotherapy is still lacking.

Although there have been several reports on gene expression signatures in malignant melanomas (6–8), only few studies have indicated molecular subtypes of clinical relevance in metastatic melanoma (7, 9). In primary melanoma, genetic profiles are correlated to anatomic site...
Translational Relevance

Disseminated melanoma responds poorly to conventional therapy; thus, novel treatment approaches are urgently needed. Stratification of melanoma patients into clinically important subclasses based on molecular patterns could potentially be of significance with regard to prognosis as well as treatment predictive purposes. In this study, global expression profiling was used to classify stage IV melanomas in four subtypes. Different subtypes were associated with distinct biological parameters such as pigmentation and immune response. Importantly, the classification displayed a significant correlation to clinical outcome, with the proliferative subtype predicting a poor prognosis. In conclusion, we suggest this molecular classification to be explored as a stratification parameter studying systemic and targeted therapy of metastatic melanoma patients.

Patients

Melanoma patients with distant metastases were enrolled in a prospective study from year 2000 and onward. In the initial analysis, 57 patients were included. Additionally, 20 melanoma patients with metastatic liver deposits were included in the study as a validation cohort. In total, we analyzed 77 patients with stage IV melanoma, all uniformly treated in the same clinical center (Department of Oncology, Haukeland University Hospital).

Individual patient characteristics are provided in Supplementary Table S1. Each patient received standard treatment with dacarbazine (DTIC) monotherapy, 850 to 1,000 mg/m² every 21 days. Biopsies were taken 1 day before the first treatment cycle. Most of the biopsies were obtained from subcutaneous metastases, but there were also biopsies from lymph nodes. All biopsies were subjected to histopathologic examination to ensure that the samples were representative. Remaining sample tissues were snap frozen immediately upon surgical removal in liquid nitrogen until later laboratory examination. All patients, except three, were stage IV (Supplementary Table S1). The clinical response was evaluated following the Unio Internationale Contra Cancrum criteria after 6 weeks and 3 months. Ten patients were unable to finish two treatments because of rapid progression of the disease or for other reasons. The study was approved by the local Ethical Committee, and each patient gave written informed consent.

Nucleic acid isolation

RNA for real-time quantitative PCR was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA for gene expression analysis was extracted using RNeasy Mini Kit and a TissueLyser (Qiagen) according to the manufacturer's instructions. The quality of the RNA was checked using an Agilent Bioanalyzer 2100 (Agilent) and all RNA samples used for analysis had a RIN value larger than 6. DNA was extracted from the biopsies using QIAamp DNA Mini Kit (Qiagen).

Gene expression profiling

Gene expression analyses were done using Illumina Bead-arrays (HumanWG-6 v2 Expression Beadchip, Illumina) and the Illumina system according to the manufacturer's instructions at the SCIBLU Genomics Centre, Lund University. The validation set consisting of 20 stage IV melanomas was analyzed using Illumina Beadarrays (HumanWG HT12 Expression Beadchip). Illumina gene expression data were loaded into the Beadstudio v3 software (Illumina). Here, data were normalized using the cubic spline method. Normalized gene expression data were then exported, and only RefSeq features with a detection P value of <0.01 in at least 80% of the samples were used in further analyses. Next, data were loaded into MeV v4 (11) where it was log 2 transformed and mean centered across assays. Gene expression data will be made available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO).

Array-based comparative genomic hybridization

One microgram of sample DNA and pooled reference DNA (Promega) was labeled, washed, and hybridized to 32k tiling resolution BAC clone microarrays produced at the SCIBLU Genomics Centre, Lund University, as previously described (12). Median background-corrected intensities were filtered to remove spots that had been flagged in the image analysis or had a signal-to-noise ratio <5 in either of the intensity channels. Normalization was done as described (13). Using estimated noise in each array, a moving average was applied to define adaptive thresholds.
for each sample (13), subsequently used to call gains and losses by the CGH-Plotter segmentation algorithm (14). Fraction of the genome altered was calculated as described (15). Array-based comparative genomic hybridization data will be made available in the National Center for Biotechnology Information GEO.

Quantitative real-time PCR

From total RNA, cDNA was synthesized using Transcriptor reverse transcriptase (Roche diagnostics), and p16INK4A expression was determined by quantitative PCR, using the LightCycler 480 system (Roche Diagnostics). The housekeeping gene β2-microglobulin (B2M) was used as expression reference. A standard expression curve based on cDNA from the AU565 cell line and a PCR product of p16INK4A was used to convert PCR cycle number into relative RNA concentrations. The concentration of p16INK4A mRNA was standardized relative to that of the housekeeping gene in each sample. This ratio was again related to expression levels in a control sample from a melanoma cell line (SK-MEL 28) that expressed CDKN2A. Expression of p16INK4A in the different samples was presented as percentage of expression in this cell line. Primer and probe sequences are available in Supplementary Data.

Promoter methylation status

The Ez DNA Methylation-Gold Kit (Zymo Research) was used for bisulfite conversion of tumor DNA. The methylation status of the CDKN2A promoters. PCR was then performed with specific methylated or unmethylated primers (Supplementary Data) using the AmpliTaq Gold DNA polymerase. The promoter status was determined by electrophoresis of the PCR products on a 3% agarose gel stained with ethidium bromide. Cp Genome Universal Methylated DNA (Chemicon International) was used as a positive control and a pool of leukocyte DNA from four healthy volunteers was used as a negative control.

Multiplex ligation–dependent probe amplification

The multiplex ligation–dependent probe amplification (MLPA) method and the SALSA MLPA KIT P024B CDKN2A/2B (MRC Holland) were used to identify deletions or amplifications in CDKN2A according to the manufacturer’s instructions. Capillary electrophoresis, data collection, and peak analysis were done on an automated DNA sequencer (ABI 3700).

Mutation screening

DNA was used as template for mutation screening of the tumor samples. PCRs amplifying the different exons of CDKN2A were done using primers and annealing temperatures as listed in Supplementary Data. The amplification was done with the DyNaZyme EXT polymerase system (FINNZYMES). For some of the patients, one PCR product covering the open reading frame from the tumor cDNA was obtained, and in these cases, the product was sequenced. In most cases, however, all the exons had to be sequenced from DNA.

Immunohistochemistry

Five-micrometer sections from 48 formalin-fixed, paraffin-embedded tumors were prepared. Immunohistochemical staining for CD3 (T-lymphocyte marker), CD20 (B-lymphocyte marker), and Ki67 (MIB-1) was done using antibodies from DAKO. For the staining procedure, the DAKO Envision horseradish peroxidase rabbit/mouse kit (DAKO) and a Dakocytomation Autostainer (DAKO) were used.

The extent of tumor-infiltrating lymphocytes was classified into three categories, brisk, non-brisk, and absent, according to Elder et al. (16) and Clark et al. (17). Lymphocyte infiltrates were classified as (a) brisk (Fig. 5C) when lymphocyte infiltrates were present throughout the substance of the vertical growth phase or were present and infiltrating across the entire base of the vertical growth phase; (b) non-brisk (Fig. 5C) when infiltration was characterized by lymphocytes located in one or more foci of the vertical growth phase (either dispersed throughout or situated focally in the periphery, so-called peripheral lymphocytes); or (c) absent (Fig. 5C), in case no lymphocytes were presented within the area of tumor tissue.

The fraction of labeled tumor cells, defined as the Ki67 labeling index, was assessed over four to five microscopic high-power fields (0.16 mm²). Images were taken from these high-power fields using Nikon microscope and camera as well as imaging software. Counting was done using Nikon imaging software.

Data sets for independent validation of melanoma subtypes

We classified samples in our validation set as well as in four independent publicly available data sets (9, 18–20) using nearest centroid correlation. Centroids based on significant genes for each subtype were computed for each of the four classes found in our data set (Supplementary Table S2). For each subtype, the 100 most significant genes were used, except for the normal-like group where all 77 significant genes were used. We then computed the Pearson correlation coefficient of each sample in the validation data sets to each of the four centroids. Samples were classified based on the centroid displaying the highest correlation (correlation >0.1). Genes were mapped between data sets using gene symbols.

As a first step, we classified our own validation set consisting of 20 stage IV melanomas. Here, data analysis was done as for the WG-6 v2 arrays. In a second step, the public data sets were used for validation. The data set from Haqq et al. (18) was downloaded as supporting information from the PNAS website. The data set from John et al. (19) was downloaded from ArrayExpress (accession no. E-TABM-403), available as raw data only. For the John et al. data, filtering and normalization were done in the Bioarray Software Environment (BASE; ref. 21). Subsequently, this data set was loaded into MeV where data were mean centered across samples. The Bogunovic et al. data (9) had
been deposited in the GEO with accession no. GSE19234. Thus, we downloaded normalized data and mean centered each gene across all 44 samples. Normalized cell line data (20) were obtained from the GEO with accession no. GSE7127 and each gene was mean centered across all cell lines.

**Immune-cell classification using an a priori defined gene set**

Using an a priori defined gene set to predict outcome has successfully been applied (22). To identify samples with a high expression of immune response genes, all genes present on our arrays with Gene Ontology (GO) term associated with the immune response–activating cell surface receptor signaling pathway (GO:0002429) were extracted. This gave us a total number of 30 genes. A sum of log 2 ratios for all 30 genes related to immune response signaling was calculated for each sample. All samples with a positive sum were regarded as being immune response positive and all samples with a negative sum were regarded as being immune response negative. The a priori gene set was also applied in the Bogunovic et al. (9) data set where the same procedure as above was used.

**Statistical analysis of melanoma subtypes**

For gene expression analysis, the 3,000 most variable RefSeq genes were selected and average linkage agglomerative hierarchical clustering with Pearson correlation distance measure was done. Four subgroups were identified by visual inspection of the hierarchical tree. In principle, the same subtypes were identified when using a smaller or larger number of genes. However, we hypothesized 3,000 to be a reasonable number characterizing the identified subtypes. Additionally, to test the reliability of the visually identified clusters, we performed support-tree analysis using jackknife resampling 1,000 times as done by Liu et al. (23).

To identify genes significantly expressed in each molecular subtype, two-group significance of microarray analyses (SAM) were done using false discovery rates of zero. For the high-immune response group, 1,368 genes were significantly differentially expressed. Corresponding numbers for the proliferative, pigmentational, and normal-like groups were 309, 978, and 77, respectively. Clustering analysis, SAM, and support-tree analysis were done in MeV (11). To determine which functional processes characterized each subtype, we used GO analysis applying the web-based software DAVID (24).

Kaplan-Meier and log-rank *P* value analyses were done in Stata v9.1 (StataCorp). All survival analyses were done using the entire follow-up time. Fisher's exact test or ANOVA was used to determine differences between subtypes and other parameters. A *t* test was used to determine if *p16\(^{INK4A}\)* gene expression was significantly lower in the proliferative group. *P* values <0.05 were considered significant.

**Results**

**Identification of melanoma subtypes by gene expression profiling**

We analyzed metastatic lesions (skin and regional lymph nodes) from 57 melanoma patients with global gene expression profiling and unsupervised hierarchical clustering based on the most variable genes (*n* = 3,000). Tumors were distributed in four groups reflecting distinct gene expression patterns (Fig. 1A). Support-tree analysis indicated more than 90% support for the identified subclasses using hierarchical clustering (Fig. 1A). The four subtypes were named (a) high-immune response, (b) proliferative, (c) pigmentation, and (d) normal-like, as reflected by the set of genes characteristic of each group.

The high-immune response subtype was distinguished by high expression of genes such as LCK, IFNGR1, HLA class I H antigen, CXCL12, and IL1R1, all involved in different immunologic processes. However, it should be noted that tumors with increased expression of immune response–related genes were present also in the pigmentation and normal-like subtypes. In contrast, the proliferative subtype displayed low expression of the same set of immune response genes as well as an elevated expression of cell cycle–associated genes such as E2F1, BUB1, and CCNA2, suggesting highly proliferative tumors. The pigmentation subtype displayed a higher expression of genes (e.g., MITF, TYR, SILV, DCT, and EDNRB) involved in melanin synthesis and melanocyte differentiation as compared with the other subtypes. Additionally, WNT5A mRNA expression was significantly (*P* < 0.01, ANOVA) lower in the pigmentation subtype. The normal-like subtype expressed genes involved in epidermis and ectoderm development, such as KRT10 and KRT17, and also displayed high expression of KIT, FGFR3, and EGF.

Although BRAF/NRAS–mutated cases were found within all subgroups, mutations were observed more frequently among tumors belonging to the proliferative subtype (*P* < 0.01, Fisher's exact test); here, no double wild-type samples were observed (Table 1).

Lymphocyte infiltration and Ki67 expression across the different subtypes are described in Supplementary Table S3. The high-immune response group was characterized by an increased number of tumors (5 of 11) revealing brisk infiltration of CD3-positive lymphocytes, whereas in the proliferative subtype, 8 of 11 tumors had a high fraction of Ki67 positive tumor cells. However, none of these parameters were statistically different across the tumor subtypes. Only a few tumors revealed brisk infiltration of CD20.

**CDKN2A status across tumor subtypes**

Tumors were analyzed by array-based comparative genomic hybridization in search for subtype-specific global genomic patterns and specific aberrations. Homozygous deletions at chromosomal band 9p21, harboring CDKN2A and other genes, were observed in eight (14%) tumors, in
Fig. 1. Molecular classification of stage IV melanoma. A, unsupervised hierarchical clustering analysis identified four distinct subtypes of stage IV melanoma. Zoom-in images of representative genes for each subtype are displayed. Melanocyte-associated genes such as MITF, TYR, DCT, and MLANA (green) have increased expression in tumors of the pigmentation subtype, whereas, for example, CCL13 and CD209 have increased expression in the high-immune response subtype (yellow). KRT17, KRT10, and KRT80 have increased expression in the normal-like subtype (blue). The proliferative low-immune response subtype is characterized by an absence of expression of immune response–related genes, here exemplified by GIMAP1 (red). Also, above each identified subtype results from support tree validation using jackknife resampling (1,000 times) are indicated. All subtypes displayed more than 90% support. B, box plot of MITF gene expression for each subtype displays significantly [false discovery rate = 0 (SAM)] higher expression in the pigmentation subtype.
all cases corroborated by 9p21 MLPA analysis. Six of the eight tumors harboring homozygous CDKN2A deletions belonged to the proliferative subtype (P < 0.01, Fisher's exact test), comprising 50% of all tumors in this subtype (Table 1; Fig. 2; Supplementary Table S1).

To further investigate the extent of CDKN2A inactivation, we searched for CpG hypermethylation in the p16INK4A and p14ARF promoters, as well as for mutations in coding regions. None of the tumors had methylation or mutation targeting p14ARF specifically. On the other hand, 12 (21%) of the 57 tumors (24% of 49 tumors without homozygous deletion) had hypermethylation of the p16INK4A promoter. In all, p16INK4A inactivation by either biallelic deletion or hypermethylation combined with hemizygous deletion was significantly associated with the proliferative subtype (P < 0.001, Fisher's exact test). Hypermethylation of p16INK4A was also common in the high-immune response and normal-like groups where homozygous deletions were absent. One somatic p16INK4A missense mutation, three germ-line p16INK4A missense variants of unclear functional effect, and one truncating p16INK4A mutation were observed, but not confined to a specific subtype (Supplementary Table S1). Real-time quantitative PCR analysis of p16INK4A transcripts revealed a relatively low expression in the majority of tumors. Moreover, its expression was significantly more reduced in the proliferative subtype (P < 0.05, t test), which is in agreement with the high proportion of homozygous CDKN2A deletions (Fig. 2).

Global genetic changes in tumor subtypes

The global genomic profiles further showed that tumors with a proliferative or pigmentation phenotype were genetically more unstable with a higher fraction of altered genome (Fig. 2). We identified one tumor with a focal MITF amplification; as expected, this tumor belonged to the pigmentation subtype. In fact, all tumors of this subtype displayed consistently high MITF expression (Fig. 1B).

Gene amplifications were noted at other chromosomal loci, including AKT3, IGFR1, ERBB2, MDM2, MAPK6, and CCND1, but these samples were not restricted to a certain tumor subtype. Notably, a PTEN homozygous deletion was identified in a tumor of the proliferative subtype. Indeed, the proliferative and pigmentation subtypes were characterized by frequent hemizygous chromosome 9p and 10 deletions, suggesting targeted deletions of the known melanoma suppressor genes CDKN2A and PTEN. The normal-like and high-immune response subtype tumors typically harbored gains rather than deletions (Fig. 2).

**Gene expression subtypes and their correlation to clinical outcome**

All patients included in the present study were enrolled in a prospective study assessing predictive markers in DTIC treatment for disseminated melanomas. They harbored skin or regional lymph node lesions from which tumor specimens for molecular analysis were collected. Thus, outcome as well as response to therapy could be correlated to molecular parameters avoiding confounding factors that characterize retrospective studies, such as diversity with respect to patient selection or therapy.

Patients with an objective response (n = 3) to DTIC treatment were distributed among the different groups (one high-immune response group, one proliferative group, and one normal-like group). In contrast, 10 of 18 patients revealing an objective response or stable disease at 6 weeks and 7 of the 9 patients with stable disease at 3 months of treatment had tumors belonging to the pigmentation subtype (P = 0.01 and P = 0.02, respectively.

### Table 1. Summary of clinical and molecular parameters in gene expression subtypes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>High-immune response (n = 15)</th>
<th>Proliferative (n = 12)</th>
<th>Pigmentation (n = 22)</th>
<th>Normal-like (n = 8)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>67% male, 33% female</td>
<td>50% male, 50% female</td>
<td>55% male, 45% female</td>
<td>38% male, 62% female</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Median age at metastases (y)</td>
<td>53</td>
<td>62</td>
<td>68</td>
<td>69</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Median age at primary diagnosis (y)</td>
<td>53</td>
<td>52</td>
<td>64</td>
<td>62</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Median Breslow thickness (mm)</td>
<td>3.5</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Median Clark classification</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>3.5</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Lactate dehydrogenase (mean IU/L)</td>
<td>386</td>
<td>203</td>
<td>293</td>
<td>271</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>BRAF/NRAS mutated (%)</td>
<td>46</td>
<td>100</td>
<td>82</td>
<td>38</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CDKN2A mutated (%)</td>
<td>13</td>
<td>50</td>
<td>9</td>
<td>13</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>9p21 homozygous (%)</td>
<td>0</td>
<td>50</td>
<td>9</td>
<td>0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>p16INK4A methylated (%)</td>
<td>33</td>
<td>17</td>
<td>9</td>
<td>38</td>
<td>&gt;0.01</td>
</tr>
</tbody>
</table>

*P values for sex, mutation, and methylation status were calculated using Fisher's exact test. For all other parameters, ANOVA was used.
Comparing patients having a stable disease to those progressing after 6 weeks of DTIC treatment, the former group had a significantly longer survival ($P = 0.002$, log-rank test; Fig. 3A). Overall survival differed significantly between the four subtypes ($P = 0.01$, log-rank test), with the proliferative subtype associated with the shortest survival ($P = 0.003$, log-rank test, comparing the proliferative subtype to all other patients; Fig. 3B). The median overall survival in our cohort was approximately 7 months, corresponding to what has been described in other studies (3), and long-term stable disease ($\sim 2$ years) was observed in six patients only. Two of the latter were confined to the pigmentation subtype and three in the high-immune response subtype. Neither CDKN2A status nor metastatic location predicted overall survival ($P = 0.31$ and $P = 0.31$, respectively, log-rank test). Potential correlations between individual biological and clinical parameters and overall survival are shown in Table 1.

**Identification and validation of gene expression subtypes in independent data sets**

To validate the gene expression subtypes described herein, we used subtype-specific gene expression centroids. In a first step, we classified our validation set of 20 liver metastases, where 7 were confined to the high-immune response, 3 to the proliferative, and 10 to the pigmentation subtype (Supplementary Table S1). All tumors could be clearly classified into one of the four subtypes. Notably, the two long-term survivors ($\sim 3$ years) with liver metastases were both classified in the high-immune response group.

In a second validation step, we used independent publicly available data sets. For each of the 92 metastases in three public data sets (9, 18, 19), we calculated Pearson correlations to each of the four subtype-specific centroids. In the Bogunovic et al. data set, 39 stage III and 5 stage IV melanomas were available (9). Here, 11 cases were classified as normal-like, 16 as pigmentation, 7 as proliferative, and 1 unclassified. Most
importantly, clinical follow-up was available for all patients, revealing a significant \( (P = 0.04, \text{log-rank test}) \) association between survival and tumor subtype (Fig. 4). In the Haqq et al. data set (18), 19 metastatic lesions could be classified as belonging to the proliferative (2), high-immune response (6), or pigmentation (11) subtype (Supplementary Fig. S1; Supplementary Table S4). For the John et al. data set consisting of 29 lymph node metastases from stage III melanomas (19), 7 high-immune response, 7 proliferative, and 15 pigmentation subtype tumors were found (Supplementary Fig. S2; Supplementary Table S4). In this data set, originally composed by selecting patients with a particular poor (< 40 months) versus long (> 40 months) survival, we found no association between tumor subgroup and prognosis.

Finally, we classified a public data set of 63 melanoma cell lines (20); here, we found all but the normal-like subtype to be represented (Supplementary Fig. S3).

**Immune response and correlation to clinical outcome**

Based on our observation that immune response–related genes play a key role in subtype classification and on recent studies, we investigated the clinical impact of immune response in stage IV melanoma. We initially analyzed tumor samples from patients treated with interferon-α and cetuximab (Fig. 3A). Among patients assessable for response, 15 patients displayed stable disease (excluding 3 objective responders) after 6 wk of treatment whereas 31 displayed progressive disease. B, Kaplan-Meier analysis based on gene expression subtypes discloses a significant difference in outcome.

**Table S4.** In this data set, originally composed by selecting patients with a particular poor (< 40 months) versus long (> 40 months) survival, we found no association between tumor subgroup and prognosis.

**Fig. 3.** Survival analysis in stage IV melanoma. A, Kaplan-Meier survival estimates based on patients that have received DTIC. Among patients assessable for response, 15 patients displayed stable disease (excluding \( n = 3 \) objective responders) after 6 wk of treatment whereas 31 displayed progressive disease. B, Kaplan-Meier analysis based on gene expression subtypes discloses a significant difference in outcome.

**Fig. 4.** Independent validation of gene expression subtypes. Forty-four stage III and IV melanomas from the Bogunovic et al. data set were classified. A, heatmap of genes included in the centroid classification. Samples are ordered according to subtype, and centroids used in the classification are included in the heatmap. Gray box, unclassified sample. B, Kaplan-Meier analysis discloses a significant difference in survival between subtypes.
findings advocating a prognostic role of immune gene expression (9), we investigated the prognostic effect of a priori defined genes related to immune response signaling. A set of 30 genes were obtained from the GO database using the GO term immune response–activating cell surface receptor signaling pathway (GO:0002429). The set included LCK, CD3E, and PTPRC among other genes anticipated to be upregulated in activated immune cells. Based on the sum of expression of the 30 genes, we categorized tumors into two groups characterized by high or low expression of immune-cell genes. This classifier defined immune response–negative cases from all four subtypes; 12 of 12 proliferative, 4 of 15 high-immune response, 9 of 22 pigmentation, and 6 of 8 normal-like tumors (Supplementary Table S3). We observed a significantly ($P = 0.001$, log-rank test) inferior overall survival in the group of tumors with a low expression of immune response genes (Fig. 5A and B). Similarly, it predicted prognosis in the combined subgroup of pigmentation and normal-like tumors ($P = 0.173$, log-rank test). This a priori defined gene signature was further validated in a set of 44 stage III and IV melanomas in the Bogunovic et al. data (9); again, a significant association ($P = 0.04$, log-rank test) between poor survival and low expression of these immune response genes was found.

To verify the findings of high expression of immune response–related genes in our tumors by an independent method, we used immunohistochemistry analysis of CD3, which was included in the gene set (Fig. 5C). A significant ($P = 0.004$, Fisher’s exact test) association between strong staining of CD3 and a positive sum of the immune response genes was observed. In addition, a brisk pattern of CD3 was significantly associated with improved survival ($P = 0.003$, log-rank test).

**Discussion**

Malignant melanoma is a cancer form presenting a distinct phenotype. For example, melanoma is among the least sensitive tumors to systemic treatment as well as to radiotherapy, and metastatic disease inevitably
becomes fatal. On the other hand, malignant melanoma belongs to the few cancer forms in which spontaneous regression has been confirmed (5), indicating immunologic responses to be of importance. Although malignant melanomas are known to harbor certain biological characteristics including high incidence of BRAF mutations (25, 26), the biology of melanomas in general remains poorly understood.

Gene expression signatures derived through supervised analysis have been shown to predict prognosis in primary melanomas (8, 27). Although specific gene expression signatures associated with radial versus vertical growth (18) and BRAF mutations (28) have been derived, we so far lack biological signatures providing a tumor taxonomy.

Using hierarchical clustering, we here show that malignant melanomas may be separated into four distinct subgroups, each characterized by a distinct gene expression profile. We identified melanoma cell lines representing all subtypes except the normal-like, perhaps reflecting that the normal-like subtype is a consequence of normal contaminated tumor tissue. Most importantly, applying our stratification to previously published gene expression data sets (9, 18, 19), we identified all four classes with a distribution resembling what we observed in our own material.

Moreover, individual subgroups were found to express a different prognosis in the advanced setting. For external validation, we used subtype-specific gene expression centroid correlations to classify 92 metastatic melanoma lesions from three independent studies (9, 18, 19). We observed no correlation to clinical outcome in the John et al. data set (19); however, the number of observations in their study is limited. In addition, 13 of 29 patients in their data set had been selected for that study based on a particularly good prognosis (progression-free disease >40 months; ref. 19). In contrast, our subgroups predicted overall survival in the Bogunovic et al. data set (9). Although the data set of Bogunovic et al. and our own in general mirrored each other with respect to prognosis of the individual subgroups, an interesting difference was observed with respect to the pigmentation group. The fact that this subgroup of patients had a good prognosis in our data set but a poor outcome in the Bogunovic et al. set could be related to a high benefit (stable disease) rate in response to DTIC treatment among our patients, as not all of the patients in the Bogunovic et al. data set received chemotherapy. Although further studies are needed to confirm this observation, it supports a hypothesis of a predictive role of the pigmentation subgroup with respect to DTIC treatment.

Even though only a few genes overlap between the Winnepenninckx et al. (8) metastasis signature and our proliferative subtype, the same mechanism seems to operate because samples in the proliferative subtype displayed consistently high expression of genes that are upregulated in the Winnepenninckx et al. metastasis signature (data not shown). Overall, there are convincing lines of evidence that support the importance of proliferation and tumor-infiltrating immune cells in clinical outcome for metastatic melanoma.

We further characterized the proliferative subtype as having a high frequency of homozygous deletions of the CDKN2A locus, indicating that such tumors develop through a distinct genetic pathway. More recently, p14ARF-specific epigenetic and genetic inactivation has been identified in melanoma (29); however, in the current study, no p14ARF-specific inactivation was found, somewhat contradicting previous results.

In support of our stratification of metastatic melanoma into four gene expression subtypes, we identified associated genetic changes for each tumor class. Although no recurrent focal amplification was found, the known melanoma oncogene MITF was amplified in one case belonging to the pigmentation subtype. Notably, all tumors belonging to this subgroup displayed consistently high MITF mRNA expression. MITF gene amplification was recently associated with poor survival in primary melanoma and subsequently confirmed in a larger cohort of metastatic melanomas where MITF amplification (or rather low level gain) indicated a poor prognosis (30, 31). Additionally, one case harbored AKT3 amplification and one case a PTEN homozygous deletion, suggesting that defects in the phosphatidylinositol 3-kinase pathway are key events in malignant melanoma; however, the tumors harboring these alterations were not confined to the same subtype.

Several of the subtype-specific copy number changes are noteworthy. For example, the proliferative subtype displayed more frequent loss of 6q where a novel melanoma tumor suppressor gene was recently mapped (32). Notably, NEDD9, which has been identified as a melanoma metastasis oncogene and also suggested to be involved in neural crest to melanocyte differentiation (33), resides within the 6p gain associated with pigmentation subtype tumors.

We found tumors belonging to the high-immune response class to harbor a good prognosis, contrasting tumors of the proliferative subtype, which express low levels of immune response genes and display a poor prognosis. The importance of immune response genes to prognosis was further underlined by the prognostic effect of an a priori defined gene set based on immune response signaling. This a priori immune-cell classifier, in addition, defined immune response–negative cases from all four subtypes, suggesting immune response heterogeneity within each melanoma subtype. Moreover, CD3 immunohistochemistry analysis verified the gene expression analysis showing a significant association between high frequency of tumor-infiltrating lymphocytes and good outcome. This is in accordance with observations made by other investigators (34). Others have identified critical chemokines to be crucial for the recruitment of CD8+ T cells in melanoma (35). Currently, vaccination against melanoma antigens is being explored as a treatment option in advanced melanomas (36, 37). Our findings suggest the expression of T-cell–related genes to be explored.
as a potential predictive factor for response to immunotherapy in melanomas.

In conclusion, we propose a novel biological classification of metastatic melanoma. We used unsupervised hierarchical clustering to describe four subtypes of stage IV melanoma displaying significant correlation to clinical outcome. More specifically, subtypes were broadly characterized by expression of immune response, pigmentation differentiation, and proliferation genes. These findings, as well as the prognostic role of this subclassification, were confirmed in independent data sets. The potential predictive roles of the pigmentation class genes in DTIC responsiveness and of immune response genes (T-cell enrichment) in response to immunotherapy warrant further exploration in larger studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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