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

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Statement of 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 regards 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 to distinct biological parameters such as pigmentation and immune response. Importantly, the classification displayed 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.
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. While spontaneous regression, likely due to immune defense activation, rarely occurs, we lack a biological rationale and predictive markers 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. Even though 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 high frequency of CDKN2A homozygous deletions (p<0.01). We observed different prognosis between the subtypes (p=0.01), with a particularly poor survival for patients harboring tumors of the proliferative subtype compared to 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 to immune response signaling was significantly associated to poor outcome (p=0.001).
Conclusions: Our data reveal a biologically-based taxonomy of malignant melanomas with prognostic impact and support an influence of the anti-tumoral immune response on outcome.
Introduction

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 regimes for metastatic melanoma have little impact on long-term survival. Single-agent dacarbazine (DTIC) has been standard treatment for many years with response rates of 7-13%, 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.

While 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 rather than to conventional histopathological classification (10), suggesting divergent tumor developmental pathways. While supervised gene expression profiling has been used to predict outcome in primary melanoma (8), further research is needed to improve our biological understanding of melanomas.

To address these issues, we used global expression profiles to stratify stage IV melanomas into distinct subtypes. Using hierarchical clustering, we identified four tumor subtypes characterized by gene signatures representing diverse biological mechanisms. In addition, the subtypes were characterized by different DNA copy number changes. These subtypes were named proliferative, high-immune response, pigmentation and normal-
like, reflecting the pattern of genes representative for each subtype. Importantly, a significant difference in clinical outcome was observed between the four tumor classes, with the proliferative group having worst prognosis. This classification of metastatic malignant melanoma may prove useful in selecting patients eligible for immunomodulating drugs as well as putative targeted therapies.
**Material and methods**

**Patients**

Melanoma patients with distant metastases were enrolled in a prospective study from year 2000 and onwards. 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, University of Bergen).

Individual patient characteristics are provided in Supplementary Table 1. Each patient received standard treatment with dacarbazine (DTIC) monotherapy, 850-1000mg/m² every 21 days. Biopsies were taken one day prior to 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 histopathological examination to ensure that the sample was representative. Remaining sample tissue was snap frozen in liquid nitrogen until later laboratory examination. All patients, except three, were stage IV (Supplementary Table 1). The clinical response was evaluated following the UICC-criteria after both 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, Carlsbad, CA) according to the manufacturer’s instructions. RNA for gene expression analysis was extracted using RNeasy mini kit and a TissueLyser (Qiagen, Hilden, Germany) according to manufacturer’s instructions. The quality of the RNA was checked using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, USA) 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, Hilden, Germany).

**Gene expression profiling**

Gene expression analyses were performed using Illumina Beadarrays (HumanWG-6 v2 Expression Beadchip) (Illumina, San Diego, CA, USA) and the Illumina system according to manufacturer’s instructions at the SCIBLU Genomics Centre, Lund University. The validation set consisting of 20 stage IV melanomas was analysed using Illumina Beadarrays (HumanWG HT12 Expression Beadchip). Illumina gene expression data was loaded into the Beadstudio v3 software (Illumina). Here, data was normalized using the cubic spline method. Normalized gene expression data was then exported, and only features with a detection p-value of <0.01 in at least 80% of the samples were used in further analyses. Next, data was loaded into MeV v4 (11) where it was log2-transformed and mean-centered across assays. Gene expression data will be made available in the NCBI Gene Expression Omnibus.

**Array-based comparative genomic hybridization**
One microgram of sample DNA and pooled reference DNA (Promega, Madison, WI, USA) 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 performed 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 (FGA) was calculated as described (15). Array CGH data will be made available in the NCBI Gene Expression Omnibus.

Quantitative real time PCR

From total RNA, cDNA was synthesized using Transcriptor reverse transcriptase (Roche diagnostics, Basel, Switzerland), and p16<sup>INK4a</sup> expression was determined by quantitative PCR, using the LightCycler®480 system (Roche Diagnostics). The housekeeping gene β-2-microglobulin (B2M) was used as expression reference. A standard expression curve based on cDNA from the AU565 cell line and a PCR product of p16<sup>INK4a</sup> was used to convert PCR cycle number into relative RNA concentrations. The concentration of p16<sup>INK4a</sup> and 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 p16<sup>INK4a</sup> in the different samples was presented as percentage of expression in this cell line. Primer and probe sequences are available in Supplementary Information.
Promoter methylation status

The Ez DNA-methylation gold™ Kit (Zymo Research, Orange, CA, USA) was used for bisulphite conversion of tumor DNA to determine the methylation status of the CDKN2A promoters. PCR was then performed with specific methylated or unmethylated primers (Supplementary Information), using the AmpliTaq Gold™ DNA polymerase. The promoter status was determined by electrophoresis of the PCR products on a 3% agarose gel stained with ethidiumbromide. CpGenome™ Universal Methylated DNA (Cemicon International, Temacula CA, USA) was used as a positive control and a pool of leukocyte DNA from four healthy volunteers was used as a negative control.

MLPA

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

Mutation screening

DNA was used as template for mutation screening of the tumor samples. PCR’s amplifying the different exons of CDKN2A were performed using primers and annealing temperatures as listed (Supplementary Information). The amplification was performed with the DyNazyme EXT polymerase system (FINNZYMES, Espoo, Finland). 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 (IHC)**

Five µm 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) were performed using antibodies from DAKO, Denmark. For the staining procedure, the DAKO Envision HRP 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: 1) brisk (Figure 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; 2) non-brisk (Figure 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 3) absent (Figure 5C), in case no lymphocytes were presented within the area of tumor tissue.

The fraction of labeled tumor cells, defined as the Ki-67 labeling index (Ki-67 LI), was assessed over 4-5 microscopic high power fields (0.16mm²). Images were taken from these high power fields using a Nikon microscope and camera as well as imaging software (Nikon, Japan). The counting was performed using Nikon imaging software.
Data sets for independent validation of melanoma subtypes

We classified samples in our validation set as well as 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 dataset (Supplementary Table 2). 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 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 performed as was done 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 number E-TABM-403), available as raw data only. For the John et al. data, filtering and normalization were performed in the Bioarray Software Environment (BASE) (21). Subsequently, this data set was loaded into MeV where data was mean-centered across samples. The Bogunovic et al. data (9) had been deposited in gene expression omnibus (GEO) with accession number GSE19234. Thus, we downloaded normalized data and mean-centered each gene across all 44 samples. Normalized cell line data (20) was obtained from the GEO with accession number GSE7127 and each gene was mean-centered across all cell lines.
**Immune-cell classification using an a priori-defined gene set**

Using *a priori*-defined gene sets 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 to immune response-activating cell surface receptor signaling pathway (GO:0002429) were extracted. This gave us a total number of 30 genes. A sum of log2-ratio 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 genes were selected and average linkage agglomerative hierarchical clustering with Pearson correlation distance measure was performed. 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, in order to test the reliability of the visually identified clusters we performed support-tree analysis using jackknife re-sampling 1,000 times as performed by Liu *et al.* (23).

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

Kaplan-Meier and log-rank p-value analyses were performed in Stata v9.1 (StataCorp, Texas, USA). All survival analyses were performed 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 less than 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 (Figure 1A). Support-tree analysis indicated more than 90% support for the identified subclasses using hierarchical clustering (Figure 1A). The four subtypes were named (1) high-immune response, (2) proliferative, (3) pigmentation and (4) 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 II antigen, CXCL12 and IL1R1, all involved in different immunological 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 but also 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 to 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 EGFR.

While 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 3. The high-immune response group was characterized by an increased number of tumors (5/11) revealing brisk infiltration of CD3 positive lymphocytes, while in the proliferative subtype eight of eleven 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 (aCGH) 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 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, Figure 2, Supplementary Table 1).

To further investigate the extent of CDKN2A inactivation we searched for CpG hypermethylation in the p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} promoters, as well as for mutations in
coding regions. None of the tumors had methylation or mutation targeting \( p14^{ARF} \) specifically. On the other hand, 12 (21%) of the 57 tumors (24% of 49 tumors without homozygous deletion) had hypermethylation of the \( p16^{INK4A} \) promoter. In all, \( p16^{INK4A} \) inactivation by either bi-allelic deletion or hypermethylation combined with hemizygous deletion was significantly associated with the proliferative subtype (\( p<0.001 \), Fisher’s exact test). Hypermethylation of \( p16^{INK4A} \) was also common in the high-immune response and normal-like groups where homozygous deletions were absent. One somatic \( p16^{INK4A} \) missense mutation, three germline \( p16^{INK4A} \) missense variants of unclear functional effect, and one truncating \( p16^{INK4A} \) mutation were observed, but not confined to a specific subtype (Supplementary Table 1). Real-time quantitative PCR analysis of \( p16^{INK4A} \) 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 (Figure 2).

**Global genetic changes in tumor subtypes**

The global genomic profiles further showed that tumors with a proliferative or pigmentation phenotype were genetically more instable with a higher fraction of altered genome (Figure 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 consistent high \( MITF \) expression (Figure 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 (Figure 2).

*Gene expression subtypes and correlations to clinical outcome*

All patients included in the present study were enrolled in a prospective study assessing predictive markers to treatment with DTIC 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, like diversity with respect to patient selection or therapy, characterizing retrospective studies.

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, ten of 18 patients revealing an objective response or stable disease at 6 weeks and seven of the nine patients with stable disease at three months of treatment had tumors belonging to the pigmentation subtype (p = 0.01 and p=0.02, respectively; Fisher’s exact test). Comparing patients having a stable disease to those progressing after six weeks of DTIC treatment, the former group had a significantly longer survival (p=0.002, log-rank test) (Figure 3A). Overall survival differed significantly between the four subtypes (p=0.01, log-rank test) with the
proliferative subtype associated to the shortest survival (p=0.003, log-rank test comparing the proliferative subtype to all other patients; Figure 3B). The median overall survival in our cohort was approximately seven months corresponding to what has been described in other studies (3), and long-term stable disease (~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

In order 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 seven were confined to the high-immune response, three to the proliferative and ten to the pigmentation subtype (Supplementary Table 1). All tumors could be clearly classified into one of the four subtypes. Notably, the two long-term survivors (~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 datasets (9, 18, 19) we calculated Pearson correlations to each of the four subtype-specific centroids. In the Bogunovic et al. dataset 39 stage III and five stage IV melanomas were available (9). Here, 11 cases were classified as high-immune response, nine as proliferative, 16 as pigmentation, seven as
normal-like and one unclassified. Most importantly, clinical follow-up was available for all patients revealing a significant (p=0.04, log-rank test) association between survival and tumor subtype (Figure 4). In the Haqq et al. dataset (18), 19 metastatic lesions could be classified as belonging to the proliferative (two), high-immune response (six) or pigmentation (eleven) subtype (Supplementary Figure 1, Supplementary Table 4). For the John et al. data set consisting of 29 lymph node metastases from stage III melanomas (19), seven high-immune response, seven proliferative and 15 pigmentation subtype tumors were found (Supplementary Figure 2, Supplementary Table 4). 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 Figure 3).

**Immune response and correlation to clinical outcome**

Based on our observation that immune response related genes play a key role to subtype classification and recent findings advocating a prognostic role of immune gene expression (9), we investigated the prognostic impact of *a priori*-defined genes related to immune response signaling. A set of 30 genes was obtained from the gene ontology (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; 12/12 proliferative, 4/15 high-immune response, 9/22 pigmentation and 6/8 normal-like tumors (Supplementary Table 3). 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 (Figure 5A-B). Similar, it predicted prognosis in the combined subgroup of pigmentation and normal-like tumors (p=0.005, log-rank test) and the pigmentation group alone (p=0.05, log-rank test), but a non-significant trend when applied to the combined subgroup of high-immune and proliferative 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 IHC analysis of CD3, which was included in the gene set (Figure 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 immunological responses to be of importance. While 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 has been shown to predict prognosis in primary melanomas (8, 27). While 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 datasets (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 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). While we observed no correlation to clinical outcome in the John et al. data set (19), the number of observations in their study is limited. In addition, 13 out of 29 patients in their data set had been selected for that study based on a particularly good prognosis (progression-free disease > 40 months) (19). In contrast, our subgroups predicted overall survival in the Bogunovic et al. data set (9). While the dataset 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 dataset 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. While 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. (9) metastasis signature and our proliferative subtype, the same mechanism seems to operate, since samples in the proliferative subtype displayed consistent high expression of genes that are upregulated in the Winnepenninckx et al. metastasis signature (data not shown). Overall, there are convincing evidence that support the importance of proliferation and tumor infiltrating immune cells on clinical outcome in metastatic melanoma.

We further characterized the proliferative subtype as having a high frequency of homozygous deletions of the CDKN2A locus, indicating 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

\( p14^{ARF} \) 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. While 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 consistent 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 PI3K 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, \( NEDD9 \) that 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 impact 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 IHC 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 expression of T-cell related genes to be explored as a potential predictive factor for response to immuno-therapy 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-related genes. These findings, as well as the prognostic role of this subclassification were confirmed in independent data sets. A potential predictive role of the pigmentation class to DTIC responsiveness and immune response genes (T-cell enrichment) toward response to immunotherapy warrants further exploration in larger studies.
References


Figure Legends

Figure 1. Molecular classification of stage IV melanomas. A) Unsupervised hierarchical clustering analysis identified four distinct subtypes of stage IV melanoma. Zoom-in panels of representative genes for each subtype are displayed. Melanocyte-associated genes such as *MITF*, *TYR*, *DCT* and *MLANA* (green panel) have an increased expression in tumors in the pigmentation subtype while, e.g., *CCL13* and *CD209* have an increased expression in the high-immune response subtype (yellow panel). *KRT17*, *KRT10* and *KRT80* have an increased expression in the normal-like subtype (blue panel). The proliferative low-immune response subtype is characterized by an absence of expression of immune response related genes here exemplified by *GIMAP1* (red panel). Also, above each identified subtype results from support tree validation using jackknife re-sampling (1,000 times) are indicated. All subtypes displayed more than 90% support. B) Boxplot of *MITF* gene expression for each subtype display significantly (FDR=0, SAM) higher expression in the pigmentation subtype.

Figure 2. Characterization of gene expression subtypes. Nine patients were still receiving DTIC after three months of treatment and displayed a stable disease; seven of these patients had tumors grouped in the pigmentation subtype. Also, six of eight tumors harboring homozygous deletions at the *CDKN2A* locus were located in the proliferative subtype. Real-time PCR validation demonstrated a low expression of the *p16INK4a* gene in the proliferative subtype. *BRAF/NRAS* mutated samples were found in all subtypes however only three samples were mutated in the normal-like subtype. DNA copy number
analysis revealed genetic pathways for each subtype displayed as most common copy number changes and FGA.

**Figure 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 six weeks of treatment while 31 displayed progressive disease. B) Kaplan-Meier analysis based on gene expression subtypes discloses a significant difference in outcome.

**Figure 4.** Independent validation of gene expression subtypes. 44 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 heatmap. Grey box indicates unclassified sample. B) Kaplan-meier analysis discloses a significant difference in survival between subtypes.

**Figure 5.** Immune-cell classification of stage IV melanoma. A) Heatmap of immune-cell related genes with samples ordered according to their gene expression subtype. B) Kaplan-Meier analysis of immune-cell positive and negative cases reveals a significant association to clinical outcome. C) Representative IHC staining of CD3 with brisk, non-brisk and absent pattern.
### 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>Stromal-like (n=8)</th>
<th>p-value*</th>
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<tr>
<td>Sex</td>
<td>67% male</td>
<td>50% male</td>
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<td>33% female</td>
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<td>Median age at metastases</td>
<td>53</td>
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<td>Median age at primary diagnosis</td>
<td>53</td>
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<td>Median Breslow thickness</td>
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<td>Median Clark classification</td>
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<td>Lactate dehydrogenase (LDH) (mean IU/l)</td>
<td>386</td>
<td>403</td>
<td>293</td>
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<td>BRAF/NRAS mutated (%)</td>
<td>46</td>
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<td>CDKN2A mutated (%)</td>
<td>13</td>
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<td>9p21 homozygous (%)</td>
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<td>p16^{INK4a} methylated (%)</td>
<td>33</td>
<td>17</td>
<td>9</td>
<td>38</td>
<td>p&gt;0.01</td>
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</table>

*p-values for sex, mutation and methylation status were calculated using Fisher's exact test. For all other parameters ANOVA was used.*
<table>
<thead>
<tr>
<th>Genes</th>
<th>High-immune response</th>
<th>Proliferative</th>
<th>Pigmentation</th>
<th>Normal-like</th>
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**B) MITF gene expression**

- Log2ratio
- Immune response
- Proliferative
- Pigmentation
- Normal-like

96% 100% 94% 97%
High-immune response 1/14
Proliferative 0/11
Pigmentation 7/18
Normal-like 1/6

Stable disease at 3 months DTIC treatment

CDKN2A homozygous

p16-INK4A gene expression

Genetic aberrations found in >50% in respective subtype

Fraction of the genome altered (FGA)
Pigmentation, n=21  p=0.01
High-immune response, n=14
Normal-like, n=8
Proliferative, n=11
Stable disease, n=15
Progressive disease, n=31  p=0.002

**A)**

- Stable disease, n=15
- Progressive disease, n=31

**B)**

- Pigmentation, n=21
- Normal-like, n=8
- High-immune response, n=14
- Proliferative, n=11  p=0.01
Survival fraction (%) vs. Time (weeks)

- Pigmentation, n=16
- High-immune response, n=11
- Normal-like, n=7
- Proliferative, n=9

A) Normal-like centroid, Proliferative centroid, Pigmentation centroid, High-immune centroid

B) Survival fraction (%)

- Normal-like, n=7
- Proliferative, n=9
- Pigmentation, n=16

p=0.04

Research. on April 14, 2017. © 2010 American Association for Cancer Research.
A) Gene expression heatmap showing high-immune, proliferative, pigmentation, and normal-like expression.

B) Kaplan-Meier survival curve showing survival fraction (%) over time (weeks) for immune response positive (n=24) and immune response negative (n=30) groups. p=0.001.

C) Images showing brisk, non-brisk, and absent immune responses.

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


Clin Cancer Res  Published OnlineFirst May 11, 2010.

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