Abstract

Purpose: To better understand the molecular mechanisms of malignant melanoma progression and metastasis, gene expression profiling was done of primary melanomas and melanoma metastases.

Experimental Design: Tumor cell-specific gene expression in 19 primary melanomas and 22 melanoma metastases was analyzed using oligonucleotide microarrays after laser-capture microdissection of melanoma cells. Statistical analysis was done by random permutation analysis and support vector machines. Microarray data were further validated by immunohistochemistry and immunoblotting.

Results: Overall, 308 genes were identified that showed significant differential expression between primary melanomas and melanoma metastases (false discovery rate <0.05). Significantly overrepresented gene ontology categories in the list of 308 genes were cell cycle regulation, mitosis, cell communication, and cell adhesion. Overall, 47 genes showed up-regulation in metastases. These included Cdc6, Cdk1, septin 6, mitosin, kinesin family member 2C, osteopontin, and fibronectin. Down-regulated genes included E-cadherin, fibroblast growth factor binding protein, and desmocollin 1 and desmocollin 3, stratifin/14-3-3, and the chemokine CCL27. Using support vector machine analysis of gene expression data, a performance of >85% correct classifications for primary melanomas and metastases was reached. Further analysis showed that subtypes of primary melanomas displayed characteristic gene expression patterns, as do thin tumors (≤1.0 mm Breslow thickness) compared with intermediate and thick tumors (>2.0 mm Breslow thickness).

Conclusions: Taken together, this large-scale gene expression study of malignant melanoma identified molecular signatures related to metastasis, melanoma subtypes, and tumor thickness. These findings not only provide deeper insights into the pathogenesis of melanoma progression but may also guide future research on innovative treatments.

The incidence of malignant melanoma is steadily increasing with a present lifetime risk of 1 in 75 among the Caucasian population (1). The underlying factors for this phenomenon are largely unknown. After diagnosis of malignant melanoma, the single most important factor for the prognosis of melanoma patients is vertical tumor thickness as described earlier by Breslow (2). It could be shown that tumors of a few-millimeter thickness already show a high potential for metastasis with a fatal outcome for the patient. In the metastatic stage, melanoma patients have only few treatment options, consisting of monochemo-therapies with dacarbazine (DTIC) or temozolomide, or polychemotherapy regimens combining DTIC with other chemotherapeutic agents such as cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (3–5). Although significant clinical response rates were achieved by these treatment modalities, there was no substantial effect on the overall survival of these patients. Unfortunately, little is known about factors that contribute to the process of melanoma progression and metastasis.

In recent years, DNA microarray technology has fostered hopes for a more complete understanding of the mechanisms of tumor progression and metastasis in a variety of tumors, including malignant melanoma (6–8). A series of studies done on colon carcinoma, pancreatic adenocarcinoma, lung adenocarcinoma, and breast carcinoma showed tumor-specific gene expression compared with normal tissues (9–13). More recently, a comprehensive microarray study on metastasis-related gene expression analyzed different primary tumors from breast adenocarcinoma, prostate adenocarcinoma and...
A large-scale gene expression study based on the analysis of melanoma cell lines of different aggressiveness was presented by Clark et al. (15). RhoC, a member of the family of Rho GTPases, was shown to be highly expressed in metastatic melanoma cells, when compared with their nonmetastatic counterparts of the same genetic background. The differences in gene expression between cell lines of both stages were most significant for genes involved in extracellular matrix and cytoskeleton organization [e.g., fibronectin, collagen subunits $\alpha_2(1)$ and $\alpha_1(III)$, matrix Gla protein, fibromodulin, biglycan, and thymosin $\beta_4$].

To identify a metastatic phenotype in primary melanomas that might help to predict the clinical outcome of melanoma patients, Bittner et al. analyzed biopsies from primary melanomas and melanoma metastases (16). Based on biostatistical and functional analyses, two major melanoma clusters were identified. Cluster I contained less aggressive melanoma tissues, and cluster II contained highly aggressive melanoma tissues. Cluster I showed reduced expression for integrin $\beta_1$, integrin $\beta_3$, syndecan, vinculin, and fibronectin. This study further underlined the particular role of cytoskeletal and extracellular matrix molecules for melanoma progression. A more recent study based on gene expression patterns of 58 primary melanomas showed that gene expression patterns in primary tumors may indeed help to predict the clinical outcome of melanoma patients (17). Gene expression patterns in primary tumors differed between patients with a 4-year distant metastasis-free survival from those who developed metastases within this time. A large series of molecules with enhanced expression in the bad prognosis group belonged to the functional groups of cell cycle regulation, mitosis, and DNA replication, such as Cdc2, Cdc6, CENPF, and proliferating cell nuclear antigen.

A comprehensive study on different stages of malignant melanoma development was published recently, analyzing specimens from benign melanocytic nevi, primary melanomas, and melanoma metastases (18). A major finding of this study was the identification of two different gene patterns found in metastases reflecting those in vertical or radial growth phase cells of primary melanomas. These findings argue for a metastatic gene pattern present in a subtype of primary tumors.

In the present report, the expression of 22,283 probe sets was analyzed in a series of laser-microdissected tissues from 41 primary melanomas and melanoma metastases using oligonucleotide microarrays. Overall, 389 probe sets (representing 308 different genes) were identified that showed significant differential expression between both disease stages. A predictive diagnostic model [support vector machine (SVM)] for discriminating primary tumors and metastases was trained, and a performance of >85% correct classifications was reached in cross-validation.

**Materials and Methods**

**Tissue specimens.** For microarray analyses, biopsy material of 19 primary melanomas and 22 cutaneous melanoma metastases was obtained from 41 different patients after surgical excision of tumors. The vertical tumor thickness of primary melanomas ranged from 0.38 to 11.00 mm according to Breslow (2), including one in situ melanoma. A complete list with detailed information about primary melanoma samples including thicknesses of biopsies is given in Supplementary Table S1A. Immunohistochemistry was done on an independent set of 20 primary melanomas and 20 melanoma metastases from either archival paraffin-embedded or frozen material, depending on the antibodies used for immunostaining. The vertical tumor thickness of these primary melanomas ranged from 0.40 to 12.20 mm according to Breslow. Metastatic lesions in both groups were cutaneous or s.c. metastases. The presented study was approved by the local Ethic Committee at the University of Rostock, and informed consent for microarray analyses of tumors was obtained from all patients.

**Laser-capture microdissection of tumor tissue.** Laser-capture microdissection was done as described by Bonner et al. (19). Cryopreparations of tumor specimens were cut into sections of 10-μm thickness and put onto slides covered with a transparent plastic foil. Sections were then stained using conventional H&E stain. Twenty to 30 sections of each tumor (primary tumors or metastases) were prepared. These were subjected to laser-capture microdissection using a laser-capture microdissection Facility (P.A.L.M. Microlaser Technologies, Bernried, Germany). Melanoma cells were microdissected under microscopic control by an experienced histopathologist. For the analysis of primary melanomas, the entire thickness of each tumor was microdissected. This included intraepidermal melanoma cells, when these cells were part of clearly demarcated tumor cell nests. Single cells in the epidermis were not included. Metastases generally had a diameter of 6 to 8 mm and were cut into halves. One half was used for laser microdissection, and the other half was used for histopathology. In metastases, central and peripheral areas were used for microdissection. Necrotic areas, when present, were avoided. Microdissected tissues were captured in Eppendorf tubes and processed for RNA extraction.

**RNA extraction, labeling, and hybridization of microarray targets.** Total RNA was extracted from tumor tissues using commercially available systems (RNEasy kit, Qiagen, Hilden, Germany). RNA probes were labeled according to the supplier’s instructions using 1 μg of total RNA (Affymetrix, Santa Clara, CA). Analysis of gene expression was carried out using the Human Genome U133A microarray (Affymetrix), which contains 22,215 probe sets (omitting control probe sets). Hybridization and washing of gene chips were carried out according to the supplier’s instructions. A laser scanner (Gene Array Scanner from Hewlett-Packard, Boeblingen, Germany) was used for reading out microarrays.

**Immunohistochemistry.** Five-micrometer sections of paraffin-embedded or frozen tissues from 20 primary melanomas and 20 melanoma metastases were prepared for immunohistochemistry. To improve antigen recognition in paraffin sections, a 1-min heat treatment was done in a pressure cooker, containing 1 liter of 10 mmol/L sodium citrate buffer (pH 6.5). The following antibodies were used for immunostaining: anti–p34/cyclin-dependent kinase 1 (Cdk1; DM114P, mouse monoclonal; Acris Antibodies, Hiddenshausen, Germany), anti-Cdc6 (CC30, mouse monoclonal; Calbiochem, Bad Soden, Germany), anti-desmocollin 1 (anti-DSC1; H44951, mouse monoclonal; Biodesic, Saco, ME), and anti-CCL27 (AF376, goat polyclonal; R&D Systems, Bad Nauheim, Germany). Immunodetection was done with a commercially available system (ZytoChemPlus AP Broad Spectrum Bulk kit, Zytomed, Berlin, Germany) using Permanent APRed (Zytomed) as chromogen. Counterstaining was done with hematoxylin. To evaluate differential expression levels of homogenously staining Cdk1 and Cdc6 in primary melanomas and metastases, the following four-point scale scoring system was used: 0, negative staining; +1, weak staining; +2, moderate staining; +3, strong staining.
evaluate differential expression levels of DSC1 and CCL27, which showed patchy staining or staining of isolated cells, the four-point scale scoring system was based on the percentage of positively staining cells in four different high power fields (×400): 0%, 0% to 5%; +1, 6% to 20%; +2, 21% to 50%; +3, 51% to 100% positively staining cells. Evaluation was done by two independent investigators.

**ImmunobLOTS.** The nonmetastasizing 1F6 melanoma cell line was kindly provided by G.V. Muijen (Institute of Pathology, Nijmegen, The Netherlands; ref. 20). Melanoma cells were lysed on ice for 30 min using radioimmunoprecipitation assay buffer. Forty micrograms of total protein extract were denatured in electrophoresis sample buffer for 5 min at 95°C and subjected to SDS-PAGE. Gels were electroblotted onto nitrocellulose membranes (Highbond ECL, Amersham, Braunschweig, Germany) and subjected to immunodetection. The following primary antibodies were used: anti-DSC1 (H44951, mouse monoclonal; Biodesign), anti-CCL27 (AF376, goat polyclonal; R&D Systems), anti-cytokeratin 10 (CK10; DM055, mouse monoclonal; Acris Antibodies), anti-CK14 (DM3066, mouse monoclonal; Acris Antibodies), anti–stratifin/14-3-3 \( j \) (ab14123, mouse monoclonal; Abcam, Cambridge, United Kingdom), anti–fibroblast growth factor binding protein (anti-FGFBP; MAB1593, mouse monoclonal; R&D Systems), anti-integrin \( \beta_4 \) (ab6136, mouse monoclonal; Abcam), anti-S100A2 (S6797, mouse monoclonal; Sigma-Aldrich, Munich, Germany), anti-osteopontin (BAF1433, biotinylated goat polyclonal; R&D Systems). Signal detection was done by appropriate anti-mouse or anti-goat horseradish peroxidase–coupled secondary antibodies (purchased from Becton Dickinson, Heidelberg, Germany), or by horseradish peroxidase–coupled streptavidin (S2438, Sigma-Aldrich). A standard enhanced chemiluminescence reaction (Amersham) was done for signal visualization.

**Pre-processing of microarray data.** For pre-processing of microarray data, a background correction, normalization on probe level, and probe set summarization were done. The background correction was done similarly to Microarray Suite 5.0 (Affymetrix, 2001), but negative values were not truncated. Probe level normalization was done using the variance stabilization method by Huber et al. (21). Finally, probe set summarization was done using a median polish fit of an additive model (22).

**Statistical analysis of microarray data and immunohistochemical staining patterns.** To find genes with statistically significant differences in gene expression between different clinical phenotypes, genes were ranked according to a regularized \( t \) score (23). False discovery rates (FDR) for the lists of top ranking genes were calculated based on 10,000 random permutations of the class labels (24). Differences in gene expression were regarded as significant when the FDR of the resulting lists did not exceed 0.05. The expression levels of these genes were visualized in color-coded heat maps. In these plots, genes were hierarchically clustered using complete linkage. The samples were grouped by phenotype, and each group is separately hierarchically clustered for better visual comparability. Using the R Software for Statistical Computing and the Bioconductor package GOstats, every gene list was further examined for significant overrepresentation of biological processes and pathways defined by gene ontology (GO) categories. To derive diagnostic signatures, SVM (25, 26), combined with a regularized \( t \) score–based feature selection filter, were used. To compensate for unbalanced group sizes, we adjusted the class weights within the SVM according to the group sizes. Predictive performance was assessed using the MCRestimate package (27). Using this package, the optimal number of genes and the optimal variable setting of the SVM were determined in a nested cross-validation setting. Cross-validation was repeated 10 times with a 10-fold outer and 10-fold inner loop. The outer loop construction ensures an unbiased estimation of predictive performance, even when using optimal variables. Predictive performances are generally contrasted with prevalence. The prevalence is the number of samples in the larger of the two group divided by the total number of samples. Thus, it is the fraction of correct classifications when assigning all samples to the bigger group. We also implemented an in silico panel diagnosis that shows the patient-specific confidence of molecular diagnosis. This was achieved by running cross-validation 1,000 times with different random assignments to the 10-fold cross-validation bins of patients and recording how many times the SVM misclassified each sample. In 10-fold cross-validation, the data is divided into 10 equally sized batches. Iteratively, one of the batches is set aside as a test set, and a classifier is learned on the rest. This is done so that each datum was exactly once in a test set. The accuracy of the classifier is finally determined by counting how many times the data in the test set was assigned to the correct label. The SVM was trained using a linear kernel and feature selection of the 100 genes with the highest absolute significance analysis of microarrays scores. To find novel molecular subentities of melanomas and metastases in our samples, an unsupervised class discovery analysis using the ISIS algorithm program was done (28). The ISIS algorithm searches for binary class partitions that are clearly separated based on a subset of genes. Due to memory and time constraints, we pre-filtered the genes to the 9,000 highest variance, expressed genes and set the default setting for the number of possible candidate splits to 3,000. Finally, the splits were further refined by only keeping splits that had >60 (0.5%) differentially regulated genes at a FDR \( \leq 0.05 \). For statistical analysis of immunohistochemical staining patterns, a \( \chi^2 \) test according to Pearson was done. Differences in staining intensities of tissue sections with \( P \leq 0.05 \) were regarded as statistically significant.

**Results**

**Molecular signatures of melanoma metastases.** Oligonucleotide microarrays harboring >22,000 probe sets were used to analyze gene expression patterns of 19 primary melanomas and 22 melanoma metastases after laser-capture microdissection of tumor cells. By this means, 308 differentially expressed genes were identified with an estimated FDR \( \leq 0.05 \) (Fig. 1A). Interestingly, the vast majority of 261 genes showed reduced expression in metastases. However, 47 genes showed up-regulation in metastases. Among the up-regulated genes were Cdc6, Cdc28/Cdk1 subunit 2 (Ck2s2), sepin 6 (STP6), mitoerin, kinesin family member 2C (KIF2C), and serine/threonine kinase 6 (STK6). This set of genes also included well-known tumor progression factors, like osteopontin (SPP1), fibronectin (FN1), and members of the MAGE family of differentiation antigens, and less-known tumor progression molecules, such as thrombospordin 4, biglycan, and NCAM1. The complete list of up-regulated genes, including information about their main functions, is given in Supplementary Table S2.

Molecules that showed down-modulation in metastases were E-cadherin (CDH1), FGFBP, keratin 10 (KRT10), DSC1 and DSC3, desmoglein 1 (DSG1), stratifin/14-3-3\( j \), chemokine CCL27, tumor protein p73-like (TP73L), p21-activated kinase 6 (PAK6), and FGF receptors 1 and 2 (FGF2 and FGF3). The complete list of down-regulated genes, including information about their main functions, is given in Supplementary Table S3. Some of these genes had been described in earlier studies as being down-regulated during tumor progression in malignant melanoma (e.g., E-cadherin; ref. 29). However, others have not yet been described to be expressed by melanoma tumors (e.g., FGFBP, KRT10, DSC1, DSC3, stratifin/14-3-3\( j \), and CCL27).

Using Fisher’s exact test, the list of 308 differentially expressed genes was further examined for significant overrepresentation of biological processes as defined by GO categories. Genes with the GO terms cell cycle (GO:7049), mitotic cell cycle (GO:0278), M phase of mitotic cell cycle, (GO:0279), mitosis (GO:7067), and chromosome condensation (GO:30261) showed statistically
Fig. 1. Molecular differentiation based on gene expression profiles between primary melanomas and melanoma metastases, different melanoma subtypes, and primary melanomas of different tumor thickness. 

A. A color-coded heat map was generated from differentially expressed genes after comparing gene expression in primary melanomas and melanoma metastases using 10,000 permutations. Only genes were included with a FDR <0.05. Overall, 308 differentially expressed genes were identified, which clearly separated both tumor stages. Increased (yellow) and decreased (blue) gene expression referring to the mean of each individual gene over all patients. Right margin, representative genes. 

B. The same analysis was done as described in (A) for the identification of differentially expressed genes in different melanoma subtypes (NM versus SSM). Overall, 60 differentially expressed genes were identified. The one ALM tested in this study showed a gene expression pattern similar to that of NM. The same analysis was done as described in (A) and (B) for primary melanomas of different tumor thickness (<1.0 mm Breslow thickness versus >2.0 mm Breslow thickness). Overall, 199 genes showed differential gene expression. The majority of these (172 genes) were up-regulated in thin (<1.0 mm Breslow thickness) melanomas, and only a minority (27 genes) was up-regulated in intermediate and thick (>2 mm Breslow thickness) melanomas.

Taken together, primary melanomas and metastases show significant differential gene expression. Functional categories of differentially expressed genes argue for a particular role of cellular proliferation, cell cycle regulation, cell adhesion, and cell-extracellular matrix interaction as central mechanisms for tumor progression in melanoma.

Molecular signatures of melanoma subtypes. Our next analyses addressed the question of whether melanoma subtypes may be differentiated by gene expression patterns. When comparing gene expression profiles of SSM and NM, a series of 67 probe sets (60 genes) was identified with a FDR <0.05 (Fig. 1B). Genes with the following GO terms showed statistically significant enrichment in the list of down-regulated genes in metastases. Figure 2 shows the hierarchical structure of these GO terms. Significantly enriched nodes/biological processes are indicated.

To address the question of whether tumor thickness of primary melanomas is reflected by a particular gene expression pattern, we performed a comparison of gene expression in primary melanomas of different thickness using the Wilcoxon rank sum test. 

Based on currently available information, the mean tumor thickness of primary melanomas at the time of first diagnosis is 0.3 to 0.5 mm (31, 32). In the presented analysis, the group of SSM included two SSM (SSM6 and SSM13) with unusual high tumor thickness of 5.67 and 5.4 mm, respectively. Interestingly, these two thick SSM showed a gene pattern similar to that of SSM and not of NM, which further supports the notion that there is indeed a clinical and molecular difference between both melanoma subtypes. In summary, NM and SSM show different gene expression patterns. Functionally, SSM show enhanced expression of genes involved in cell-cell contact and cell-cell communication.

Molecular signature of tumor thickness in primary melanomas. The most important prognostic factor for malignant melanoma is vertical tumor thickness, also termed Breslow thickness. To address the question of whether tumor thickness of primary tumors is reflected by a particular gene expression pattern,
thin primary tumors (≤1.0 mm Breslow thickness) were compared with intermediate and thick primary tumors (>2.0 mm Breslow thickness). These cutoff points were chosen based on an earlier study correlating tumor thickness with the patients’ prognosis in 5,000 melanoma patients (33). When comparing both groups, a list of 240 probe sets (representing 199 genes) was identified which showed differential expression with an estimated FDR <0.05 (Fig. 1C). Interestingly, 116 (58%) of these 199 genes overlapped with differentially expressed genes of primary melanomas and metastases.

Genes with the GO terms cell communication (GO:7154), cell adhesion (GO:7155), and ectoderm development (GO:7398) were significantly enriched in the list of genes with enhanced expression in thin melanomas. These categories included DSG1, connexin 43 (GJA1), epidermal growth factor receptor (EGFR), ANXA8, and CD24. Among the up-regulated genes in intermediate and thick melanomas were BCL2A1, cadherin 19 (CDH19), BH-protocadherin (PCDH7), regulator of G-protein signaling 20 (RGS20), thymosin-like 8 (TMSL8), catenin z-like 1 (CTNNAL1), and Ets variant gene 1 (ETV1). Overall, genes with the GO terms regulation of cellular process (GO:50794), regulation of signal transduction (GO:9966), and cell communication (GO:7154) were significantly enriched in the list of genes with enhanced expression in intermediate and thick melanomas. Taken together, these findings argue for the development of a metastatic signature in primary tumors with increasing tumor thickness, which supports the concept of tumor thickness as a major prognostic factor.

Molecular classification by SVM. We then addressed the question, whether gene expression profiles may accurately predict the tumor stage (primary melanoma or metastasis) of our samples and calculated the estimated accuracy of this prediction. For this purpose, SVM were applied (34). In an unbiased validation, using nested cross-validation, our predictor reached an accuracy of 85% correct classifications (with a prevalence of 54%). Classifiers of this accuracy could be designed with as little as 30 genes. Mainly NM were misclassified (Fig. 3), and most of them were misclassified in all runs. Misclassified samples were NM1, NM2, NM3, Meta1, and Meta18. For a couple of samples (Meta2, Meta19, ALM1, and SSM13), the SVM diagnosis results were ambiguous between different cross validation runs, indicating that for these patients the expression profiles do not contain sufficient information to decide whether patients had primary tumors or metastases. In these analyses, the accuracy for distinguishing SSM and NM was 89% (with a prevalence of 72%) using 15 genes. The accuracy for distinguishing SSM and Meta was 91% (with a prevalence of 63%) using 50 genes. NM could not be distinguished from Meta with an accuracy higher than prevalence.

Taken together, these analyses showed that by use of a supervised classification method, a high level of prediction...
accuracy for tumor stages could be achieved, which further argues for particular biological stages represented by different melanoma subtypes or primary melanomas and metastases.

**Unsupervised class discovery.** To find novel molecular subentities of melanomas or metastases, we did an unsupervised class discovery analysis using the ISIS algorithm (28). The ISIS algorithm finds bipartitions in a set of samples that show a clear separation regarding the expression of a subset of genes. By use of this technique, a total of 20 bipartitions (splits) were produced (Supplementary Fig. S1). Examples of differentially expressed genes of major splits (splits 9, 11, 15, and 19), and tumor samples forming clusters within these splits are shown in Table 1. Gene patterns in split 15 were able to differentiate between primary melanomas and metastases. In accordance with the above described findings, three NM (NM1, NM2, and NM3) grouped together with the majority of metastases. Interestingly, tumor thickness alone was not sufficient to attribute primary tumors to the metastasis cluster because the two thick SSM (SSM6 and SSM13) did not group together with NM1-3 and metastases in cluster 1.

These analyses also provided evidence that SSM might be divided into two subentities. Split 11 showed a tumor cluster consisting of SSM1 and SSM7 to SSM9, all primary tumors with low tumor thickness (i.e., 0.45 mm, 0.6 mm, in situ melanoma, and 0.95 mm, respectively). Top genes within split 11 were lectin domain 3B (CLEC3B), clusterin, dermatopontin, and COLA1, functionally all related to the cell-extracellular matrix interaction. The four thin primary SSM were also part of a cluster in split 9. Split 19 showed a cluster of NM1 to NM3 and several metastases, which differed in gene expression patterns from all SSM, further underlining the molecular differences in melanoma subtypes. Taken together, unsupervised class discovery supported our findings of a molecular differentiation not only between primary tumors and metastases but also between subtypes of NM. Moreover, unsupervised clustering provided evidence for a molecular differentiation between thin and thick SSM.

**Validation of microarray data by immunohistochemistry and immunoblotting.** Immunohistochemistry of different melanoma tissues was done to validate microarray data by an independent method. A detailed list of the specimens and staining patterns is given in Supplementary Table S4. Figure 4 shows representative tissue sections from 20 primary melanomas and 20 metastases immunostained for Cdc6, Cdk1, DSC1, and CCL27/CTACK. These genes have been chosen because they represent new interesting molecules for melanoma pathogenesis. Cdc6 and DSC1 had not been described in melanoma cells before, and Cdk1 and CCL27 had not been shown to be differentially expressed in metastases compared with primary melanomas. All four genes are involved in central mechanisms that contribute to melanoma pathogenesis [i.e., deregulated cell cycle control (Cdk1 and Cdc6), loss of cell adhesion (DSC1), and high immunogenicity of primary tumors (CCL27/CTACK)]. In microarray experiments Cdc6 and Cdk1 showed up-regulation, DSC1, and CCL27/CTACK showed down-regulation in metastases compared with primary melanomas. Both Cdc6 and Cdk1 showed strongly positive immunohistochemical staining of metastases and moderate to weak staining of primary melanomas. Differences in staining intensities between metastases and primary melanomas were statistically significant with \( P < 0.001 \), as determined by \( \chi^2 \) test. Interestingly, both molecules showed mainly cytoplasmic expression in melanoma cells. This unexpected observation was supported by earlier reports, which showed that Cdc6 is

<table>
<thead>
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<th>Split no.</th>
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<th>Tumor cluster 2</th>
<th>Dependency</th>
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<td>9</td>
<td>FLJ12289; RNASE4; GAS1; MAGEA3; MAGEA6; MMP1; STK6</td>
<td>SSM1, SSM2, SSM4, SSM7-9, SSM12, SSM13 NM1, NM2 ALM1 Meta3, Meta4, Meta8, Meta11, Meta12, Meta17, Meta20</td>
<td>SSM3, SSM5, SSM6, SSM10, SSM11 NM3, NM4, NM5 Meta1, Meta2, Meta5, Meta6, Meta7, Meta9, Meta10, Meta13-16, Meta18, Meta19, Meta21, Meta22</td>
<td>Thickness</td>
</tr>
<tr>
<td>11</td>
<td>MBP; SERPINE2; CLEC3B; CLU; DPT; OSR2; COL1A1; PECAM1; TNXB</td>
<td>SSM1, SSM7-9</td>
<td>SSM2, SSM3-6, SSM10-13 NM1-5 ALM1 Meta11</td>
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<td>NM1-3 ALM1 Meta2-17, Meta19-22</td>
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<td>—</td>
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NOTE: Binary splits were generated by unsupervised clustering. The complete list of splits is provided in Supplementary Fig. S1. †, up-regulated gene expression in cluster 1 compared with cluster 2. ‡, down-regulated gene expression in cluster 1 compared with cluster 2.
nuclear in G1 phase of cell cycle but translocates to cytoplasm after activation and phosphorylation and is mainly cytoplasmic in S phase of cell cycle (35, 36). Cytoplasmic staining of Cdk1 has also been reported in advanced lesions and metastases of esophageal adenocarcinoma (37).

In contrast to Cdc6 and Cdk1, DSC1 and CCL27/CTACK showed a more focal staining of tumor areas or isolated cell clusters. Both DSC1 and CCL27/CTACK showed higher percentages of positively staining cells in primary melanomas compared with metastases. These differences were statistically significant with \( P < 0.010 \) for DSC1 and \( P < 0.001 \) for CCL27. Interestingly, DSC1 had not been described to be expressed in melanoma cells before. Figure 4 shows representative samples immunostained for both molecules.

Immunohistochemical analyses were extended by immunoblots for DSC1, CCL27, and a further series of molecules identified in the presented microarray study, which had not yet been described in melanoma cells (FGFBP, CK10, CK14, stratifin/14-3-3\( \alpha \), and integrin \( \beta_4 \)). As shown in Fig. 5, protein extracts of 1F6 melanoma cells showed moderate to strong expression of these proteins. Immunoblots for osteopontin and S100A2 were included as controls. The latter are known to be expressed by melanoma cells in vitro and in vivo and have also been detected in our microarray analyses. Taken together, these experiments further supported the presented microarray data, which identified new molecules not yet described in melanoma cells.

**Discussion**

A large-scale gene expression study is presented analyzing primary melanomas and melanoma metastases. To focus on gene expression in melanoma cells, tumor cells were excised from tissue biopsies using laser-capture microdissection. A major aim of the study was the identification of genes with enhanced expression in metastatic lesions, which might be of interest as targets for future innovative treatment approaches. Based on the analysis of >22,000 probe sets, 389 probe sets (representing 308 different genes) were shown to be differentially expressed between primary melanomas and metastases. Of these, a majority was down-regulated in the metastatic stage. However, 57 probe sets (representing 47 different genes) were up-regulated in the metastatic stage. Gene expression profiles were also able to differentiate between melanoma subtypes (SSM versus NM) and between tumors of different tumor thicknesses (tumors \( \leq 1.0 \) mm compared with tumors >2.0 mm tumor thickness).

Using different statistical approaches, it could be shown that primary tumors were clearly distinguishable from metastases by their molecular gene patterns. These findings argue that specific biological processes are active at different tumor stages. Major biological functions as derived from GO categories involved in this process were cell proliferation, cell cycle regulation, and mitosis, represented by the majority of up-regulated genes in metastases. These findings are in accordance with a large body of data implicating that cancer development and progression are based on deregulated cellular proliferation and cell cycle control (38, 39). Indeed, in a variety of tumors activating...
mutations in oncogenes, such as phosphatase and tensin homologue deleted on chromosome 10, Akt kinase, and Ras, inducing cellular proliferation have been identified. However, such mutations have rarely been detected in human melanomas and melanoma metastases (1, 40). There is a considerable body of evidence from experimental mouse melanoma models that activating Ras mutations in combination with an inactivated INK4a/ARF (CDKN2a) tumor suppressor locus induce spontaneous melanomas with consecutive distant metastases in mice (40–42). These findings argue for a particular role of deregulated cell cycle control in the pathogenesis of malignant melanoma. However, little is known about the molecules and pathways involved in these processes. In the presented study, a series of candidate molecules were identified. Among these were Cdc6, Cdk1, mitosin (CENPF), SPT6, and KIF2C. In line with this, enhanced expression of Cdc6, Cdk1 (CKS2), CENPF, and KIF2C in primary melanomas had been shown to be associated with bad prognosis, referring to a 4-year distant metastasis-free survival of investigated patients (17). Enhanced gene expression of Cdc6 and Cdk1 in metastases in our study was paralleled by overexpression of corresponding proteins, as shown by immunohistochemistry in an independent set of melanoma tumors. Cdc6 is an essential factor for DNA replication regulated by E2F in mammalian cells. It had been also described as a marker molecule for progressive cervical cancer and showed enhanced expression in a subset of non–small cell lung carcinomas compared with normal tissue (43, 44). The Cdk complex cdc28-Cln is of central importance for cell division in yeast. The yeast cdc28 molecule corresponds to Cdk1 in the mammalian system and is a major regulator in G2-M phase of the cell cycle. Here, we show that melanoma metastases express high levels of Cdk1 mRNA and protein compared with primary melanomas. These findings may open interesting perspectives for future melanoma treatment approaches because a series of new small-molecule inhibitors targeting cyclins and Cdkks have already been, or will be, used in clinical trials in the near future (45). Among these are in particular inhibitors of Cdk1, such as flavopiridol and roscovitine. These molecules have been tested in chronic lymphocytic leukemia, non-Hodgkin’s lymphoma, and kidney cancer with remarkable response rates. Mitosin, also termed centromere protein F, is a multifunctional protein for cell proliferation and is of importance for the function of the mitotic spindle checkpoint (46). It is active in the G2-M phase of the cell cycle. Interestingly, mitosin has been shown to be an independent predictor of recurrence in breast carcinomas (47).

A second major functional group identified, when comparing gene expression in primary melanomas and metastases, included molecules involved in cell-cell adhesion and cell-extracellular matrix interaction. This group comprised up-regulated and down-regulated genes in metastases. Prominent up-regulated genes involved in these processes were osteopontin, fibronectin, biglycan, and thrombospondin 4. Osteopontin, fibronectin, and biglycan have been described as progression factors for melanoma in recently published large-scale microarray studies from other groups analyzing either different melanoma tissues (16, 18) or melanoma cell lines of different aggressiveness (15). Molecules with down-regulated expression in metastases were E-cadherin, FGFBP, CK10, DSC1, DSC3, stratifin/14-3-3σ, and CCL27. These findings were partly overlapping with studies of Herlyn et al., who proposed a model for melanoma progression based on a tightly regulated up-regulation and down-regulation of molecules involved in cell-cell adhesion and cell-extracellular matrix interaction (29, 48). It was shown that benign melanocytes express high levels of E-cadherin, which mediates contact to epidermal keratinocytes via E-cadherin-connexin 43 interactions. During transition from benign melanocytes to melanoma cells, E-cadherin is down-regulated, and N-cadherin is up-regulated. In accordance with this, it could be shown in the presented study that both E-cadherin and connexin 43 were down-regulated in NM compared with SSM. A series of further molecules (e.g., tenascin, ICAM-1, Mel-CAM) and members of the integrin family (e.g., αvβ3, αvβ1, and α4β1 integrins) involved in cell adhesion had been described to play a role in tumor progression and tissue invasion in melanoma (29, 49). Cell adhesion molecules also formed a major group of down-regulated molecules in thick tumors in the mentioned gene expression study of primary melanomas (17).

Interestingly, some of the overexpressed molecules in primary melanomas compared with metastases identified in this study had not been described to be expressed in melanoma cells before (e.g., FGFBP, CK10, CK14, DSC1, DSC3, stratifin/14-3-3σ, and CCL27) but had been shown to be expressed by epidermal keratinocytes. Therefore, we did immunohistochemistry and immunoblotting to further validate our findings. By this means, it could be shown that melanoma cells in vivo and in vitro express the mentioned adhesion and cytoskeletal molecules as well as the CCL27 chemokine. In summary, the presented data together with those of other groups underline the concept of local tumor progression and distant metastasis in melanoma based on specific losses and gains of adhesion molecules and molecules facilitating migration and tissue invasion.

An important finding of the presented study was the molecular differentiation of tumors of different tumor thicknesses. We chose melanomas of less than 1 mm tumor thickness and compared these with melanomas thicker than 2 mm. These cutoff points had been shown earlier to be of relevance for the prognosis of melanoma patients (33). Both tumor stages could clearly be differentiated from each other based on their gene expression profiles. The application of unsupervised clustering methods even allowed further subclassification of a group of very thin SSM. Interestingly, there were a considerable number of genes overlapping between the metastasis signature and the signature for tumor thickness. In fact, 58% of differentially expressed genes in intermediate and thick versus thin melanomas overlapped with those differentially expressed genes comparing metastases with primary melanomas. These findings are highly suggestive for a progressive change in gene expression patterns during tumor progression in melanoma. Moreover, these molecular data support the current concept of tumor thickness of primary tumors as the major prognostic factor for melanoma patients (2, 5, 33). These overlapping genes might even be regarded as a metastatic signature in primary melanomas as described for other tumor entities (13, 14).

Among the genes that showed up-regulation in gene expression in intermediate and thick melanomas were BCL2A1, different cadherins, and thymosin β. Interestingly, BCL2, cadherins, thymosin β, and Rho molecules had been shown to contribute to melanoma progression in a series of...
independent studies (15, 29, 50). In a further recently published gene expression study, vertical growth phase of primary melanomas was compared with horizontal growth phase (18). In accordance with our findings, down-modulated genes involved functional categories of cell adhesion and extracellular matrix molecules, such as DSC2, MMP10, CDH3, and integrin α2.

Based on gene expression profiles identified in our study, different subtypes of melanomas could also be distinguished. Interestingly, two SSM with tumor thicknesses comparable with that of the NM showed gene pattern of SSM and not of NM. This argues against a differentiation between both subtypes merely based on different tumor thicknesses. These findings are in accordance with epidemicologic data summarized in a recent publication on prognostics factors of melanoma, showing that risk profiles of both tumor subtypes remain different even after correction for tumor thickness (51).

The differences between the two major subtypes of melanomas (SSM and NM) involved genes of cell adhesion, cell communication, epidermis development, and morphogenesis. SSM compared with NM showed enhanced expression of FGFR2 and FGFR3, connexin 43, ANXA8, and DSG1. Some of these genes (e.g., FGFR2, connexin 43, and DSG1) had been shown to play a role in melanoma development (28). Among those with enhanced expression in NM were MMP16, BCL2A1, ICAM1, and CEACAM1, emphasizing the role of molecules supporting tissue invasion, proliferation, and adhesion mechanisms for tumor progression in NM. In support of these findings, ICAM1 and CEACAM1 had been shown earlier to contribute to progressive tumor growth in melanomas (30).

Taken together, the presented data show that melanoma metastasis represents a specific biological stage of tumor progression with a particular gene pattern. A majority of up-regulated genes in metastases fit the current pathogenic concepts of tumor progression and may serve as targets for innovative treatment approaches. Moreover, we were able to show that melanomas of different thicknesses and different melanoma subtypes are represented by particular gene expression patterns. Further studies should be initiated to analyze whether gene expression patterns in primary melanomas may predict the prognosis of patients, and whether gene expression patterns in metastases may be used for treatment monitoring in clinical trials.

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References

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