Signaling Networks in Cutaneous Melanoma Metastasis Identified by Complementary DNA Microarrays

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Background: Melanoma is a complex multigenic disease, susceptibility to which is determined by several parallel and stepwise progressive pathways affecting growth control, differentiation, cell adhesion, and survival. Melanoma and human cancers in general undergo a continuous development from benign to malignant states, as most thoroughly documented in the multistep mole-to-melanoma transition.

Objective: To examine how high-throughput microarrays are being used in expression profiling to identify regulated genes, patterns, and pathways that may lead to functional characterization and tumor subclassification.

Design: Ten melanoma metastases were analyzed by DNA array technology for important regulated candidate genes, with subsequent confirmation by real-time reverse transcription polymerase chain reaction.

Results: Hepatocyte growth factor receptor c-met, growth factor receptor–bound protein 10, B-raf proto-oncogene, and several mitogen-activated protein kinase kinase genes were significantly up-regulated in melanoma metastases and several melanoma cell lines relative to normal human melanocytes ($P=.03$). Among the up-regulated genes, phosphorylated growth factor receptor–bound protein 10 is known to serve a molecular switch turning on the mitogen-activated protein kinase pathway in response to hepatocyte growth factor receptor binding.

Conclusions: As suggested by the DNA arrays, we found the mitogen-activated protein kinase kinase/extracellular-regulated kinase pathway to be activated in most of the cutaneous melanoma metastasis specimens. These findings are in the context of the current microarray technology in melanoma research. Additional steps are needed to gain insights into the pluralistic signaling milieu of this malignancy as we enter the postgenomic era.

Arch Dermatol. 2005;141:165-173

MICROARRAY PLATFORMS share in essence the same principle—a miniaturized slide that carries multiple probes. They differ, however, in the nature of the arrayed probe and the way the probes are created (Table 1). There are several variants of the DNA microarray technology for analysis of transcription profiles, including complementary DNA (cDNA) (>250 base pairs long), which are immobilized to a solid surface such as glass or nylon membrane using robotic spotting, and oligonucleotides (20-80 mer oligodeoxynucleotides), which are synthesized in situ (on chip) or by conventional methods followed by on-chip immobilization.1,4 Protein/peptide arrays are solid-phase ligand-binding assays that use immobilized proteins/peptides on surfaces, including glass, membranes, microtiter wells, mass spectrometer plates, and beads or other particles (Table 1). Protein arrays enable the parallel screening of thousands of protein interactions that encompass protein–antibody, protein–protein, protein–ligand, and protein–drug interactions; enzyme-substrate screening; and multianalytic diagnostic assays.5 Tissue microarrays contain tissues arrayed on modified glass slides. This microarray technology allows the rapid visualization of molecular targets in hundreds of tissue specimens at the same time, at the DNA, the RNA, or the protein level (Table 1). In particular, the signal is visualized on a per-cell basis (in situ).6,7 By revealing the cellular localization, prevalence, and clinical significance of candidate genes, tissue microarrays are ideally suited for genomic-based diagnostic and drug-target discovery. In recent years, comparative genomic hybridization has also emerged as a novel array-based tool.
platform for genome-wide comprehensive analysis of chromosomal imbalances. Thus, array technologies represent a spectrum of high-throughput means to identify molecular targets associated with biological and clinical phenotypes by comparing samples representative of distinct pathophysiological states. However, irrespective of the type of the array platform, an appropriate experimental design, accurately annotated clinical specimens, and proper controls are key to the successful use.

STEPWISE TUMORIGENESIS MODEL OF MELANOMA DEVELOPMENT AND PROGRESSION

Melanoma is an ideal model to study the molecular mechanisms of tumor progression, as it develops in about half of the cases through a series of architectural and phenotypically distinct stages and becomes progressively aggressive, culminating in metastasis (Figure 1).

Five distinct stages have been proposed in the evolution of melanoma on the basis of histological criteria: (1) common acquired and congenital nevi without dysplastic changes; (2) dysplastic nevi with structural and architectural atypia; (3) radial growth-phase melanoma; (4) vertical growth-phase melanoma; and (5) metastatic melanoma. Considerable progress has been made in understanding the biological, pathological, and immunological aspects of human melanoma progression. Epidemiological and experimental studies have suggested that intense exposures to UV radiation during early childhood may lead to melanoma in adults, but molecular and genetic studies have shown few autosomal abnormalities, infrequent mutational spectra, and very few epistatic and epigenetic mechanisms. At the cellular level, it has become clear that dysregulated homeostatic control of the cutaneous microenvironment occurs through alterations in the expression of specific proteins. These include growth factors and their receptors, adhesion molecules and their ligands, proteases and their substrates, and transcription factors and their target genes. As in most other human tumors, there are significant alterations in the regulatory network involved in signal transduction in human melanoma.

The prognosis of melanoma is based on histological criteria such as tumor thickness (measured by the Breslow index), level of invasion (Clark level), presence of ulceration, and number of mitoses per square millime-

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Abbreviations: bp, base pairs; cDNA, complementary DNA.
Biological signaling pathways interact with each other to form complex networks. If we want to understand cellular behavior and its responses to external signals or to influence cellular function in a predictable manner, we have to understand the pathways through which these signals are mediated into and within the cell. In most cases, changes in cellular behavior involve the abrogation or the block of transcriptional events, which are specific for each signal in its cellular context. Hence, expression profiling can be used for the functional classification of dysregulated genes. This is often referred to as “guilt by association.” This method is based on the observation that genes with related expression patterns—genes that presumably are coregulated—are likely to be functionally related and involved in the same biological processes or physiological pathways. Disruption of the INK4a/ARF tumor suppressor locus is a common genetic alteration in many different types of human cancers, including malignant melanoma. Activation of receptor tyrosine kinase pathways as well as Ras and phosphatidylinositol 3-kinase has been described in subsets of human melanomas. Using a mouse model for cutaneous melanoma, Bardeesy et al investigated whether activated Ras can cooperate with p53 loss to cause melanoma, whether such melanomas are biologically comparable to those arising in INK4a (delta2/3−/−) mice, and whether tumor-associated mutations emerge in the p16(INK4a)-Rb pathway in those melanomas. These studies validated the role of p53 inactivation in melanoma development and suggested that the Rb and p53 pathways function to suppress melanocyte transformation in vivo, which had previously been disputed. Similarly, the BRAF (B-raf proto-oncogene) mutation is common in cutaneous melanoma metastases. These mutations include exons 11 and 15 and suggest that anti-Ras/Raf strategies may be effective for treating metastatic melanoma; however, these mutations were absent in uveal melanoma.

Earlier genomic and proteomic investigations by our group showed that mitogen-activated protein kinase kinase (MEK)–1, MEK2, and MEK3, as well as extracellular-regulated kinase (ERK) 1/2 (p44/42) were highly upregulated in subsets of uveal melanoma metastases without the prerequisite of activating BRAF mutations. Results from our recent studies suggest that subgroups of cutaneous malignant melanoma metastases have alternate pathways of regulating extracellular matrix and thereby show variation in their metastatic potential (S.N., A.M., Á.G., U.R.H., unpublished data, December 2002). Earlier expression profiling studies have shown a putative role for RhoC and Wnt5a in cell motility and invasiveness of melanoma. However, further investigation is required to unravel when and how the metastatic cell makes such decisions (Figure 2) and what its impact would be on diagnosis and therapy.

For elucidating signal transduction pathways in melanoma, we analyzed differential gene expression between normal human melanocytes (NHMs) and 10 cutaneous melanoma metastases of different patients with the use of cDNA array technology focusing on oncogenesis and apoptosis.

METHODS

CELL CULTURE

Normal human melanocytes were obtained from PromoCell (Heidelberg, Germany) and cultured in phorbol myristate acetate–free medium (PromoCell). Cell culture was maintained in a 37°C incubator and a moist atmosphere of 5% carbon dioxide. The human melanoma cell lines BLM, M13, MV3, and SK-Mel-28 were cultured in Dulbecco Modified Eagle Medium (Gibco BRL Life Technologies, Taufkirchen, Germany) supplemented with 10% fetal calf serum, 100 U/mL of penicillin, and 100 pg/mL of streptomycin.

TUMOR SPECIMENS

The cutaneous melanoma metastases came from different body sites and had diameters greater than 5 mm. Biopsy specimens were snap frozen in liquid nitrogen and homogenized using a dismembrator (Braun AG, Melsungen, Germany). One half of the homogenate was transferred into ice-cold lysis buffer containing 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.9), 50 mM sodium fluoride, 15 mM Trition X-100, 5 mM EDTA, 100 mM sodium chloride, and 1 tablet of protease inhibitor cocktail per 10 mL of buffer (Roche, Mannheim, Germany) for Western blot analysis. The other half of the pulverized specimen was immediately transferred into TriZol reagent (Invitrogen, Karlsruhe, Germany) for total RNA isolation according to the manufacturer’s instructions. Contaminating DNA was removed by treatment with DNase I (Invitrogen). The RNA quality was controlled by agarose gel electrophoresis.

MICROARRAY HYBRIDIZATION

Several membrane arrays containing apoptosis- and cancer-related genes (Atlas human apoptosis cDNA array and Atlas human cancer cDNA array 1.2K; Clontech, Heidelberg, Germany) were hybridized comparing NHMs, different melanoma
cell lines, and patient-derived metastases. After DNase treatment, 1 μg of total RNA was reverse transcribed using superscript (Invitrogen) and SMART cDNA primers (Clontech). We used 100 ng of SMART cDNA for long-distance reverse transcription polymerase chain reaction (RT-PCR) with SMART primers. The PCR reaction consisted of 21 cycles and was within the linear amplification range. The reaction mix was removed from the PCR product with the use of Microcon-100 columns (Millipore, Tokyo, Japan). We used random-primated labeling for 100 ng of SMART-PCR products with phosphorus 33-o-deoxyctydine triphosphate (ICN, Eschwege, Germany). The 32P-labeled probes were purified with the use of Microcon-100 columns. Prehybridization and hybridization of membrane microarrays were performed in DIG Easy Hyb solution (Roche) at 42°C.

**MICROARRAY IMAGE AND DATA ANALYSIS**

The radioactive intensity of each spot was detected by a phosphoimager (BAS-1500; Fuji, Raytest GmbH, Straubenhardt, Germany) and recorded with AIDA software (Raytest, Germany). To compare the results, normalization of each spot was performed. As reference spots, 9 different housekeeping genes were used. The ratio of gene expression between the experimental samples and NHM was computed. No gene regulation was present at a ratio of 1, and an up- or a down-regulation was observed when the normalized ratio was greater than or equal to or less than 2, respectively.

**STATISTICAL EVALUATION**

We used significance analysis of microarrays, which identifies genes with statistically significant changes in expression by assimilating a set of gene-specific unpaired t tests. Each gene is assigned a score on the basis of its change relative to the standard deviation of repeated measurements for that gene’s expression level. Genes with scores greater than a threshold are deemed potentially significant. The percentage of such genes identified by chance is the false discovery rate. To estimate the false discovery rate, nonsense genes are identified by analyzing permutations of the measurements. The threshold can be adjusted to identify smaller or larger sets of genes, and false discovery rates are calculated for each set. Subsequently, J-Express version 1.1, a Java software application, was used to perform the hierarchical agglomerative cluster analysis to group similar data (average linkage).

**SUPERVISED DATA ANALYSIS**

We also performed supervised data analyses, including data mining, functional categorization, and pathway construction. The statistically significant genes defined within meaningful clusters were queried for in the SOURCE database, which contains gene reports that include aliases, chromosomal location, functional descriptions, gene ontology annotations, gene expression data, and links to external databases. Functional categorization based on gene ontology annotations and an analysis of the cause-consequence relationship were performed using available information on signaling pathways in cancer. Finally, the results of expression profiling were mapped to known biological pathways to gain further functional insights.

**QUANTITATIVE PCR BASED ON THE USE OF FLUOROGENIC PROBES**

Briefly, in a 5’ nuclease assay, a specific oligonucleotide probe that is labeled with a reporter fluorescent dye at the 5’ end and a dark quencher at the 3’ end is annealed to the target sequence located between the 2 primer-binding sites. The 5’ exonuclease-mediated release of the reporter dye from the probe during the extension phase results in an increase in the fluorescent signal of the reporter dye. This is proportional to the amount of amplicon produced and is monitored in real time during PCR amplification using the PRISM 7300 sequence detector (Applied Biosystems Inc, Foster City, Calif). The primers and probes used for detection of c-met and 18S were ordered as TaqMan gene expression assay (Applied Biosystems Inc), and the PCR analysis was carried out in a 23-μl mixture. All samples were amplified simultaneously in triplicates. The data were analyzed by using a standard curve with 18S as the endogenous control.

**WESTERN BLOT ANALYSIS**

Cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer. The samples were pretreated with ultrasound, and cell extracts were centrifuged at 14,000g for 20 minutes at 4°C. The protein concentration of the cellular extracts was determined using the advanced protein assay reagent (TEBU, Frankfurt, Germany). Twenty micrograms of the protein extract was electrophoresed on 4% to 12% NuPage Bis-Tris-Glycin gels (Invitrogen) for 2 hours at 120 V. Proteins were blotted onto polyvinylidene fluoride membranes (Bio-Rad, Munich, Germany) at 25 V for 90 minutes using a tank-blot system. The membranes were blocked with 5% nonfat dry milk powder in TBST buffer consisting of 10mM Tris hydrochloride (pH 7.5), 150mM sodium chloride, and 0.05% polysorbate 20 (Twee 20) overnight at 4°C; washed 3 times with TBST buffer; and incubated with the appropriate primary anti-human rabbit polyclonal antibody (growth factor receptor–bound protein 10 [Grb10], phosphorylated Grb10, p42/44, and phosphorylated p42/44, all from Santa Cruz, Heidelberg, Germany) in TBST buffer with 1% nonfat dry milk overnight at 4°C. The membranes were washed 3 times with TBST buffer and incubated with the respective secondary antibody (anti-rabbit IgG, dilution 1:5000; Cell Signaling Technology, Beverly, Mass) and visualized using the chemiluminescence detection system (Pierce Biotechnology Inc, Rockford, Ill) following the supplier’s instructions.

**RESULTS**

For elucidating signal transduction pathways in melanoma, we analyzed differential gene expression between NHMs and cutaneous melanoma metastases using cDNA array technology focusing on oncogenesis and apoptosis. A selection of genes that demonstrate altered expression in a consistent pattern was selected after significance analysis of microarrays and unsupervised hierarchical clustering (Figure 3). In particular, c-met, GRB10, mitogen-activated protein kinase kinase 6 (MAPKK6), MAPK7, E2F transcription factor 3, and several other promitotic genes were up-regulated in melanoma metastases (Figure 3). The c-met proto-oncogene encodes the receptor for the hepatocyte growth factor (HGF)/scatter factor, which is known to mediate mitogenic, migratory, and invasive effects in several different cell types. The ligand for c-met is HGF/scatter factor, and although normal HGF/scatter factor–Met signaling is required for embryonic development, abnormal c-met signaling has been strongly implicated in tumorigenesis, particularly in the development of invasive and meta-

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Shc, Crk/CRKL, and the large adapter protein Gab1. These adapter proteins in turn recruit several signal transducing proteins to form an intricate signaling complex. The c-terminal end of the receptor. This docking site mediates the binding of several adapter proteins such as Grb2, SHC, Crk/CRKL, and the large adapter protein Gab1. These adapter proteins in turn recruit several signal transducing proteins to form an intricate signaling complex. The c-terminal end of the receptor. This docking site mediates the binding of several adapter proteins such as Grb2, SHC, Crk/CRKL, and the large adapter protein Gab1. These adapter proteins in turn recruit several signal transducing proteins to form an intricate signaling complex. Therefore, the presence of the Met/HGF receptor may contribute to the acquisition of an invasive phenotype.41-44

To confirm c-met expression at the transcriptional level, we used real-time RT-PCR in several melanoma cell lines (MV3, BLM, and M13) and in cutaneous melanoma metastases (Figure 4). In contrast, insulin-like growth factor I (IGF-1) receptor; IGF-2; IGF-binding proteins 2, 4, and 6; and other apoptotic genes were down-regulated (Figure 3).

Next, we analyzed the cause-consequence relationship of these genes within and across clusters using available published information on signaling pathways in cancer (Figure 5). Genes defined within clusters with meaningful profiles were queried for the SOURCE gene report, which include aliases, chromosomal location, functional descriptions, gene ontology annotations, gene expression data, and several pathway-related databases. This information is valuable to understand interrelations between different pathways and elute to translational research. Given the up-regulation of GRB10 and its potential significance in melanoma pathogenesis, we confirmed the up-regulation of Grb10 in 4 analyzed melanoma cell lines (SK-Mel 28, MV3, BLM, and M13) and 3 of 4 cutaneous melanoma metastases using Western blot analysis (Figure 6). Growth factor receptor–bound protein 10 belongs to a small family of proteins, including Grb7 and other adapter proteins to form an intricate signaling complex. These adapter proteins in turn recruit several signal transducing proteins to form an intricate signaling complex.
Figures 4 and 5. Real-time reverse transcriptase polymerase chain reaction (PCR) detection of c-met using TaqMan gene expression assay (Applied Biosystems Inc, Foster City, Calif). To confirm complementary DNA arrays, ABI prism 7300 (Applied Biosystems Inc) was performed. The bar graph shows expression of normalized c-met compared with normal human melanocytes (NHM) in 3 different melanoma cell lines (BLM, MV3, and M13) and 4 melanoma metastases. c-met was up-regulated in most investigated samples, with NHM used as the calibrator.

Figures 6 and 7. Western blot analysis of growth factor receptor–bound protein 10 (Grb10) and phosphorylated Grb10 (P-Grb10). To confirm protein expression, protein extracts of normal human melanocytes (NHM), different melanoma cell lines, and 4 melanoma metastases were probed with Grb10 and P-Grb10 antibodies. The Grb10 and P-Grb10 were up-regulated in most samples compared with NHM. Reprinted with permission from Mirmohammadsadegh et al.©2004, Karger Publishers, Basel, Switzerland.

Grb14.46 These molecules are commonly referred to as adapter proteins, because they have no catalytic function but contain numerous protein-binding motifs; thus, they are predicted to mediate interactions between disparate proteins. It is thought that Grb10 brings together activated receptors with downstream signaling components of several pathways (eg, MEK-1 and -2 and Raf-147 and Akt48) and may also interact with protein ubiquitination.49
Recent research on global transcript analysis has identified previously unrecognized subtypes of cutaneous melanoma and predicted phenotypic characteristics that may be of importance to disease progression. Bittner et al reported the discovery of 2 subsets of melanomas identified by mathematical analysis of gene expression. A series of uveal melanoma showed several highly repressed genes that were inversely correlated with the same genes from the major cluster of cutaneous melanoma samples, proving the different nature of these 2 entities. However, because the patients in this study were uniformly characterized by poor prognosis, future work should determine whether these new subsets discriminate patients with distinct natural histories. Recent studies further divided uveal melanoma into 2 distinct, previously unrecognized entities that were indistinguishable by clinicopathologic features. Similarly, patterns of gene expression that correlated with progression to metastatic disease have recently been defined. In particular, 2 sets of differentially expressed genes were identified. The first set is involved in extracellular matrix assembly and the expression that correlated with progression to metastatic melanoma and provided prognosis prediction with 4 antibodies (Ki67, p16INK4a, p21CIP1, and Bcl-6) in an independent series of 72 vertical growth-phase melanoma specimens. Two potential problems with the application of microarray technology to primary human tumor specimens are tissue availability and cellular heterogeneity of samples. Archival formalin-fixed, paraffin-embedded tissue is a vast source of clinical material; however, standard processing methods limit the use of such tissue in high-throughput protein analysis. To overcome this limitation, more efficient methods for using formalin-fixed, paraffin-embedded tissue need to be developed. Second, the presence of variable levels of normal cells such as stroma, blood vessels, and lymphocytes could mask expression patterns attributable to cancer cells. For instance, the macular components of nevi and radial growth-phase tumors are very thin and contain limited numbers of tumor cells. Especially, the flat nature of these lesions usually predispenses them to contamination with nontumor cells. This has been overcome with the advent of laser capture microdissection. However, from an alternative perspective, the reductionist laser-capture microdissection approach of analyzing highly homogenous cell populations could miss important interactions between the carrier and stroma cells. According to this line of reasoning, an accurate analysis of cancer pathology must address the full mixture and interactions of cancer cells, surrounding stroma, infiltrating inflammatory cells, and vascular elements (ie, interstitial, intracellular, and intercellular compartments). These so-called normal cells associated with a neoplasm are clearly distinct from populations of normal cells residing in non-tumor regions. Support for this contention is provided by the recent finding that tumor endothelial cells exhibit a unique signature. Hence, an argument for the genomic analysis of more heterogeneous cancer tissue samples is gaining momentum at present. Therefore, we have purposely used melanoma metastases that contain the melanoma cells within their natural stroma.

Given the subsets of cutaneous melanoma metastases with phosphorylated Grb10 and transcriptional up-regulation of c-met, B-RAF, Raf-1, MAPKK6, MAPKK7, and MEK2 and down-regulation of IGF-I receptor, IGF-I, and IGF-II, we suggest that Grb10 may have a critical regulatory function in melanoma metastasis in promoting IGF-I receptor–independent Raf/MAPK signaling to elicit cell cycle progression, proliferation, and metastasis. In addition, the presence of c-met/Grb10–mediated activation of the Raf/MEK/ERK pathway seems to override IGF-I receptor–mediated activation of Grb10. This inability to be overridden may be important to disease progression. Moreover, because IGF-I receptor down-regulation enhances the sensitivity to cytotoxic drugs and irradiation, this subset of cutaneous melanoma metastases could be more responsive to chemotherapy compared with melanoma metastases, where the IGF-I receptor plays a more central role. Further experiments will be required to address these issues in detail to uncover subsets of melanoma metastases with alternative pathway preferences leading to invasion and metastasis and to decipher the critical role of adaptor molecules as decision makers in the selection of those pathways. Microarray technology offers a rapid, high-throughput technology that may help to answer clinically important questions about cancer, such as which tumors will behave aggressively, which will remain indolent, and which are likely to respond to selected therapies.

Expression profiling using microarray platforms has several limitations and pitfalls that need to be considered during experimental design and analysis, to avoid false-positive and false-negative results (Table 2). Use of adequate technical and biological replicates is a prerequisite to any microarray experiment. Few replicates will identify a smaller percentage of differentially expressed genes. The inclusion of dye swap labeling is critical. The analysis of the microarray results represents the next challenge. First, the estimation of the differential expression of each gene from the digital image is performed. Exploratory graphical methods such as hierarchical clustering are already being used to assist in identifying structures within the data, but further methods are needed, including seriation and multidimensional scaling. Appropriate statistical models also need to be refined. Issues of quality control arise, and the final inference needs to take into account the propagating of uncertainties that arise at each analytical step. Constructing a meaningful classifier based on microarray gene expression data has recently emerged as an important problem for cancer classification. Difficulties arise from the fact that each sample contains expression data of a vast number of genes that may interact with one another. The selection of a small number of critical genes is fundamental to correctly analyze the vast amount of
data. It is essential to use a multivariate approach for data. Therefore, effective dimension reduction methods and multivariate approaches are key to tumor classification. Clustering has been the primary analytical tool used to define disease subtypes from microarray experiments of cancer. Assessing cluster reliability, therefore, poses another level of complication in analyzing microarray data. An issue of equal importance is the need for downstream validation of identified targets. Several techniques such as Northern blot analysis and real-time RT-PCR are being used to validate microarray experiments.

The appropriate use of high-throughput DNA assay platforms offers an efficient means of identifying patterns of regulated genes and associated pathways, leading to functional characterization and tumor subclassification. If we transfer the power of recent biotechnology achievements to individual patients, we will not only benefit from an era of unprecedented opportunity for addressing scientific questions to uncover the molecular basis of individual cancers but also improve the treatment and most likely the outcomes of our patients with melanoma.

Accepted for Publication: November 8, 2004.

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