

The gene expression signatures of melanoma progression

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Because of the paucity of available tissue, little information has previously been available regarding the gene expression profiles of primary melanomas. To understand the molecular basis of melanoma progression, we compared the gene expression profiles of a series of nevi, primary melanomas, and melanoma metastases. We found that metastatic melanomas exhibit two dichotomous patterns of gene expression, which unexpectedly reflect gene expression differences already apparent in comparing laser-capture microdissected radial and vertical phases of a large primary melanoma. Unsupervised hierarchical clustering accurately separated nevi and primary melanomas. Multiclass significance analysis of microarrays comparing normal skin, nevi, primary melanomas, and the two types of metastatic melanoma identified 2,602 transcripts that significantly correlated with sample class. These results suggest that melanoma pathogenesis can be understood as a series of distinct molecular events. The gene expression signatures identified here provide the basis for developing new diagnostics and targeting therapies for patients with malignant melanoma.

bioinformatics | human | microarray | metastasis | laser capture

In the current staging system for cutaneous melanoma, vertical thickness of the primary tumor is the dominant prognostic factor, belying the fact that a subset of thin tumors metastasize, whereas some thick tumors do not undergo metastasis (1). The original melanoma tumor progression model is characterized by an initial radial growth phase, encompassing *in situ* and minimally invasive tumors (2). This phase is followed by the development of vertical growth phase, which has been postulated to be the first point at which the tumor gains metastatic capacity. However, metastasis occurs, although with decreased frequency, in patients whose primary melanoma pathology exhibits only a radial growth pattern (3). Previous transcriptome analysis in melanoma defined a cluster of genes expressed in a majority of metastatic melanomas (4); however, this cluster was not related to radial or vertical growth, and precursor nevi (moles) and primary melanomas were not examined. Likewise, mutations in *B-RAF* occur commonly in both nevi (5) and melanoma (6), and, thus, do not distinguish progressive stages in melanoma progression. In this study, we used cDNA expression array profiling to characterize the global patterns of transcript modulation that underlie the various phases in the known tumor progression pathway of melanoma.

Methods

Study Subjects. Samples from melanoma patients and nevus volunteers presenting to the Melanoma Center were obtained with informed consent under a protocol approved by the UCSF Institutional Review Board. After biopsy, all samples were frozen in OCT freezing medium over dry ice. Subsequently, samples were processed for hematoxylin/eosin staining and confirmed by pathologic review. Only samples comprised of >95% tumor cells were analyzed.

Isolation and Purification of Total RNA from Biopsy Specimens.

Depending on the sample size, 5–12 20- μ m sections were cryotomed, homogenized [Polytron 1200C, Brinkmann, Westbury, NY, at setting 4 for 30 sec] with 600 μ l of RNA lysis buffer plus 1% 2-mercaptoethanol, and RNA was isolated by using RNeasy columns (Qiagen, Valencia, CA). Samples from a large primary melanoma, PM09, were subjected to laser-capture microdissection (Arcturus Instruments, Mountain View, CA) before RNA preparation.

RNA Amplification and Labeling. One microgram of total target RNA, side by side with 1 μ g of universal human reference RNA (Stratagene), was linearly amplified through two rounds of modified *in vitro* transcription (7). Amplified RNAs were converted to aminoallyl-modified cDNA and coupled to *N*-hydroxysuccinimidyl esters of Cy3 or Cy5 (Amersham-Pharmacia, Piscataway, NJ) (8), and then hybridized to a microarray slide at 65°C for 12–16 h (9); www.microarrays.org). Slides were then washed and immediately scanned with Axon-imager 4000b (Axon Instruments, Foster City, CA), by using GenePixPro3 software.

Microarrays. The 20,862 cDNAs used in these studies were from Research Genetics (Huntsville, AL). On the basis of Unigene build 166, these clones represent 19,740 independent loci.

Microarray Data Analysis: Hierarchical Clustering. Gene expression was analyzed with CLUSTER (10) by using the average linkage metric and displayed using JAVATREEVIEW (which can be accessed at <http://genetics.stanford.edu/~alok/TreeView>). GENEPIX (Axon Instruments) median of ratio values from the experiment were subjected to linear normalization in NOMAD (which can be accessed at <http://derisilab.ucsf.edu>), log-transformed (base 2), and filtered for genes where data were present in 80% of experiments, and where the absolute value of at least one measurement was >1.

SIGNIFICANCE ANALYSIS OF MICROARRAYS (SAM). After linear normalization, log (base 2) transformation, and hierarchical clustering, the resulting cluster data table was imported into the SAM software package. Groups were defined based on the comparison performed; for example, group 1 = radial growth phase, and group 2 = vertical growth phase in Fig. 1. Data were censored if more than one data value was flagged in each group to eliminate poor quality array data. Delta was chosen to limit the output gene list so that fewer than 1% predicted false-positives would be included.

Abbreviations: SAM, SIGNIFICANCE ANALYSIS OF MICROARRAYS; MMP, matrix metalloproteinase; CDH3, cadherin 3.

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anoma with a predicted median false-discovery rate of 2.3 (Table 3). Up-regulated in this gene set were genes known to play an important role in melanoma progression, including SPP1 (osteopontin) (12, 13), and CXCL1 (melanoma growth-stimulating activity) (14), and RAB32 (15). Conversely, genes with potential tumor suppressor activity such as WIF1 (16), ECM2 (17), and SLIT3 (18, 19) were down-regulated. Interestingly, examination of PM09 gene set 1 did not distinguish between nevi and primary melanomas (data not shown).

Finally, we performed a multiclass SAM comparison of skin, nevi, and primary and metastatic melanoma. The algorithm identified 2,602 genes (multiclass gene set) with a predicted median false-significant rate of 1.6 that significantly distinguish the sample classes (Fig. 4). SAM results were visualized by using CLUSTER and TREEVIEW of the multiclass gene set.

This analysis demonstrates that unique gene expression patterns characterize each stage of melanoma progression. Fig. 4B.2 shows that melanocytic lineage markers S100B and MLANA (Melan-A) (Fig. 4B.2) are unable to distinguish between any of the multiclass sample groups. The biomarkers identified in Fig. 3 (e.g., CXCL1) not only distinguish primary tumors from moles but also are useful in identifying metastases, and are not expressed in nevi or normal skin (Fig. 4B.3). Biomarkers identified here are also able to distinguish metastatic tumors from all other tissue classes examined.

Among the genes overexpressed in metastatic lesions were several clones representing a reverse transcriptase homolog of an endogenous retrovirus (Fig. 4B.4). In addition, up-regulation of candidate oncogenes such as nuclear receptor coactivator receptor protein 3 (20) and PHIP, a pleckstrin homology domain-containing protein (21) was observed (data not shown). By contrast, genes lost in the transition to metastatic growth (Fig. 4B.5) are implicated in maintenance of normal melanocyte differentiation, including ZNFN1A5 (22) and HPS1 (23).

Discussion

In this study, we examined the gene expression signatures of known points in the tumor progression model of malignant melanoma, from nevus to primary melanoma to metastatic melanoma. Our studies indicate that metastatic melanoma is characterized by two different gene expression signatures, with common features compared with the signatures of radial and vertical growth phases of primary melanoma. Furthermore, gene expression profiling was used to assign specific gene expression signatures to distinct points in the melanoma tumor progression pathway (Fig. 4).

An unexpected finding of this study was that the gene expression signature of the radial growth phase of PM09 was recapitulated in some metastases. Radial growth phase melanomas are considered by some investigators to have little or no propensity for metastasis. These results challenge this notion, suggesting that a small but clinically significant proportion of melanoma metastases may arise from the radial growth phase of primary melanomas. Our hypothesis is supported by the analysis of patient MM14, whose metastasis displayed the type I (radial) signature at the molecular level, whereas the nodular primary displayed the type II (vertical) signature. Nodular melanomas characteristically lack the radial growth phase, and some investigators have postulated that the radial growth phase may be destroyed by the expansile growth of the vertical growth portion. Further analysis is required to determine the specific molecular events that promote tumor metastasis from radial growth phase melanomas.

In addition, the profiling results from PM09 are supported by the analysis of CDH3 and MMP10 immunostaining in a panel of 25 independent primary melanomas. For both markers, protein levels were significantly higher in radial growth compared with vertical growth.

Our study sheds light on the current debate regarding the genesis of the metastasis signature in primary tumors. Recent reports demonstrated the presence of a common metastasis signature in a subset of primary tumors whose origin was different from the metastases studied (24), and that the gene expression signature correlated with metastases was already present in primary breast cancers (25–27). Our study extends these observations by showing that gene expression signatures present in metastases are observed at the *in situ* or minimally invasive stages that constitute the radial growth phase of primary melanoma (2).

Moreover, the losses in gene expression noted in vertical growth could suggest that the radial growth-dissected samples consisted of genes predominantly expressed in keratinocytes and not melanoma cells. However, our immunohistochemical analyses (Fig. 1), and several previously published studies, demonstrate expression of many PM09 gene set 1 biomarkers in melanomas, including integrin $\alpha 2$ (28), laminin $\gamma 2$ (29), MMP10 (30), and CDH3 (31). Intriguingly, in an independent study, decreased CDH3 immunostaining correlated with melanoma tumor progression (31), which is similar to our results. Furthermore, the presence of expression of a large proportion of this gene signature in the second subtype of metastatic melanomas, obtained from fine needle aspirates of melanoma metastases, which are predominantly (95%) composed of melanoma cells and devoid of keratinocytes, argues against keratinocyte-specific gene expression alone. Taken together, these results strongly suggest the relevance of the PM09 gene set 1 to the melanocytic lineage.

These results have potentially important implications for the development of melanoma therapy, because many vaccine therapies are currently directed at genes expressed only in type II metastatic melanoma, although we demonstrate that type I metastatic melanoma is also frequent. Further studies are indicated to identify target antigens in the therapy of patients with type I metastatic melanoma.

Examination of the gene expression signatures of nevi versus primary melanoma, suggests the potential utility of these biomarkers as an adjunct to the problematic pathologic diagnosis of melanoma (32). In addition, distinct signatures specific to the metastatic stage were identified (Fig. 4), including activation of several clones of endogenous retrovirus polymerases, which is consistent with reports of human endogenous retrovirus (HERV) retrovirus reactivation in melanoma (33). In this study, whereas primary melanocytes did not shed retroviral particles and were resistant to infection with retrovirus, cell lines derived from melanoma metastases tested produced HERV-K type viruses. We find that long interspersed nuclear element (LINE)-1 and HERV-K type polymerase transcripts are activated in clinical metastatic samples. LINE-1, but not HERV-K, activation permits retrotransposition, which could be one molecular mechanism for genomic damage in advanced melanoma.

Our work indicates that distinct gene expression signatures exist for the known points in the tumor progression pathways of malignant melanoma. In fact, our study implicates different gene sets in the transition from nevus to primary melanoma, and from primary to metastatic melanoma. In addition, a gene expression signature derived from the radial growth phase but absent in the vertical growth phase of a primary melanoma is present in a subset of metastases. Finally, our results provide a basis for developing new molecular diagnostics and targeted therapies for melanoma patients.

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