Lack of Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase Signaling Shows a New Type of Melanoma

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Abstract

The majority of human melanomas harbor activating mutations of either N-RAS or its downstream effector B-RAF, which cause activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase and the ERK MAPK cascade. The melanoma-relevant effectors of ERK activation, however, are largely unknown. In this work, we show that increased ERK activation correlates strongly with mutational status of N-RAS or B-RAF in 21 melanoma cell lines. Melanoma lines that were wild-type for RAS/RAF showed low levels of ERK activation comparable with primary human melanocytes. Through supervised analysis of RNA expression profiles, we identified 82 genes, including TWIST1, HIF1α, and IL-8, which correlated with ERK activation across the panel of cell lines and which decreased with pharmacologic inhibition of ERK activity, suggesting that they are ERK transcriptional targets in melanoma. Additionally, lines lacking mutations of N-RAS and B-RAF were molecularly distinct and characterized by p53 inactivation, reduced ERK activity, and increased expression of epithelial markers. Analysis of primary human melanomas by tissue microarray confirmed a high correlation among expression of these epithelial markers in a heterogeneous sample of 570 primary human tumors, suggesting that a significant frequency of primary melanomas is of this “epithelial-like” subtype. These results show a molecularly distinct melanoma subtype that does not require ERK activation or epithelial-mesenchymal transformation for progression. [Cancer Res 2007;67(4):1502–12]

Introduction

Advanced melanoma is one of the most intractable entities of medical oncology. Although curable through surgery at early stage, melanoma is noteworthy for its marked therapeutic resistance and proclivity for early metastasis. Recently, however, an improved understanding of the pathogenesis of the disease has provided cautious optimism. Mutational activation of the B-RAF serine/threonine kinase or its upstream activator, the N-RAS small GTPase, occurs at a frequency of 40% to 60% and 10% to 20%, respectively, in a mutually exclusive manner and at an early stage of melanomagenesis (1–3). Although RAS engages multiple downstream effectors [e.g., RAF, phosphatidylinositol 3-kinase (PI3K), and Ral guanine nucleotide exchange factors; ref. 4] that can contribute to oncogenesis, the finding that N-RAS and B-RAF mutations are mutually exclusive in melanoma has prompted the notion that RAF mediates much of the transforming activity of RAS activation in this tumor type. RAF activation induces phosphorylation and activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 kinases, which phosphorylate and activate the ERK1/2 MAPKs, which activate Ets family and other transcription factors. Recent studies have shown that essentially all genes deregulated by RAF activation are through the MEK-ERK pathway (5), supporting MEK1/2 as the principal target of RAF activation. Despite evidence that the ERK MAPK cascade is required for the maintenance of established melanoma (6, 7), the critical gene targets of this pathway have not been fully elucidated. Previously, we identified key targets of RAS and RAF signaling (8–10) through differential gene expression and candidate gene approaches. In this work, we elected to take an unbiased genome-wide approach to identifying the relevant targets of RAS-RAF-MEK signaling in melanoma.

In addition to RAS/RAF mutations, the majority of melanomas also harbor inactivation of the INK4a/ARF tumor suppressor loci, which encodes two negative regulators of proliferation, p16INK4a and p14ARF (alternate reading frame; reviewed in ref. 11). Both proteins play nonoverlapping tumor suppressor roles in critical tumor suppressor pathways: p16INK4a regulates the cyclin-dependent kinase (CDK) 4/6-cyclin D-retinoblastoma pathway, whereas ARF regulates MDM2-p53. Genetic data suggest that INK4a/ARF inactivation is the preferred mechanism of retinoblastoma and p53 pathway inactivation in most melanoma (3, 12–14). In particular, p53 loss is less common in melanoma compared with other human cancers, occurring in 10% to 25% of cases (3, 15–17). RAS and RAF activation have been shown to induce p16INK4a and/or ARF expression in cultured cells, suggesting that INK4a/ARF activation functions to antagonize the transforming activities of oncogenic ERK activation, and work in murine models has confirmed synergy between RAS activation, and germ-line p16INK4a and/or ARF loss in melanoma formation (18, 19). Given the high frequency of RAS/RAF mutation and INK4a/ARF inactivation, melanoma seems relatively homogeneous in molecular genetic terms compared with other human cancers. Because B-RAF and N-RAS mutations are observed in >80% of all melanomas, one might conjecture that
activation of ERK is a requirement for the growth of all advanced melanoma. This would imply that the 10% to 20% of melanomas that do not harbor B-RAF or N-RAS mutation would have arisen by distinct genetic mechanisms, for example, by activation of tyrosine kinases upstream of RAS [e.g., HER2/epidermal growth factor receptor and fibroblast growth factor (FGF) receptor], which cause persistent activation of the RAS-ERK signaling network.

In this work, we provide evidence to refute this hypothesis. We characterized a panel of melanoma cell lines and primary cultures of normal human melanocytes (NHM) through biochemical and RNA expression analysis and correlated these characteristics with alterations in gene expression by unsupervised and supervised analysis. These analyses led to the discovery of a downstream set of ERK-regulated transcripts in melanoma cell lines. Moreover, we have shown that melanoma cell lines lacking N-RAS/B-RAF mutation showed low ERK activity and decreased expression of ERK-regulated gene transcripts. Instead, lines with low ERK activity showed a significantly higher frequency of p53 inactivation and increased expression of epithelial markers, such as CD24, cytokeratin 8/18, E-cadherin, and P-cadherin. An analysis of the expression of these epithelial markers on a 570-sample tissue microarray (TMA) of primary melanomas likewise suggested the existence of this tumor subtype in vivo. These analyses suggest the existence of a heretofore unappreciated form of melanoma ("epithelial-like") that is molecularly distinct from the more common form of RAS/RAF mutant melanoma.

Materials and Methods

Cell culture and mutational analysis. Cell lines were obtained from indicated sources and cultured as described (Supplementary Table S1). Genomic DNA was isolated using Genomic Tips kits (Qiagen, Valencia, CA), and the mutational status of B-RAF (exons 11 and 15) and N-RAS (codons 12, 13, and 61) were determined by direct sequencing of PCR amplification products as described (20).

Western blot analyses. Protein lysates (30 μg) from exponentially growing cultures of cells were harvested and transblotted as described (8); immunoblotted with antibody to activated phosphorylated ERK (pERK) 1/2 (Cell Signaling, Danvers, MA), ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), activated phosphorylated AKT (pAKT; Cell Signaling), AKT (Cell Signaling), MCAM (Abgenix, Fremont, CA), E-cadherin (BD Transduction Laboratories, San Jose, CA), Rhodamin/β3 (Upstate, Waltham, MA), CDK6 (LabVision Corp., Fremont, CA), and β-actin (clone AC-15; Sigma, St. Louis, MO); and detected with appropriate antimouse or antirabbit horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ) by enhanced chemiluminescence (Amersham).

Flow cytometry. Exponentially growing cells were detached using 0.5 mmol/L EDTA for 3 to 5 min at 37°C and stained with FITC-conjugated antibodies to the CD24 adhesion protein (24C02; Neomarkers, Fremont, CA) and analyzed using a FACScan analytic flow cytometry. Appropriately gated cells were analyzed (10,000 cells counted per staining) and compared with unstained or isotype controls.

Microarray analysis. To synthesize labeled cDNA, reverse transcription reactions were carried out using 5 μg mRNA as described (21). Briefly, experimental mRNA was labeled with Cy5-dUTP and the pooled cell line control was labeled with Cy3-dUTP using the Agilent (Santa Clara, CA) low RNA input linear amplification kit and hybridized overnight at 65°C to an Agilent 4×1 complete human genome (G4112A) 60-mer oligonucleotide microarray. Arrays were washed and scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) and images were quantified (using GenePix Pro 5.1) and uploaded to the University of North Carolina microarray database6 where the data are publicly available. All array data were normalized locally and globally as described (21). Replicate arrays were done for four of the lines, which were averaged and treated as a single array. Data analyses were done as described in the Supplementary Materials and Methods. Briefly, for analyses done using significance analysis of microarrays (SAM), data were excluded for genes with poor spot quality or with a mean intensity greater than twice the median background. Hierarchical clustering analyses were conducted using the program Cluster7 to do median-centered, average-linkage clustering, which were visualized using TreeView. All data shown in the heat map figures are median centered.

ERK inhibition. Briefly, three cell lines (SKMEL24, SKMEL28, and WM2664) that showed elevated pERK levels were treated for 24 and 48 h with 30 μmol/L U0126 (Promega, Madison, WI), a MEK1/2-specific inhibitor to block ERK1/2 activation, with parallel control cultures treated with carrier (DMSO) and protein lysates or RNA extracted as described above and Western blots or RNA microarray analyses done as described in the Supplementary Materials and Methods.

Melanoma TMA construction and analysis. Melanoma tissue section blocks were stained with H&E and first reviewed by a pathologist to define the selective areas to be punched. Immunofluorescent staining of slides was as described previously (22) with modifications as described in the Supplementary Materials and Methods. Primary antibodies used were as follows: S100, a specific marker for melanoma (AM508; Biogenex), E-cadherin (BD Transduction Laboratories), P-cadherin (BD Transduction Laboratories), N-cadherin (Invitrogen, Carlsbad, CA), CD24 (Neomarkers), and cytokeratin 18 (Neomarkers). Automated Image Acquisition and Analysis (AQUA) was done as essentially as described previously (22) with modifications as described in the Supplementary Materials and Methods and the targets measured in both cytoplasmic and nuclear compartments within the S100 tumor mask on a scale of 0 to 4050 and expressed as target signal intensity relative to the respective cytoplasmic or nuclear compartment area.

Results

Description and analysis of cell lines and melanocyte cultures. We collected and characterized 17 melanoma cell lines (Supplementary Table S1) and three independent NHM cultures for N-RAS and B-RAF mutation status, ERK and AKT activation status, INK4a/ARF status, G1 checkpoint response, proliferative rates, and RNA expression profiles. An additional line (SKMEL23) lacking N-RAS or B-RAF mutation was analyzed where indicated. To analyze ERK and AKT activation, we did immunoblot analyses to determine the levels of pERK, total ERK, pAKT, and total AKT (Fig. 1). Although total ERK and AKT levels were comparable in all lines, the cell lines were markedly heterogeneous with regard to steady-state pERK and pAKT levels. When compared with NHMs, the majority of lines with B-RAF and N-RAS mutations displayed moderate to highly elevated pERK (12 of 15) with the highest pERK expression associated with B-RAF activation (6 of 7). In contrast, lines wild-type (WT) for both genes showed the lowest pERK levels that were comparable with that seen in NHMs. A similar pattern was noted across the panel with regard to pAKT levels: the majority of B-RAF and N-RAS mutant lines showed elevated pAKT (10 of 15), with higher levels of activation associated with B-RAF mutations, whereas lines WT for RAS/RAF expressed little or no pAKT. These data show that a subset of melanoma cell lines does not require persistent ERK or AKT activation for growth.

p53 function and INK4a/ARF status. To interrogate unequivocally p53 activity, we combined assays to infer p53 function, including G1 checkpoint response to ionizing radiation (IR), INK4a/ARF Taqman analysis, microarray analysis, candidate gene sequencing, and Western blotting, which yielded consistent results.

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6 http://genome.unc.edu/
7 http://rana.lbl.gov/EisenSoftware.htm
G1 checkpoint response suggests that lack of IR-induced G1 arrest expression of p53 gene targets in lines with defective DNA damage elsewhere). In addition, consistent with the observation that ARF is overexpressed in cell lines lacking p53 function, G1 checkpoint-defective cell lines showed increased expression of the ARF transcript, except in two lines with homozygous deletion of the INK4a/ARF locus (Supplementary Fig. S1). The finding of increased expression of ARF, but not p16INK4a, and decreased expression of p53 gene targets in lines with defective DNA damage G1 checkpoint response suggests that lack of IR-induced G1 arrest is a surrogate marker for inactivation of p53 function (Supplementary Fig. S1) and is consistent with observations from other cell line systems (24). These results are in accord with the published data about p53 and INK4a/ARF expression across the panel of cell lines (summarized in Supplementary Table S1). All (4 of 4) of the melanoma cell lines that were WT for RAS/RAF showed a defect in the p53 response to IR versus only 6 of 13 for the RAS/RAF mutant lines (P = 0.03). These data suggest that melanoma cell lines, which are WT for RAS/RAF, are also characterized by a defective p53 response to DNA damage, which is an otherwise uncommon feature of melanoma (3, 15–17).

**Unsupervised expression analysis.** We first did RNA expression profiling on the 17 melanoma cell lines and three NHM cultures by unsupervised analysis. Using predefined criteria, 1,944 genes passed filtering and were analyzed by hierarchical clustering (Fig. 2; entire gene list is shown in Supplementary Table S2). Two large subgroups of melanoma could be discerned, which were distinguished by expression of microphthalmia-associated transcription factor (MitF), MitF transcriptional targets and additional genes (e.g., Pax3 and ENDRB) that specify melanocyte differentiation (Fig. 2A, cluster C). A similar microarray pattern has been reported in an analysis of uncultured melanoma metastases (25), suggesting that our cell line panel is representative of the major subtypes of patient-derived metastatic tumors. Cell lines that exhibited high expression of MitF clustered with NHMs, whereas cell lines with lower expression of MitF clustered with amelanotic lines, such as PMWK and SKMEL24. MitF-expressing lines showed increased expression of classic melanoma markers, such as tyrosinase, MLAN-A/MART1, and Silver/PMEL17; many of which are known transcriptional targets of MitF (26). Of note, tyrosinase-related protein (TYRP) 1, a commonly used melanoma marker, showed a different pattern of expression (Fig. 2A, cluster B) from MitF (Fig. 2A, cluster C). TYRP1 clustered with other genes that are more highly expressed in NHMs than melanomas, such as c-KIT, P-cadherin, and E-cadherin (27, 28). Whereas all the lines WT for RAS/RAF showed low MitF expression, TYRP1 expression was increased in two of these lines (RPMI-8322 and PMWK).

Mutations in B-RAF versus N-RAS did not correlate with MitF expression across the panel of cell lines (Fig. 2A, cluster C) nor were B-RAF mutant and N-RAS mutant lines discriminated by unsupervised analysis. The lines WT for RAS/RAF, however, showed an expression profile that was distinct from the RAS/RAF mutant lines and the NHMs (Fig. 2A, far right clade). The WT lines harbored increased expression of several epithelial markers (Fig. 2A, cluster A). This latter observation was made explicit by clustering the lines based on selected genes that are known transcriptional epithelial markers (29) versus melanoma and melanocyte markers (Fig. 2B). The WT lines showed increased expression of epithelial transcripts, such as cytokeratins 8/18, E-cadherin, P-cadherin, CD24, and amphiregulin (AREG), but showed decreased expression of many genes previously associated with melanoma progression [e.g., MCAM/MUC18, N-cadherin, and integrin β3 (ITGB3)]. Consistent with a recent report linking TWIST1 expression to epithelial-mesenchymal transition (EMT; ref. 30), we also noted a strong inverse correlation between TWIST1 expression and E-cadherin expression and other epithelial markers across the panel of melanoma cell lines. Lines WT for RAS/RAF in particular showed low TWIST1 expression and increased expression of epithelial markers, such as E-cadherin, indicating that these metastatic tumor lines developed in the absence of EMT (Fig. 2B).

These differences in gene expression discovered by unsupervised analysis were confirmed in representative cell lines by Taqman for TWIST1 and COTL1 (data not shown) and at the protein level for E-cadherin, MCAM, and CD24 (Fig. 3A–C). Therefore, cell lines WT for RAS/RAF are readily discernible by unsupervised analysis from
lines harboring N-RAS or B-RAF mutations, with some features more typical of NHMs (E-cadherin and TYRP1 expression) and other features more typical of epithelial cells (CD24 and cytokeratin expression). We will henceforth refer to this group as the epithelial-like melanoma subtype.

Supervised analysis with regard to RAS/RAF mutation and pERK. We did several types of supervised analysis using SAM (31) with regard to RAS/RAF mutational status and pERK levels, yielding similar results. Whether we classed lines with N-RAS and B-RAF mutations together (two class comparison) or treated each group separately (multiclass comparison), a gene list was obtained that distinguished the WT from the RAS/RAF mutant lines with a low (<5%) false discovery rate (FDR). For example, the multiclass comparison identified 511 genes that correlated with RAS/RAF mutation (Supplementary Table S3), including genes associated with melanoma progression (e.g., N-cadherin, FGF2, and TWIST1).

This list, however, did a poor job of discriminating B-RAF mutant from N-RAS mutant tumor types with a high misclassification rate. In an effort to find transcripts that distinguished the N-RAS and B-RAF mutant lines, we omitted the epithelial-like lines from the SAM analysis but still obtained small gene lists with high FDRs (~8%). These results indicate similarity in the expression profiles of N-RAS and B-RAF mutant cell lines, consistent with the results of unsupervised analysis (Fig. 2A). The ability to easily discern WT from RAS/RAF mutant cell lines, but not RAS from RAF mutant lines, is in accord with one previous report from Bloethner et al. (32), but in contrast to another report from Pavey et al. (33). The reasons for the apparent discrepancy among these studies are unclear but likely are technical: all three analyses were done on different microarray platforms using different bioinformatic approaches. Importantly, however, whereas there was a highly significant overlap between the epithelial-like specific

Figure 2. Unsupervised analysis of melanocytes and melanoma cell lines. A, hierarchical clustering was done using 1,944 genes that showed variable expression in 17 melanoma cell lines and 3 NHM cultures. Melanoma cell lines with mutations in N-RAS (red), those with B-RAF (blue), and those with WT for N-RAS and B-RAF (black). Selected clusters are shown (source data is included in Supplementary Table S2). Cluster A, genes highly expressed in the WT cell lines; cluster B, genes expressed predominantly in NHMs; cluster C, genes associated with increased MiT expression; cluster D, genes expressed at increased levels in RAS/RAF mutant lines and at decreased levels in WT lines and NHMs. Several other genes important in melanoma biology (e.g., Sox10, Pax3, etc.) are noted on the heat map as well.
gene lists in this analysis and the Bloethner study (see discussion), there was virtually no overlap between the gene list that distinguished N-RAS and B-RAF mutant lines in any of the three studies. Given the lack of independent reproducibility, it is likely that the N-RAS versus B-RAF distinguishing gene lists of each group are not generalizable.

To study the explicit link between ERK activation and gene expression, we grouped the cell lines by pERK level from Fig. 1 and did SAM comparisons. A list of 351 genes passed SAM with FDR < 5% (Supplementary Table S4) that was similar if we treated pERK as a multiclass (high, medium, or low) or continuous variable. This list significantly overlapped with the list obtained by supervising to RAS/RAF mutation: 111 of 281 genes that correlated with increased pERK levels also correlated positively with B-RAF or N-RAS mutation (P < 0.0001). The pERK list includes previously identified RAS-RAF-MEK-ERK targets [e.g., hypoxia-inducible factor 1a (HIF1a); ref. 34, Rho/Rnd3 (35), SPRY2 (34), and IL-8 (36, 37)] as well as many genes that correlate with adverse outcome or progression in melanoma, such as N-cadherin (28), FGF2 (37, 38), and TWIST1 (39). These data suggest that increased levels of pERK are associated with the expression of a suite of genes involved in melanoma vertical growth and metastasis.

Supervised analysis with regard to MEK inhibition. Although we identified a correlation across a large panel of cell lines between pERK levels, presence of B-RAF or N-RAS mutation, and the expression of genes of importance in melanoma pathogenesis, this correlation does not establish the causal relationship between ERK activation and up-regulation of gene expression. In an effort to establish which of these genes are dependent on persistent ERK activation in melanoma, we did microarray analysis on three B-RAF mutant lines with high baseline pERK levels that had either been treated or untreated with U0126, a potent and specific MEK1/2 inhibitor (40). Treatment with U0126 for 24 or 48 h produced a marked decrease in expression of pERK (Fig. 4B) but did not cause significant morphologic change in the cell lines (data not shown).

To identify transcripts that were functionally dependent on ERK activity, we did not pretreat cells with inhibitors of protein synthesis, such as cycloheximide. This approach identifies genes that are not only direct biochemical targets of ERK but also downstream targets of transcription factors, such as E2F and HIF1a, which are controlled by ERK.

As expected, ERK inhibition modulated the expression of a large set of transcripts in B-RAF mutant cell lines. Using stringent criteria, a significant change was noted in 1,470 genes by SAM, representing 3% of the transcripts on the array (Supplementary Fig. S2; Supplementary Table S5). Of the 1,470 genes, 863 decreased in expression after U0126 treatment. We observed marked decreases in many known RAS and/or ERK targets, such as SPRY2, HIF1a, MKP3/DUSP6, and cyclin D1 (Supplementary Table S5). Many of these genes are known targets of the c-Jun

**Figure 2 Continued.** B, epithelial marker genes are elevated in WT melanoma cell lines. Dendrogram of select known epithelial marker and melanoma progression genes. Note the increased expression of several epithelial marker genes (cytokeratin 8/18, desmoplakin, CD24, AREG, and E-cadherin) and the decreased expression of melanoma progression genes (MCAM, N-cadherin, ITGB3, FGFR2, and TWIST1).
NH$_2$-terminal kinase (JNK) MAPK pathway (e.g., c-Jun and Fra2), suggesting cross-talk between the MAPK pathways. Importantly, however, most genes that were noted to correlate with pERK expression across the panel of cell lines (Supplementary Table S4) did not significantly decrease in expression after U0126 treatment. For example, MCAM expression closely correlated with pERK expression and RAS/RAF mutation but did not change after 48 h of U0126 treatment at either the RNA or protein level (Fig. 4B). These results suggest that most transcripts, such as MCAM that correlate with increased pERK and RAS/RAF mutation across the cell line panel, are not downstream targets of ERK in these lines.

To refine our estimate of ERK target transcripts in melanoma, we combined the analyses of transcripts that correlate with pERK across the panel of cell lines with the analysis of transcripts down-regulated by MEK inhibition. Toward this end, we restricted the SAM analysis on the U0126-treated samples to the 281 genes identified in Supplementary Table S4, which positively correlated with pERK expression. Combining these analyses identified genes that correlated with ERK activation across a large set of cell lines but also that were decreased in expression by ERK inhibition. As variables, such as S phase and growth, did not correlate with pERK across the panel of cell lines, we believe that this approach excludes genes that are only indirectly related to pERK expression through other transcription factors, such as E2F. In addition, as these SAM analyses were done on independent data sets, this approach further limited chance false discovery. By combining the

Figure 3. Validation of protein expression of genes deregulated in WT melanomas by unsupervised analysis. A, E-cadherin expression is increased in WT melanoma cell lines. Protein lysates from the indicated melanoma cell lines were resolved and immunoblotted with E-cadherin antiserum. B, MCAM expression is decreased in WT melanoma cell lines. Protein lysates from the indicated melanoma cell lines were resolved and immunoblotted with MCAM antiserum. C, CD24 is elevated in WT melanoma cell lines. Flow cytometry was done on WT cell lines (Mel 505 and PMWK) and cell lines harboring mutant B-RAF (SKMEL24 and WM2664) with FITC-conjugated anti-CD24.
U0126 and pERK genes lists in this way, we found that of the 281 genes that positively correlated with pERK expression, 90 transcripts (82 unique) significantly decreased with U0126 treatment (Fig. 5; Supplementary Table S6). A true ERK target might not have passed the U0126 screen because the filtering was overly stringent or the target mRNA was highly stable and therefore would not sufficiently change with 48 h of drug treatment. Therefore, although the list in Fig. 5 represents genes that were reduced by transient MEK inhibition and correlated with pERK expression across a large battery of cell lines, it is no doubt an underestimate of the true “ERK-ome” in melanoma.

Melanoma TMA analysis. To determine the frequency of epithelial-like melanoma in primary human tumors, we analyzed a 570-sample TMA of melanomas representing all clinical stages. TMAs were immunostained for expression of epithelial markers that showed discordant mRNA expression between epithelial-like melanoma and RAS/RAF mutant melanoma: E-cadherin, P-cadherin, N-cadherin, cytokeratin 18 (CK18), and CD24. These markers were chosen because of the availability of validated antibodies for immunohistochemical staining with paraffin-embedded tissue samples, known pathologic significance in melanoma, and/or increased mRNA expression in epithelial-like cell lines in this work as well as an independent analysis (CD24 and CK18; ref. 32). Expression was quantitated as described (41) using AQUA. To calculate correlations among the markers, expression values were median centered, normalized to SD, and then compared by multivariate analysis with adjustment for multiple comparisons. The resulting correlation matrix is shown in Supplementary Table S5. Table 1 shows the Spearman correlation coefficient between each pair of markers. The degree of correlation between paired markers is very high, with r values range from 0.85 to 0.95. When the same antibody is used on different spots from the same tumors, r values are between 0.8 and 0.95. When the same antibody is used on different spots from the same tumors, r values are between 0.6 to 0.8. For related markers, like estrogen receptor and progesterone receptor in breast cancer, r values are typically between 0.3 and 0.4. A N-cadherin was inversely associated with P-cadherin expression by multivariate analysis (Pearson r = −0.15) in accord with previous findings (28). These data show a positive correlation among four epithelial markers in melanomas of all stages, with particularly strong coexpression of CD24 and P-cadherin, thus supporting the existence of epithelial-like melanoma in a large, unselected set of primary human tumors.

Discussion

Through a comprehensive analysis of a large panel of melanoma cell lines and cultured melanocytes, we have identified a form of melanoma not associated with ERK activation, as well as a list of genes that are downstream targets of pERK activation in melanoma. We were not, however, able to discern reproducibly a difference in the expression profile or related molecular characteristics of cell lines harboring N-RAS versus B-RAF mutations. This result was surprising as RAS is known to activate many non-RAF effector targets that regulate transcription factor function and cause changes in gene expression (e.g., PI3K-AKT and Ral-GDP; see ref. 4). Consequently, we expected that the gene expression profiles would differ between the N-RAS and B-RAF groups of cell lines. Perhaps, the lack of obvious difference between the two groups can

Figure 4. Validation of targets deregulated through the RAF-MEK-ERK pathway. A, expression levels of TWIST1 and COTL1 (coactosin L1) mRNA were determined by Taqman quantitative real-time PCR from three cell lines harboring B-RAF mutations (SKMEL28, WM2664, and SKMEL24) treated with U0126. The mean fold decrease in expression after 24 and 48 h of U0126 treatment compared with vehicle (DMSO) alone–treated cells. B, SKMEL28, WM2664, and SKMEL24 cells were treated with U0126 or DMSO alone for 24 or 48 h, and protein lysates were resolved and immunoblotted with the indicated antibodies. Transient MEK inhibition decreases expression of pERK, RhoE/Rnd3, and CDK6 but not MCAM.
Figure 5. Dendrogram of supervised analyses by pERK levels and U0126 treatment: After limiting the analysis to the 281 genes identified in Supplementary Table S4 that positively correlate with pERK levels, SAM was done on data from 14 arrays with and without U0126 treatment. Hierarchical clustering of the 90 transcripts (82 unique) that significantly decreased with U0126 treatment. For clarity, unknown and duplicate transcripts are omitted. The full gene set is shown in Supplementary Table S6.
be explained, in part, by concurrent activation of other RAS effector pathways by other genetic mechanisms in the B-RAF mutant cell lines. For example, PTEN tumor suppressor inactivation, a negative regulator of PI3K activity, was shown to frequently associate with melanomas harboring B-RAF mutations (43). Loss of PTEN results in activation of the PI3K-AKT pathway, whereas RAS directly binds and activates PI3K. Therefore, our inability to distinguish N-RAS from B-RAF mutant lines may reflect compensating genetic events not equally distributed between the groups of cell lines, such as PTEN loss. The similarity in gene expression between N-RAS and B-RAF mutant cell lines, however, emphasizes the marked distinctiveness of the epithelial-like melanomas.

By merging the lists of genes that correlate with high ERK activity across the panel of cell lines with those genes that are decreased by transient ERK inhibition, we identified 82 unique transcripts that are downstream of activated ERK (Fig. 5; Supplementary Table S6). This merged list contains genes of known pathologic significance in melanoma not previously recognized as ERK targets [e.g., TWIST1 (39), intercellular adhesion molecule-1 (44), and FGFR2 (37, 38)], known ERK targets not previously recognized to be of significance in melanoma (e.g., RhoE/Rnd3 and SPRY2), and known RAS-RAF-ERK targets relevant to melanoma biology (e.g., IL-8 and HIF1α). Perhaps of greatest interest, however, was the identification of several novel genes that are neither known ERK targets nor of established pathologic significance in melanoma. These included genes involved in actin organization and cell motility (e.g., PREX1 and COTL1), angiogenesis (e.g., angiomotin-like 1), and immune response (e.g., CD58 and CD200). Understanding whether any of these novel ERK targets are of pathologic relevance in melanoma is a present major focus of our research efforts.

One gene of this list in particular bears mention. Hock et al. (39) have shown previously that TWIST1 expression is associated with melanoma progression and correlates with worsened clinical outcome. We have confirmed and extended this observation by showing that TWIST1 expression is increased in most melanoma cell lines when compared with NHMs (Fig. 24), is inversely correlated with expression of E-cadherin and other epithelial markers (Fig. 2B), correlates with pERK levels (Supplementary Table S4), and is markedly down-regulated by ERK inhibition (Figs. 4B and 5). This finding is of interest, given the recent demonstration by Yang et al. (30) that TWIST1 expression is associated with features of EMT, loss of E-cadherin expression, and metastasis in a breast cancer model. Our data, therefore, support a hypothesis where N-RAS or B-RAF mutation may facilitate melanoma metastasis by inducing EMT through an ERK-mediated increase in TWIST1.

Whereas melanoma cell lines harboring mutant B-RAF or N-RAS showed several common features, elevated ERK and AKT activation and overlapping RNA expression profiles, the epithelial-like melanoma cell lines comprised a molecularly distinct subset. These lines showed several characteristics that are uncommon in classic melanoma: (a) decreased expression of MitF and MitF gene targets; (b) decreased ERK activation and decreased expression of ERK-mediated gene targets; (c) decreased levels of pAKT; (d) loss of p53 function; (e) increased expression of epithelial markers (e.g., P-cadherin, E-cadherin, keratins 8/18, and CD24); and (f) decreased expression of FGFR2 with increased expression of FGFR9/FGFR3. In aggregate, these data suggest that these epithelial-like lines represent a molecularly distinct and heretofore unappreciated subtype of melanoma. As pathologic misdiagnosis, contaminations, and mislabeling occur with passed human cell lines, we considered the possibility that these epithelial-like lines are not derived from melanomas but rather from some other type of cancer. This is unlikely for several reasons: (a) we have analyzed five independent lines from disparate institutions; (b) all of the WT lines expressed S100 and/or TYRP1, sensitive markers of melanoma and (c) a recent smaller microarray analysis using a different microarray platform on an independent set of cell lines found a highly similar pattern of gene expression in WT melanomas (ref. 32;
the gene signatures are compared in Supplementary Table S7). Therefore, the pattern of gene expression in the epithelial-like melanoma lines described by both groups seems generalizable and not the result of data overfitting or inadequate sampling.

This analysis does not allow us to determine the frequency or clinical characteristics of epithelial-like melanoma in vivo. If the tumor entity is defined as those which are p53 mutant, lack N-RAS or B-RAF mutation, and overexpress CD24 and P-cadherin, then several lines of evidence suggest that epithelial-like melanoma is not rare. For example, individual reports have shown that 5% to 20% of advanced human melanoma exhibit keratin 8/18 (45, 46) and E-cadherin/P-cadherin expression (28, 47) and p53 inactivation (3, 15–17), and lack RAS/RAF mutation (2, 3, 33, 43), although we are not aware of a prior correlation among these phenotypes. Moreover, in a large panel of primary human tumors, we noted a strong correlation in the expression of P-cadherin and CD24, with significant heterogeneity in the expression of these markers across the sample (Fig. 6A). Although it is not clear from our analysis where to choose a ‘cut point’ to consider a tumor ‘positive’ for CD24 by immunohistochemistry, it is worth noting that ~8% of tumors showed expression 2-fold greater than median. In aggregate, these observations suggest that epithelial-like melanomas are reasonably frequent (5–20%) in unselected primary tumors and that P-cadherin and/or CD24 may be useful immunohistochemical markers of this subtype.

Likewise, the prognostic implications of the epithelial-like subtype are not clear from this work, although several lines of published evidence suggest that it is a favorable subgroup compared with classic RAS/RAF mutant melanoma. First, P-cadherin and E-cadherin have both been correlated with improved outcome in melanoma (28, 47). Second, as mentioned, several of the ERK-dependent gene targets identified in this analysis, which are expressed at higher levels in the N-RAS/B-RAF mutant lines, are known prognostic markers of poor outcome in melanoma. Third, p53 overexpression (suggesting inactivating point mutation) has been correlated with favorable outcome in melanoma, whereas INK4a/ARF loss has been associated with worsened outcome (12, 48). Fourth, RAS/RAF mutational activation has been correlated with vertical growth and progression, and the frequency of RAS/RAF mutant tumors increases with progression (49).

It is therefore surprising that CK18 expression and CD24 expression did not correlate with improved univariate survival in the TMA data set (data not shown). In fact, cut points for considering a tumor CD24 positive could be chosen for which CD24 expression seemed to be an adverse marker. A potential explanation for this discrepancy is that CD24 might mark for an adverse form of the epithelial-like subtype: CD24 has been reported to facilitate metastasis and is a strong marker of adverse outcome in carcinomas (50). Likewise, P-cadherin, whose expression strongly correlates with CD24, only seems to be a marker of good prognosis in early-stage tumors (47). It is important to note, however, that the post-hoc selection of cut points in a quantitative TMA analysis is not statistically rigorous (51); therefore, the clinical significance of CD24 and CK18 expression in melanoma will need to be verified on an independent set of primary tumors. Additionally, it is worth noting that seven of the eight WT cell lines studied in this report or by Bloethner et al. (32) were derived from vertical growth or metastatic tumors. Therefore, these data establish that neither ERK activation nor EMT is required for metastasis in melanoma, although possibly both facilitate the process.

In summary, we have shown that ERK induces several genes involved in proliferation, growth, and motility in B-RAF/N-RAS mutation-positive melanoma, and many of these genes are associated with adverse outcome and vertical growth. A subset of these genes may serve as diagnostic markers that predict melanoma response to RAF and MEK inhibitor therapeutic approaches. We also have identified a novel subtype of melanoma that is characterized by p53 inactivation, lack of RAS/RAF mutation, and increased expression of epithelial markers that may permit their immunohistochemical detection (e.g., P-cadherin and CD24). This may identify a clinically distinct subset of melanoma that will require therapeutic strategies distinct from those being considered for RAS/RAF mutant melanomas.

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References


Lack of Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase Signaling Shows a New Type of Melanoma

Janiel M. Shields, Nancy E. Thomas, Melissa Cregger, et al.


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