Identification of a Novel Subgroup of Melanomas with KIT/Cyclin-Dependent Kinase-4 Overexpression

Keiran S.M. Smalley,1 Rooha Contractor,1 Thienngaa K. Nguyen,1 Min Xiao,1 Robin Edwards,2 Viswanathan Muthusamy,3 Alastair J. King,1 Keith T. Flaherty,3,4 Marcus Bosenberg,5 Meenhard Herlyn,1 and Katherine L. Nathanson1,5

1The Wistar Institute, 2Department of Pathology and Laboratory Medicine, 3Abramson Cancer Center, Divisions of Hematology-Oncology and Medical Genetics, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; 4Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont; and 5GlaxoSmithKline, Collegeville, Pennsylvania

Abstract

Although many melanomas harbor either activating mutations in BRAF or NRAS, there remains a substantial, yet little known, group of tumors without either mutation. Here, we used a genomic strategy to define a novel group of melanoma cell lines with co-overexpression of cyclin-dependent kinase 4 (CDK4) and KIT. Although this subgroup lacked any known KIT mutations, they had high phospho-KIT receptor expression, indicating receptor activity. Quantitative PCR confirmed the existence of a similar KIT/CDK4 subgroup in human melanoma samples. Pharmacologic studies showed the KIT/CDK4-overexpressing subgroup to be resistant to BRAF inhibitors but sensitive to imatinib in both in vitro and in vivo melanoma models. Mechanistically, imatinib treatment led to increased apoptosis and G1 phase cell cycle arrest associated with the inhibition of phospho-ERK and increased expression of p27KIP1. Other melanoma cell lines, which retained some KIT expression but lacked phospho-KIT, were not sensitive to imatinib, suggesting that KIT expression alone is not predictive of response. We suggest that co-overexpression of KIT/CDK4 is a potential mechanism of oncogenic transformation in some BRAF/NRAS wild-type melanomas. This group of melanomas may be a subpopulation for which imatinib or other KIT inhibitors may constitute optimal therapy. [Cancer Res 2008;68(14):5743–52]

Introduction

As melanoma treatment moves into the era of targeted therapy, there is a growing need to unravel the underlying genetic complexity and cellular signaling heterogeneity of this tumor. It is hoped that an understanding of how genetic and signaling profiles dictate pharmacologic response will allow for the selection of optimal patient populations for clinical trials. Following the discovery that many melanomas harbor activating mutations in either BRAF or NRAS, there has been much interest in targeting the BRAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway (1, 2). Although it has been shown that the presence of the BRAF mutation is predictive of response to MEK inhibitors (3), this is unlikely to be the only determinant (4, 5). In a recent study, we showed that there was little correlation between the inhibition of phospho-ERK levels and of melanoma cell line growth following MEK inhibitor treatment (6). This observation suggested that there were likely to be other changes in the pathways responsible for cell proliferation that may be more predictive of response to these small molecule inhibitors when identified. In the current study, we have turned our attention to melanoma lines that respond poorly to BRAF inhibitors. Entry into the cell cycle is regulated at the G1 restriction checkpoint, a process that becomes deregulated in cancer cells. Progression through the G1 into S phase of the cell cycle is driven by cyclin-dependent kinases (CDK) 4 and 6, which interact with cyclin D1, as well as by CDK2, which interacts with cyclins A/E (7). Uncontrolled growth of melanoma cells results from constitutive MAPK activity leading to increased cyclin D1 and reduced p27KIP1 expression (8).

There seem to be differences in the genetic profiles of melanomas that originate from skin that is either chronically sun-damaged (as defined by the appearance of solar elastosis) or skin that lacks sun-induced damage. Thus, melanomas that arise on skin with chronic sun-induced damage have a low incidence of BRAF mutations and instead showed increased cyclin D1 copy number. Frequent amplifications of cyclin D1 also occur in distinct histologic subtypes of melanoma. Thus, 44% of acral melanomas, 19% of lentigo malignant melanomas, and 6% of superficial spreading melanomas are known to have increased cyclin D1 copy number (9). There is also evidence for amplification (10) and mutation of CDK4 (11) in small subgroups of melanomas (12). Other distinct subgroups of melanoma have been shown to harbor oncogenic mutations in the receptor tyrosine kinase KIT (13). Again, these aberrations are restricted to certain groups of melanoma, with KIT dysregulation being reported in 36% of acral melanomas and 28% of melanomas arising on chronically sun-damaged skin (13). There are no cell lines derived from these rare melanomas, making it difficult to perform the preclinical studies essential for guiding clinical trial design.

The current study has identified a novel subset of BRAF inhibitor–resistant melanoma cell lines with high expression of both KIT and CDK4. This subgroup of melanomas lack KIT mutations, but have high KIT signaling activity and show sensitivity to imatinib treatment. Similar patterns of CDK4/KIT expression were also found in clinical melanoma specimens. We therefore suggest that pharmacogenomic analysis of melanoma populations may be a suitable strategy for the further subclassification of melanoma leading to more “personalized” therapy approaches.

Materials and Methods

Cell culture. Human melanoma cells and melanocytes were isolated and cultured as described in ref. 14. The lentiviral vector shRNA constructs for
KIT were obtained from Dr. Levi Garraway (the Broad Institute, Cambridge, MA). The WM1382 cell line was derived from a superficial spreading melanoma. Histopathologic data was not available for the WM8 line.

**Adherent cell proliferation analysis.** Cells were plated into a 96-well plate at a density of \(2.5 \times 10^5\) cells/ml and left to grow overnight. Cells were treated with increasing concentrations of SB590885 (GlaxoSmithKline), or imatinib mesylate (Hospital of the University of Pennsylvania) in triplicate. After 72 h, the levels of growth inhibition were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (6). Data show the mean of at least three independent experiments ± SE.

**Western blot analysis.** Proteins were extracted and blotted as described in ref. 14. After analysis, Western blots were stripped once and reprobed for β-actin to show even protein loading. Antibodies to phospho-ERK, total-ERK, phospho-KIT, and total KIT were from Cell Signaling Technology, the antibody to CDK4 was from Fisher Scientific.

**Three-dimensional spheroid growth.** Melanoma spheroids were prepared using the liquid overlay method. Briefly, 200 μL of melanoma cells (25,000 cells/mL) were added to a 96-well plate coated with 1.5% agar (Difco). Plates were left to incubate for 72 h, by which time cells had organized into three-dimensional spheroids. Spheroids were then harvested using a P1000 pipette. The medium was removed and the spheroids were implanted into a gel of bovine collagen I containing EMEM, 1-glutamine, and 2% fetal bovine serum. Normal 2% melanoma medium was overlaid on top of the solidified collagen. Spheroids were treated with either 3 or 10 μmol/L of imatinib before being left to grow for 72 h. Spheroids were then washed twice in PBS before being treated with calcein-AM and ethidium bromide (Molecular Probes) for 1 h at 37°C, according to the manufacturer's instructions. After this time, pictures of the invading spheroids were taken using a Nikon-300 inverted fluorescence microscope.

**In vivo melanoma xenograft studies.** The study protocol was approved by the Wistar Institute Animal Care and Use Committee. Each group consisted of five severe combined immunodeficient (SCID) CB-17 mice (Charles River Laboratories). Ten mice were injected s.c. with WM1382 cells (\(2 \times 10^6\)) in Matrigel into the lower back. When animals had developed melanoma nodules of \(\geq 5\) mm in diameter, the study drug administration was initiated (day 1). The SCID mice were randomly assigned to two experimental groups of five animals each: (a) 200 μL vehicle (distilled water), and (b) 100 mg/kg imatinib mesylate (in 200 μL distilled water) twice daily by oral gavage over a period of 14 days. Tumors were measured twice a week using digital calipers. Tumor volume was calculated as a product of the three dimensions. Tumor shrinkage was calculated as a fold.
change relative to the starting volume. At treatment day 14, 1 h after the final drug application, all animals were euthanized.

For the specimens analyzed in Fig. 3 and Supplemental Table S1, 17 melanoma tumor samples were collected from 15 patients as described in ref. 10. The sample set was derived following the previous identification of high CDK4 overexpression in three of the samples (10). One of the samples represented subungual primary melanoma (MMF). Specimens MMH and WM1382 were derived from clinically apparent metastases from either superficial spreading or nodular primary melanomas. No mucosal melanomas were analyzed, and no acral melanomas aside from the subungual cases listed above were analyzed. No lentigo maligna melanomas or desmoplastic/spreading or nodular primary melanomas. No mucosal melanomas were analyzed, and no acral melanomas aside from the subungual cases listed above were analyzed. No lentigo maligna melanomas or desmoplastic/spreading or nodular primary melanomas.

**Melanoma tumor samples.** Melanoma tissue samples were collected according to institutional review board–approved protocols in compliance with Health Insurance Portability and Accountability Act guidelines at the Memorial Sloan-Kettering Cancer Center (New York, NY), Dana-Farber Cancer Institute (Boston, MA), and University of Vermont (Burlington, VT). For the specimens analyzed in Fig. 3C and Supplemental Table S1, 17 melanoma tumor samples were isolated from 15 patients as described in ref. 10. The sample set was derived following the previous identification of high CDK4 overexpression in three of the samples (10). One of the samples represented subungual primary melanoma (MMF). Specimens MMH and MMI were synchronous antecubital and axillary metastases from the same patient with primary melanoma MMF. All the remaining tumor samples were derived from clinically apparent metastases from either superficial spreading or nodular primary melanomas. No mucosal melanomas were analyzed, and no acral melanomas aside from the subungual cases listed above were analyzed. No lentigo maligna melanomas or desmoplastic/neurotropic melanomas were analyzed. All tumor specimens were collected immediately after surgical excision and were rapidly frozen in optimal cutting temperature compound and left to grow overnight before being treated with either SB590885 (1 μmol/L), or U0126 (10 μmol/L, Sigma) for 24 h, or with imatinib (3–10 μmol/L) for 24 to 48 h. Cells were analyzed as previously described (6).

**Cell cycle analysis.** Cells were plated into 10-cm dishes at 60% confluency and left to grow overnight before being treated with either SB590885 (1 μmol/L), or U0126 (10 μmol/L, Sigma) for 24 h, or with imatinib (3–10 μmol/L) for 24 to 48 h. Cells were analyzed as previously described (6).

**Table 1. BRAF, NRAS, and KIT mutational status of the melanoma cell line panel**

<table>
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<th>Cell line</th>
<th>BRAF</th>
<th>NRAS</th>
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<th>KIT Ex 13</th>
<th>KIT Ex 14</th>
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Abbreviation: WT, wild-type.

**Quantitative PCR on melanoma lesions.** RNA was isolated from microdissected tumor samples using the RNeasy Plus kit (Qiagen) according to the manufacturer's recommendations. One microgram of total RNA was used in a reverse transcription reaction using the RETROscript kit (Ambion) according to the manufacturer's recommendations. Real-time quantitative PCR was performed by using 2 μL of 100-fold diluted cDNA template and 0.2 μmol/L of gene-specific primers against KIT (forward-5’ TCA TTG AGA GTT TTG TCT TGG A 3’ and reverse-5’ ACT TAC GCC GCT TAT GTA TT A 3’) in a 25 μL PCR reaction using JumpStart SYBR green kit (Sigma) according to the manufacturer’s instructions in an ABI 7700. The reactions were performed in duplicate, dissociation curve analysis was performed to ensure a single product, the CT values obtained were normalized to glyceraldehyde-3-phosphate dehydrogenase levels and quantification was performed using the comparative CT method.

**Stem cell factor ELISA.** Cells were cultured for 24 h in serum-free tumor medium. Supernatants were harvested and measured using a commercially available stem cell factor (SCF) ELISA kit (R&D Systems). As a positive control, we used conditioned medium from a human skin fibroblast line transduced with a lentiviral vector SCF construct.

**Expression profiling data.** Melanoma samples were prepared for analysis on the Affymetrix U133A array platform (16). Cell lines represented in Figs. 1A and 3A are (in order): FOM103, FOM99, FOM1041, FOM1131, WM1321, WM9833A, WM239A, WM88, WM51, WM3268V, WM1799, WM1727A, WM858, WM983B, WM983C, WM1361B, 1205Lu, WM902B, WM46, WM75, WM164, WM164, WM39, WM35, WM3248, WM793, WM278, WM8, WM1382, WM3211, WM361A, WM1366, and WM1346. The data generated from these arrays has been published previously (16, 17) and have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus web site.8 Data are accessible using Gene Expression Omnibus Series accession GSE4845. Analysis was performed using GeneSpring software, in which the data from three V600E mutated melanoma cell lines (WM35, WM793, and 1205Lu) were compared with that from the WM8 and WM1382 melanoma cell lines.

**Mutational testing.** Exons 11 and 15 of BRAF and exon 3 of NRAS were screened for mutations by PCR-based sequencing as previously described (18, 19). Mutation screening of KIT for exons 11, 13, and 17 was performed as described in ref. 13. Mutation screening of exon 9 was performed with primers and conditions described in ref. 20. Primers were designed using the Primer3 software9 for exons 14 F 5’ gccttgattgcaaaccctta and R 5’ gcttgatgtagcaacccctta, and 18 F 5’ catttcagcaacagcagcat and R 5’ caagaagaagaccagccata. PCR was carried out for 35 cycles (30 s at 95°C, 30 s at 56°C, and 60 s at 72°C) on a PCRExpress Thermo Hybaid thermal cycler.

**Statistical analysis.** Unless otherwise stated, all experiments show the mean ± SE of at least three independent experiments. Statistical significance was measured using Student’s t test, in which P < 0.05 was judged to be significant.

**Results**

**Identification of melanoma cell lines with high CDK4 expression.** Initial screening of our microarray data identified a panel of melanoma cell lines that were wild-type for BRAF/NRAS with increased expression of CDK4 (Fig. 1A). From these data, we identified two cell lines, WM8 and WM1382, with increased levels...
of CDK4. These cell lines harbored neither a BRAF nor an NRAS mutation (Table 1).

Consistent with the lack of BRAF/NRAS mutations, both the WM8 and WM1382 cell lines expressed only low levels of phospho-ERK in Western blotting experiments (Fig. 1B). However, further exposure of the blots to film did reveal the presence of some basal phospho-ERK in these lines (data not shown). Increased expression of CDK4 was confirmed in the WM8 and WM1382 cell lines at the protein level (Fig. 1B), and was also correlated with an increase in cyclin D1 expression.

**CDK4-overexpressing melanoma lines are resistant to the BRAF inhibitor SB590885.** To determine whether CDK4 overexpression led to BRAF inhibitor resistance, a panel of melanoma cell lines were treated with increasing concentrations of the BRAF inhibitor SB590885 (Fig. 2A; ref. 21). Compared with three melanoma cell lines with BRAF V600E mutations (1205Lu, WM35, and WM983B), the two CDK4-overexpressing lines were highly resistant to the BRAF inhibitor SB590885 (Fig. 2A; ref. 21). Similarly, SB590885 had little effect on the cell cycle profile of the WM1382 cells but induced a profound G1 arrest in the BRAF-mutated 1205Lu cells (Fig. 2B). Interestingly, the U0126 did induce some cell cycle arrest in the WM1382 cells, suggesting that these cells may have low basal phospho-ERK activity that could be responsible for cell cycle entry (Fig. 2B).

**CDK4-overexpressing melanomas retain constitutive KIT expression.** As melanoma lines that overexpress CDK4 are resistant to BRAF inhibitors, we next attempted to identify novel therapeutic targets in the BRAF inhibitor–resistant subgroup of melanomas. To address this issue, we performed a microarray analysis comparison between three BRAF–mutated melanoma cell lines (WM35, WM793, and 1205Lu) and two CDK4-overexpressing lines (WM8 and WM1382). The analysis was designed to identify any gene with a 3-fold or greater expression in the CDK4-overexpressing population. The gene with the highest fold upregulation in the CDK4 population was the receptor tyrosine kinase KIT (859-fold higher in the CDK4-overexpressing cell lines).

Stratification of KIT expression according to mutational status (melanocyte, BRAF V600E–mutated melanoma, NRAS/BRAF wild-type melanoma, or NRAS-mutated melanoma) revealed the upregulation of KIT mRNA expression in the BRAF/NRAS wild-type melanoma panel (Fig. 3A). The cell lines that we identified (WM8 and WM1382) were the same two lines with high CDK4 expression.

![Figure 2. The CDK4-overexpressing melanoma cell lines are resistant to the antiproliferative effects of the BRAF inhibitor SB590885. A, the CDK4-overexpressing melanoma lines are resistant to SB590885 in an MTT assay. Cells were treated with increasing concentrations of SB590885 (1 nmol/L–10 μmol/L) for 72 h before being treated with MTT. B, SB590885 preferentially reduces S phase entry in BRAF V600E–mutated melanoma cell lines. The BRAF V600E–mutated cell line (1205Lu) and the CDK4-overexpressing cell line (WM1382) were treated with U0126 (10 μmol/L) or SB590885 (1 μmol/L) for 24 h, before being fixed, stained with propidium iodide, and analyzed using flow cytometry.](https://www.aacrjournals.org/cancerres/article-pdf/68/14/5746/5263517/5746.pdf)
It was also noted that there were several BRAF V600E–mutated melanomas that also maintained some KIT expression (Fig. 3A; data not shown).

Western blotting revealed that KIT was highly expressed in the CDK4-overexpressing melanoma cell lines at the protein level and the high levels of phospho-KIT seen indicated that the receptor was constitutively active (Fig. 3B). Expression of KIT was also maintained in a subgroup of melanoma cell lines (WM39, WM46, WM902B, and SK-MEL-28) that harbored the BRAF V600E mutation (Fig. 3B). However, in this instance, the receptor was not constitutively active, as shown by the lack of phospho-KIT expression. To investigate the potential mechanism of the KIT receptor activation, we performed an ELISA experiment looking for secretion of SCF (Supplemental Fig. S1) and noted a lack of SCF.

Figure 3. CDK4-overexpressing melanomas also show high KIT expression. A, CDK4-overexpressing melanoma lines have higher KIT mRNA expression. Microarray analysis showing KIT mRNA expression in a panel of primary human melanocytes (mel), V600E-mutated melanoma cell lines (BRAF V600E), NRAS/BRAF wild-type melanomas and NRAS-mutated melanomas (NRAS). B, CDK4-overexpressing melanoma cell lines have high KIT expression and phospho-KIT activity. Left, protein expression of total KIT (tKIT) and phospho-KIT (pKIT) receptor across a panel of human melanocytes (mel), and human melanoma cell lines (WM35, WM793, 1205Lu, C8161, WM164, 451Lu, WM983A, WM983B, WM8, and WM1382). Right, expression of KIT in melanoma lines harboring the BRAF V600E mutation. Western blots showing the expression of total KIT and phospho-c-KIT across a panel of melanoma cell lines (WM39, WM46, WM902B, and SK-MEL-28) harboring the BRAF mutation. C, human melanoma samples with increased CDK4 expression also have higher KIT expression. Data show quantitative reverse transcription-PCR results for KIT expression in 14 melanoma samples without CDK4 amplification (Control) or in 3 samples with CDK4 amplification confirmed by array CGH (CDK4). Data shown are −log2 values normalized to KIT expression in normal human melanocytes (defined as zero). Bars, mean KIT expression values for the control (non–CDK-amplified) and CDK4-amplified groups.
Figure 4. CDK4/KIT-overexpressing melanomas are sensitive to imatinib treatment. A, CDK4/KIT-overexpressing cells were sensitive to the growth-inhibitory effects of imatinib in an MTT assay. Cells were treated with increasing concentrations of imatinib (10 nmoL–10 μm/L) for 72 h before being treated with MTT. Absorbances were read at 570 nm and expressed as a percentage of control absorbance. Points, means of three independent experiments; bars, SE. Only the WM8 and WM1382 cell lines were sensitive to imatinib treatment. B, imatinib reduces the viability and survival of melanoma cells grown as three-dimensional collagen-implanted spheroids. Preformed spheroids that either overexpressed CDK4 (WM1382) or harbored the BRAF V600E mutation (1205Lu) were embedded into collagen and overlaid with medium. Cells were then treated with imatinib (3 and 10 nmoL/L) for 72 h, before being treated with calcein-AM and propidium iodide. Green, viable cells; red, dead cells. Lack of green staining also indicates loss of viability. C, imatinib treatment induces the regression of established CDK4/KIT melanoma xenografts. CDK4/KIT-overexpressing (WM1382) cells were grown as tumor xenografts in SCID mice. After tumor establishment, mice were dosed twice daily with either vehicle (distilled water) or imatinib mesylate (100 mg/kg in distilled water) by oral gavage for 14 d. Tumor volumes were measured twice a week. Left, photographs of representative vehicle and imatinib-treated tumors taken after day 14 of treatment. Right, growth curves were normalized to the start volumes. Imatinib treatment led to significant regression of the established xenografts.
secretion from any of the melanoma cell lines tested, including the WM8 and WM1382 cell lines.

Certain subgroups of mucosal and acral lentiginous melanomas harbor activating mutations in KIT (13). Mutational analysis testing of KIT at the known mutation hotspots of exons 11, 13, 14, 17, and 18 revealed that the WM8 and WM1382 cell lines were wild-type (Table 1). A recent study from our group identified a high amplification of CDK4 in a series of uncultured melanoma samples (10). Further study of 17 of these samples showed an increase in KIT expression in the 3 samples with CDK4 amplification compared with the nonamplified group (Fig. 3C; Supplemental Table S1). Similar to the CDK4/KIT-amplified melanoma cell lines, these three samples were also negative for both BRAF and NRAS mutations (Supplemental Table S1).

Inhibition of KIT following the use of imatinib has selective antitumor effects on the CDK4/KIT-overexpressing melanoma cell lines. The high expression of phospho-KIT in the CDK4-overexpressing melanoma lines suggests that KIT activity is essential for their proliferation and survival. Imatinib is a receptor tyrosine kinase inhibitor which selectively blocks the activity of Bcr-Abl, the platelet-derived growth factor receptor (PDGFR), and KIT (22). In a series of MTT assays, it was found that the two CDK4/KIT-overexpressing melanoma lines were markedly growth-inhibited following 72 hours of drug treatment with imatinib.
The panel of melanoma cell lines that harbored the BRAF V600E mutation in the absence of any significant KIT expression (WM793, 1205Lu, WM983B, and WM35) were resistant to the effects of imatinib and showed very little growth inhibition (Fig. 4A). Likewise, the melanoma cell lines with a BRAF V600E mutation that also maintained KIT expression (SK-Mel-28, WM39, WM46, WM902B) were also resistant to imatinib (Fig. 4A).

Our previous studies have indicated that melanoma cell lines that are sensitive to targeted therapy agents in two-dimensional monolayer cultures often become highly drug-resistant when grown as three-dimensional collagen-implanted spheroids (5). Here, we show that the CDK4-overexpressing WM1382 melanoma cell line is highly sensitive to imatinib under three-dimensional culture conditions (Fig. 4B). Treatment of the WM1382 spheroids with imatinib (3 and 10 μmol/L) for 72 hours was associated with reduced cell invasion into the surrounding collagen as well as marked decreases in cell viability.

Next, we grew the WM1382 cells as tumor xenografts in SCID mice. After tumor establishment (5 × 5 mm), mice were dosed twice daily with either vehicle (distilled water) or imatinib mesylate (100 mg/kg in distilled water) by oral gavage. After 14 days, it was found that imatinib treatment had suppressed tumor growth and led to a significant level of regression (Fig. 4C), demonstrating the utility of imatinib treatment in CDK4/KIT-overexpressing melanomas.

Imatinib blocks the proliferation of CDK4-overexpressing melanoma cells through the inhibition of KIT-mediated MAPK signaling. Next, we turned our attention to the mechanism of action of imatinib in our CDK4/KIT-overexpressing melanoma population. Treatment of the BRAF V600E–mutated 1205Lu cells with imatinib had very little effect on the cell cycle profile and did not induce any apoptosis (Fig. 5A). Increasing concentrations of imatinib (3 and 10 μmol/L) induced a G1 phase cell cycle arrest and some apoptosis in the CDK4-overexpressing WM1382 cells, with 27% apoptosis being induced following treatment with 10 μmol/L of imatinib (Fig. 5A).

Treatment of the WM1382 cells with imatinib led to a progressive reduction in the level of KIT receptor activity, as shown by reduced phospho-KIT expression (Fig. 5B). At the same time, imatinib also inhibited the low basal level of phospho-ERK activity in the WM1382 cells, indicating that imatinib blocked the MAPK pathway of the CDK4/KIT-expressing cells. Consistent with the ability of imatinib to block MAPK signaling, it was found that drug treatment also increased the expression of the CDK inhibitor p27KIP1 (Fig. 5B). To show the ability of the KIT receptor to stimulate phospho-ERK signaling, we treated primary human melanocytes with the KIT ligand for increasing periods of time (0–30 minutes) and showed a robust increase in phospho-ERK signaling (Supplemental Fig. S2).

Imatinib inhibits the activity of multiple receptor tyrosine kinases. To determine whether the specific effects of imatinib were mediated through KIT inhibition, we generated lentiviral shRNAs against KIT that produced effective knockdown (>85%) of protein levels (Fig. 5C). Infection of the WM8 cell line with the KIT shRNA led to very high levels of apoptosis (data not shown), and significantly reduced the growth of WM1382 cell lines (Fig. 5D), confirming the role of KIT in the survival and growth of CDK4-overexpressing melanoma lines.

Discussion

The past 2 years have seen progress in defining new subcategories of melanoma based on patterns of gene amplification and mutation (3, 12, 13). How these different mutational profiles and molecular subgroupings can be translated into novel strategies for treatment has barely been explored. Our initial screen of a panel of early passage melanomas identified two cell lines with very high expression of CDK4 that lacked BRAF and NRAS mutations. Pharmacologic studies showed that the CDK4-overexpressing melanoma lines were resistant to BRAF inhibition. Typically, BRAF inhibitors block cell cycle entry through the increase of cyclin D1 expression and through the suppression of the CDK inhibitor p27KIP1 (5, 8). As the CDK4-overexpressing melanomas have much lower phospho-ERK levels and higher baseline expression of both CDK4 and cyclin D1, high activity in the MAPK pathway seemed to be less critical for growth in these lines.

We next used existing microarray data to identify KIT expression as a possible pharmacologic target within the CDK4-overexpressing subgroup. The receptor tyrosine kinase KIT is a critical regulator of growth, differentiation, migration, and proliferation in the hematopoietic, germ cell, and melanocytic systems (23, 24). Functional KIT/SCF signaling is essential for melanocyte development and plays an important role in pigmentation. Thus, dysfunctional KIT signaling is associated with pigmentary defects, resulting from impaired melanocyte survival and migration (25).

Activating mutations in KIT are well described in cancer and are implicated in the development of gastrointestinal stromal tumors, some forms of leukemia (acute myelogenous leukemia), and testicular seminomas (26). The role of KIT in melanoma is more complex, with a number of studies demonstrating the loss of receptor expression during tumor progression (27). In some melanoma cell lines, the forced overexpression of KIT leads to the induction of apoptosis (28). The recent years have seen renewed interest in the role of KIT in melanoma following the work by Bastian and colleagues identifying activating KIT mutations in defined histologic subsets of melanomas (13).

A number of small-molecule receptor tyrosine kinase inhibitors have been developed that target KIT activity, the best studied of which being imatinib mesylate (Gleevec), a receptor tyrosine kinase inhibitor with activity against Bcr-Abl, PDGFR, and KIT (22, 29). Although imatinib is now routinely used in the treatment of patients with chronic myeloid leukemia and gastrointestinal stromal tumor, its activity in melanoma has been very disappointing (30). Recent phase II clinical trials of imatinib in patients with metastatic melanoma, uns selected with regard to KIT mutation or amplification, revealed no objective responses, poor survival rates, and significant toxicity (30). Expression of KIT alone across our cell line panel was not indicative of an imatinib response. Of the cell lines tested, only the BRAF/NRAS wild-type cell lines with CDK4/KIT overexpression showed any antiproliferative response following imatinib treatment. Responsive cell lines were found to have high phospho-KIT expression, whereas the nonresponding lines lacked any phospho-KIT. We therefore suggest that the presence of KIT alone is not necessarily predictive of response to imatinib therapy.

It is unclear at this juncture how the high phospho-KIT activity is maintained in the CDK4-overexpressing melanoma cell lines. Neither of the CDK4-overexpressing cell lines were found to harbor activating KIT mutations at known mutational hotspots, nor did any of these lines secrete autocrine SCF. One possible explanation for the constitutive activity of KIT in this system comes from studies on epidermal growth factor receptor signaling, which have shown that increased receptor expression through gene amplification/overexpression could also lead to increased signaling activity (31). Indeed, there is evidence that overexpression of
epidermal growth factor receptor is predictive of response to epidermal growth factor inhibitors in non–small cell lung carcinoma (32, 33).

Imatinib is known to inhibit the activity of at least three receptor tyrosine kinases, all of which are expressed in melanoma (34). Functional studies revealed that imatinib treatment led to reduced S phase entry and apoptosis in the CDK4/KIT-overexpressing melanoma line WM1382. To determine whether these effects were through the inhibition of KIT rather than Bcr-Abl and/or PDGFR, we stably knocked down KIT expression in the WM8 and WM1382 cell lines using a lentiviral shRNA construct. We found that KIT knockdown markedly reduced the growth of WM1382 and led to a total loss of cell viability in the WM8 line.

Having shown that the CDK4/KIT-overexpressing melanoma lines possess a limited amount of phospho-ERK activity and could be partly growth arrested, we next investigated whether KIT signaling regulated MAPK signaling in these cell lines. Studies of melanocytes showed that SCF treatment led to a robust increase in phospho-ERK expression, demonstrating that the receptor can activate this pathway. In CDK4-overexpressing melanoma cells, treatment with increasing concentrations of imatinib led to a progressive decrease in phospho-KIT and phospho-ERK. Treatment of the CDK4-overexpressing cells with imatinib was also found to increase p27(kip1) expression, suggesting that imatinib was working primarily in these cells through inhibition of the MAPK pathway (3, 8). This finding raises the intriguing possibility that all melanomas rely on MAPK signaling activity, even in the absence of BRAF and NRAS mutations, suggesting that MAPK inhibition needs to be part of any optimized future melanoma treatment strategy.

These findings lead us to propose an alternative model for melanoma progression on a BRAF/NRAS wild-type background whereby a limited MAPK signaling via KIT can cooperate with increased CDK4/cyclin D1 to drive cell cycle entry. We hypothesize that the combination of overexpression of CDK4 and KIT may play a similar role to the high MAPK activity driven through either an that the combination of KIT/CDK4 is sufficient to fully transform human melanocytes. Although there is evidence of increased melanoma formation in CDK4 R24C knock-in mice, there is also a requirement for other factors (35). Most published studies suggest that inactivation of the p53 pathway and the INK4A/retinoblastoma protein axis are also required to achieve full oncogenic transformation (36, 37). Indeed, previous work from our own group suggests that CDK4 and MDM2 amplifications occur in parallel (10).

The current study has identified a new panel of melanomas that lack BRAF/NRAS mutations and instead have coamplification of CDK4 and KIT. Unlike previously identified subgroups of acral and mucosal melanomas, this novel group was discovered in metastases from superficial spreading and nodular melanomas, not in mucosal or acral lentigious melanomas that have higher rates of KIT amplification. As this is not an exhaustive pathologic study, it is currently difficult to judge the prevalence of this genetic profile across the whole melanoma population. Based on our data, we suggest that melanomas with KIT/CDK4 overexpression may also be suitable for imatinib treatment. It is hoped that the continued molecular subclassification of melanoma will lead to the identification of focused patient groups with the best likelihood of clinical response to defined agents.

Disclosure of Potential Conflicts of Interest

A.J. King: Employment, GlaxoSmithKline. The other authors disclosed no potential conflicts of interest.

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References

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