Imatinib Targeting of KIT-Mutant Oncoprotein in Melanoma

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Abstract

Purpose: Melanoma subtypes based on anatomic location and UV light exposure can be further classified based on genetic alterations recently identified. Mutations and gene amplification in *KIT* have been described in a significant percentage of mucosal and acral melanomas. We recently reported a patient with metastatic mucosal melanoma harboring a known *KIT* mutation treated with imatinib mesylate who experienced a major response. Biological effects of KIT inhibition in these melanomas remain poorly understood. We sought to investigate further the effects of imatinib in these melanoma subsets.

Experimental Design: Mucosal melanoma cells were analyzed for *KIT* aberrations by genomic sequencing, quantitative PCR, and single nucleotide polymorphism analyses. Imatinib effects were assayed by viability measurements and apoptotic cytotoxicity. Tumor cell lysates were assayed by Western blots to determine effects on multiple signaling pathways after imatinib exposure.

Results: Mucosal melanoma cells exhibited imatinib sensitivity correlating with *KIT* mutational status. Imatinib dramatically decreased proliferation and was cytotoxic to a *KIT* mutated and amplified cell culture. Exposure to drug affected the mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT, JAK-STAT, and antiapoptotic pathways.

Conclusions: Rational targeting of KIT in melanoma offers a unique and potent clinical opportunity. *In vitro* analyses revealed major sensitivity to KIT kinase inhibition by imatinib, with potent induction of melanoma cell apoptosis. Biochemical studies identified changes in signaling molecules regulating proliferation and survival responses, which may serve as mediators and/or biomarkers of *in vivo* treatment efficacy. Pathways affected by KIT inhibition provide a model for understanding components in effective melanoma cell death and insights into targeting for resistance mechanisms.

Melanomas arising from mucosal surfaces and from the palms, soles, and nailbeds do not result from the usual risk factors of sun exposure and family history (1). Few effective treatment options exist for patients who develop metastatic disease. Mucosal melanomas are rare and can arise in the sinuses, oropharynx, vagina, and anal regions (1). Acral melanomas (~5% of all melanomas) arise on non-hair-containing surfaces, such as palms, soles, and nailbeds (2). Given their unique distribution and similar incidence across races, mucosal and acral melanomas have been shown to harbor different genetic alterations and biological behavior compared with more common cutaneous melanomas (3, 4). Nonetheless, common with all subtypes of melanoma, few effective treatment options exist for patients who develop metastatic disease.

Recently, gain-of-function *KIT* mutations were reported in 21% of mucosal melanomas, 11% of acral melanomas, and 16.7% of melanomas arising in chronically sun-damaged skin as indicated by the presence of solar elastosis (3). Other cases showed increased *KIT* copy number or amplification. In a separate report, 15% of anal melanomas harbored a *KIT* oncogenic mutation (5). Most mutations affect the juxtamembrane region of the KIT protein, which predicts responsiveness to imatinib mesylate (6).

The receptor tyrosine kinase KIT acts on a downstream signaling cascade leading to key intracellular signals controlling cellular proliferation and survival (7, 8). Oncogenic *KIT* mutations resulting in ligand-independent kinase activity have been reported in 75% to 80% of gastrointestinal stromal tumors (GIST; ref. 9). Imatinib mesylate inhibits enzymatic activity of several tyrosine kinases including KIT and the platelet-derived growth factor receptors. Importantly, clinical

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Translational Relevance

Melanoma is a historically chemotherapy- and radiation-resistant tumor. Mucosal melanomas specifically are believed to be highly resistant to treatments, with no standard therapies available. Recent identification of *KIT* mutations and amplification in this patient population has offered promise for clinical investigation with known KIT-targeting kinase inhibitors. Recently, we reported a patient treated with imatinib for metastatic, mucosal, *KIT*-mutant melanoma, who experienced rapid and profound clinical improvement and marked response by positron emission tomography/computed tomography at 4 weeks. The present study of mucosal melanoma cell cultures reveals major sensitivity to KIT kinase inhibition by imatinib, with potent induction of melanoma cell apoptosis, and effects on multiple key signaling pathways. This delineates the possible oncogene addiction concept in this disease while serving as a foundation for studying key downstream signaling events for clinical translation. This investigation offers insight into understanding the necessary molecular components of effective melanoma cell death as well as provides a platform to study for resistance mechanisms. Such improved appreciation in melanoma provides a basis for rational therapeutics.

studies have shown stable or responding disease in 75% to 90% of patients receiving imatinib for metastatic or inoperable GIST (10–12). Imatinib mesylate also inhibits the ABL tyrosine kinase and suppresses BCR-ABL-dependent cell proliferation in chronic myelogenous leukemia by competitively inhibiting ATP binding to ABL at submicromolar concentrations (13, 14). Clinical response rates approach 100% for patients in chronic phase chronic myelogenous leukemia.

Previous clinical trials testing the efficacy of single-agent imatinib for the treatment of melanoma were disappointing. Two phase II trials did not reveal objective responses in any of 41 patients (15–17). The authors concluded that imatinib had no therapeutic effect in melanoma and should no longer be investigated. However, prompted by the recent discovery of *KIT* aberrations in mucosal melanoma, and prior clinical success of imatinib therapy in GIST patients with similar *KIT* mutations, we sought to investigate imatinib in mucosal melanoma cells, particularly those containing mutated KIT. Recently, we reported a patient treated with imatinib for metastatic, mucosal, *KIT*-mutant melanoma, who experienced rapid and profound clinical improvement and marked response by positron emission tomography/computed tomography at 4 weeks (18). To better understand the biological effects of tyrosine kinase inhibition as a targeted therapeutic for this melanoma subtype, we further studied the effects of imatinib on mucosal melanoma cell cultures derived from patient metastases.

Materials and Methods

Genotyping. DNA was extracted and purified as described previously (19). *KIT* exons were amplified by genomic PCR and screened for mutations by denaturing high-performance liquid chromatography (Transgenomic WAVE System) as reported (19). Suspected mutations were confirmed by direct bidirectional DNA sequencing on an ABI 310 using the Big-Dye terminator method. PCR primers for *KIT*, *BRAF*, and *NRAS* mutational analyses were *KIT* exon 9 forward GCTGAGGTTTTC-CAGCACTC and reverse AATTGCAGTCCTTCCCCTCT, *KIT* exon 11 forward CCAGAGTGCTCTAATGACTG and reverse ACCCAAAAAGGT-GACATGGA, *KIT* exon 13 forward CATCAGTTTGCCAGTTGTGC and reverse ACACGGCTTTACCTCCAATG, *KIT* exon 17 forward TGTATT-CACAGAGACTTGGC and reverse GGATTTACATTATGAAAGTCACAGG, *BRAF* exon 11 forward TCTGTTTGGCTTGACTTGACTT and reverse CGAACAGTGAATATTTCCTTTGAT, *BRAF* exon 15 forward TGCTTG-CTCTGATAGGAAAATG and reverse AGCATCTCAGGGCCAAAAAT, *NRAS* exon 1 forward CACTAGGGTTTTCATTTCCATTG and reverse TCCTTTAATACAGAATATGGGTAAAGA, and *NRAS* exon 2 forward AATTGAACTTCCCTCCCTCC and reverse TGGTAACCTCATTTCCCCA. PCR products were sequenced and analyzed with Sequencher sequence analysis software (Gene Codes). For real-time relative quantitative PCR, *KIT* exons were amplified in 25 μL reaction volumes containing 50 ng genomic DNA, 12.5 μL SYBR Green PCR Master Mix, and 0.5 μL of 10 μmol/L primers with the 7500 Real-time PCR System (Applied Biosystems). PCR cycling conditions were as follows: an initial denaturation step at 95°C for 10 min and 40 cycles of denaturation (95°C, 30 s) and annealing/extension (58°C, 33 s). β-Actin was used as endogenous control, and auto-Ct was selected in analyzing relative quantity of PCR products. Primers used were gh*KIT* exon 12 forward ATTTTGAAACTGCACAAATGGTC and reverse GCATTTTAGCAAAAAG-CACAACT and β-actin forward CTCCATCATGAAGTGTGACGTGGA and reverse CAGGAAAGACACCCACCTTGATCT.

Single nucleotide polymorphism analyses using Affymetrix GeneChip mapping arrays (250K). Genomic DNA was isolated from cell cultures using DNeasy tissue kit (Qiagen). Human Mapping 250K Sty single nucleotide polymorphism array was done in the Molecular Diagnostics Laboratory Microarray Core at Dana-Farber Cancer Institute according to manufacturer's instructions (Gene Chip Mapping 500K Assay Manual; Affymetrix),[9] except that the MJ Research thermocycler was set to the "Block" mode, and denaturation cycles were done at 92°C. Four PCR analyses were completed for each sample. PCR product (120 μg) was fragmented, labeled, and hybridized to the array. Comparisons of gene copy number were analyzed using dChip software.[10]

Cell cultures. Tumor samples were obtained from patients on Dana-Farber Cancer Institute/Harvard Cancer Center institutional review board-approved protocols. Cell cultures were established and expanded from fresh tissues after mechanical and enzymatic digestion. Cell cultures were maintained in DMEM containing 20% (v/v) FCS. Mutational analyses of *NRAS* and *BRAF* were done by PCR and sequencing. *KIT* copy number was assessed by quantitative PCR and single nucleotide polymorphism arrays (Dana-Farber Cancer Institute Core Facility). These primary cell cultures were used within the first 5 to 10 passages.

Cell viability assay. Imatinib (supplied by Novartis Pharmaceuticals) effects on tumor cell proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (20). Briefly, 1×10^5 cells per well were treated with imatinib concentrations of 50 nmol/L, 100 nmol/L, 250 nmol/L, 500 nmol/L, 1 μmol/L, and 10 μmol/L. Proliferation was determined at 4 h, 1 day, 2 days, 3 days, and 5 days. After removal of culture medium, 50 μL DMEM (without phenol red) containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well and processed according to the manufacturer's recommendations (Roche). Blue formazan was generated from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by living cells in a 2 h incubation at 37°C, and

[9] https://www.affymetrix.com/support/downloads/manuals/500k_assay_manual.pdf

[10] http://biosun1.harvard.edu/complab/dchip/

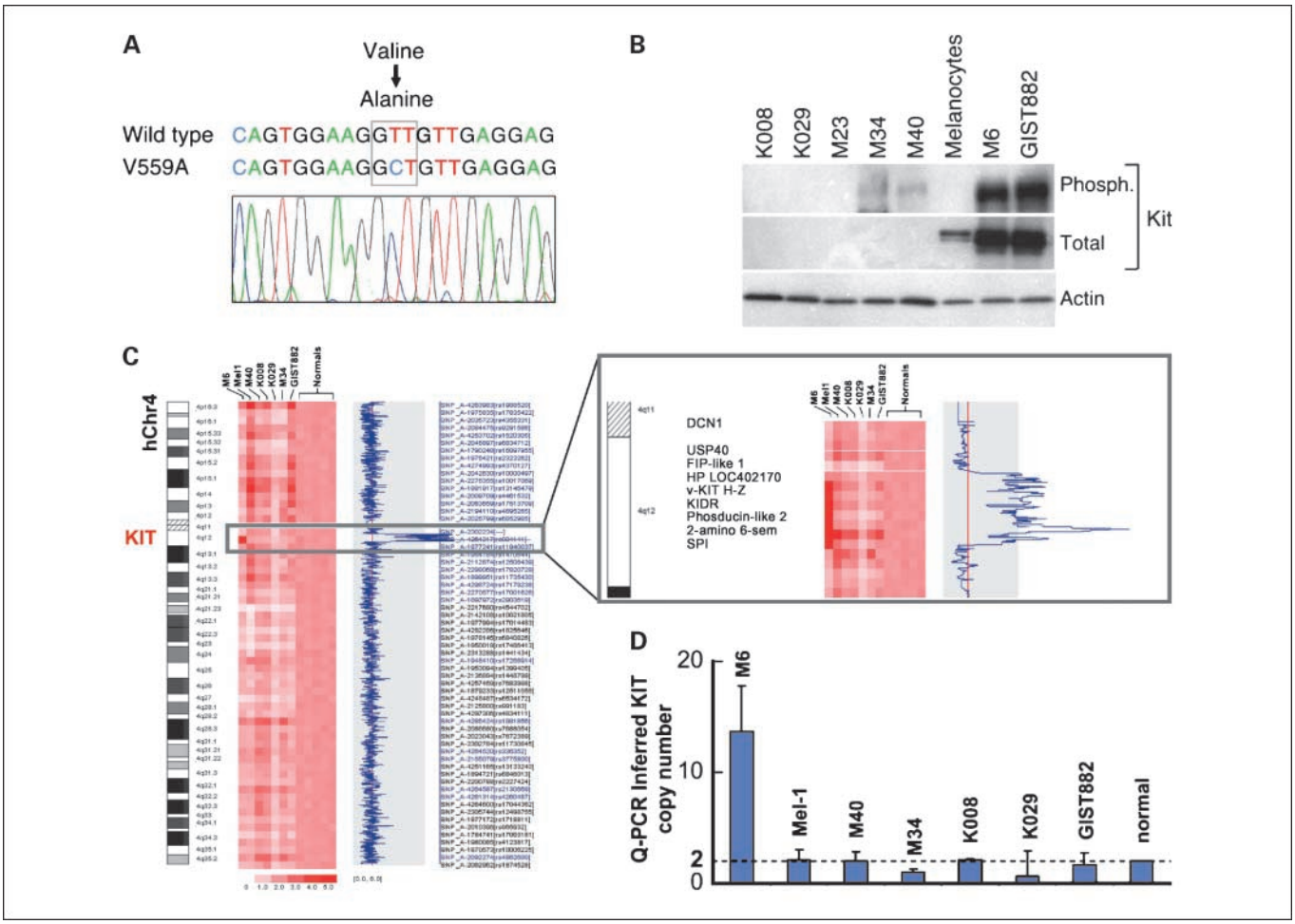

Fig. 1. KIT mutation identified in mucosal cells from patient M6 by direct bidirectional DNA sequencing on an ABI 310 using the Big-Dye terminator method (*A*). Phospho-KIT and total KITexpression in cutaneous melanoma cells from patients K008, K29, M23, and M34, mucosal melanoma cells from patient M40, melanocytes, mucosal melanoma cells from patient M6, and GIST cell line GIST882 (*B*). Mucosal melanoma cells from patient Mel1 also did not express KIT (data not shown). *KIT* amplification detected by single nucleotide polymorphism array analysis and quantitative PCR in mucosal and cutaneous melanoma cells. Genomic DNA from indicated cells were digested by StyI processed for hybridization to Affymetrix human genome 250K single nucleotide polymorphism arrays (*C*). Copy number analysis using dChip of cell line data versus normal controls indicates an ~10-copy gain of a region surrounding *KIT* on hCHR4q12. Magnification of the hCHR4q12 region. Genomic quantitative PCR detection of *KIT* exon 12 copy number versus internal control β-actin (normalized as two copies per genome) for experiment done in triplicate (with normalized SE; *D*).

solubilized with 150 μL isopropanol (in 0.04 mol/L HCl), and resulting absorbance was determined at 570 nm.

Apoptotic cytotoxicity assay. Following treatment with imatinib, cells were harvested and stained with 1 μL Annexin V-FITC (R&D Systems) according to the manufacturer's protocol. Apoptotic cells were analyzed by flow cytometry (Cytomics FC500; Beckman Coulter).

Western blot analysis. Cells were seeded in DMEM containing 20% fetal bovine serum in six-well plates. At 60% to 70% confluence, cells were then treated with imatinib for 2 h to 5 days at various concentrations. Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Roche Diagnostics). Lysates were resolved by SDS-PAGE (4-20% Tris-HCl), electrotransferred to polyvinylidene difluoride membranes, blocked in TBS with 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk for 1 h, and then incubated with the primary antibodies in TBS-Tween 20 with 5% nonfat dry milk overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and detection was with chemiluminescence substrate (SuperSignal West PicoLumino Reagent; Pierce) followed by autoradiography. Autoradiographs were subjected to scanning densitometry and quantitated by analysis software (Alpha Innotech). The following antibodies were used: KIT and BCL-2 (DakoCytomation); phospho-KIT (pY703; Biosource International); total AKT/protein kinase B, phospho-AKT/protein kinase B (Ser^{473}), total mammalian target of rapamycin (mTOR), phospho-mTOR (Ser^{2448}), phospho-70S6K (Thr^{389}), total p70S6K, S6 ribosomal protein (5G10), phospho-S6 ribosomal protein (Ser^{240}/Ser^{244}), phospho-STAT3 (Tyr^{705}), caspase-3, and caspase-9 (Cell Signaling Technology); phospho-STAT1 (pY701) and horseradish peroxidase-conjugated secondary antibody (Invitrogen); total p44/42 mitogen-activated protein kinase, phospho-p44/42 mitogen-activated protein kinase (Thr^{202}/Tyr^{204}), MCL-1, cyclin D1, cyclin E, proliferating cell nuclear antigen (PC10), STAT1, and STAT3 (Santa Cruz Biotechnology); survivin (Novus Biologicals); actin (Sigma); and livin/ML-IAP (Active Motif).

Results

KIT ***aberrations in mucosal melanoma cells.*** Early-passage mucosal melanoma cell cultures were established from three patients and one (M6; from a deceased patient) exhibited an activating *KIT* exon 11 (V559A) mutation (Fig. 1A). M6 cells showed overexpression of phospho-KIT and total KIT when compared with melanocytes and melanoma cells from other

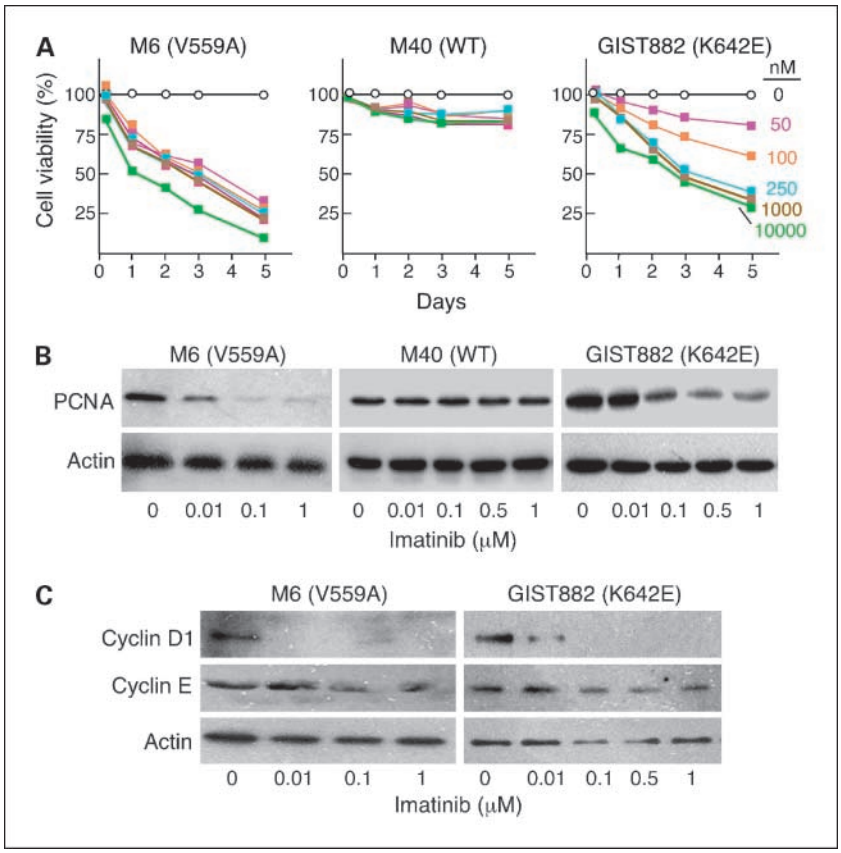

Fig. 2. Imatinib mesylate has dramatic antiproliferative effects on the *KIT* mutated and amplified mucosal melanomas from patient M6. Concentrations of imatinib mesylate tested were 50 nmol/L, 100 nmol/L, 250 nmol/L, 500 nmol/L, 1 μmol/L, and 10 μmol/L compared with untreated. Samples were analyzed at time points 4 h, 1 d, 2 d, 3 d, and 5 d. The M6 mucosal melanoma cells had dramatic antiproliferative effects at all drug concentrations (*A*) in contrast to the mucosal melanoma cells from patient M40 (*KIT* wild-type), which had no antiproliferative effects. Similar effects to M6 were observed in the GIST882 cell line that harbors a known imatinib mesylate-sensitive K642E *KIT* mutation. Data are percentage of absorbance of imatinib mesylate-treated groups in comparison with the untreated group. The proliferation marker proliferating cell nuclear antigen is reduced as a function of imatinib mesylate concentration in M6 and GIST882 cells (*B*). Imatinib mesylate induces G_1 arrest of M6 cells. Cyclin D1 is reduced after 72 h of drug exposure for M6 and GIST 882 cells but with little effect on cyclin E levels (*C*).

mucosal and cutaneous melanoma patients (Fig. 1B). The *KIT* mutation in M6 cells appeared homozygous by sequencing, and the *KIT* locus was highly amplified (~12-fold) as shown by single nucleotide polymorphism array analysis (Gene Expression Omnibus accession no. GSE8164[11]; Fig. 1C) and quantitative genomic PCR (Fig. 1D). Cell cultures from *KIT* wild-type mucosal melanomas (Mel1 and M40) and the GIST 882 line did not exhibit major changes in *KIT* copy number. *BRAF* and *NRAS* mutations were not found in any of the mucosal cell cultures. Two cutaneous melanoma cell lines (K029 and M34) harbored V600E *BRAF* mutations. In addition, there were no mutations in *PDGFRA*, which is an alternate to *KIT* oncogenes in some GISTs.

In vitro *activity of imatinib mesylate.* Cell lines treated with imatinib showed substantial antiproliferative activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in the *KIT*-mutant M6 but not in the *KIT* wild-type mucosal melanomas (Fig. 2A). This antiproliferative response was at least as potent as the response observed for the GIST882 cell line that has a well-characterized imatinib-sensitive K642E mutation in *KIT* exon 13. Imatinib mesylate induces G_1 arrest in M6 cells as depicted by reductions in the proliferation marker proliferating cell nuclear antigen (Fig. 2B) as well as reduction in cyclin D1 levels with little effect on cyclin E (Fig. 2C). Annexin V staining revealed activation of apoptosis by imatinib in M6 cells (Fig. 3A). Further evidence of apoptosis included loss of caspase-3 and caspase-9 (Fig. 3B) in both M6 and GIST882 cells (*KIT* K642E mutation). Imatinib mesylate had no effects on three cutaneous melanoma cell lines known to be *KIT* wild-type or in *KIT* wild-type mucosal melanoma cells (M40) (data not shown).

Imatinib effects on signaling pathways in the *KIT*-mutant mucosal melanoma cells were evaluated by Western blotting. Imatinib substantially inhibited phospho-KIT in M6 cells, similar to the effect in GIST882 cells. As expected, imatinib treatment had little effect on total KIT protein levels (Fig. 4A). Imatinib treatment also inhibited the MEK/mitogen-activated protein kinase pathway, as shown by marked reduction of phospho-p44/42 (extracellular signal-regulated kinase 1/2; Fig. 4B), and the phosphatidylinositol 3-kinase/AKT pathway, as shown by reduction in phospho-AKT and phospho-mTOR (Fig. 4C). There were no changes in the M40 KIT wild-type cells (data not shown). In both M6 and GIST882 cells, dramatic reduction in phospho-STAT1 and phospho-STAT3 were observed after 2 and 72 h, respectively, of imatinib treatment, whereas expression of total STAT1 and STAT3 were unchanged (Fig. 5A). Imatinib inhibited phosphorylation of p70S6K, showing cumulative effects from several upstream pathways (Fig. 5B). Phospho-S6, a translational regulator, was also inhibited in imatinib-treated M6 and GIST882 cells. There were no changes in the M40 KIT wild-type cells (data not shown).

To examine components of the apoptosis machinery in M6 mucosal melanoma cells exposed to imatinib, BCL-2 family members and inhibitor of apoptosis proteins were examined. Imatinib reduced expression of BCL-2, MCL-1, ML-IAP, and

[11] http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zxoltywskyaiur-u&acc=GSE8164

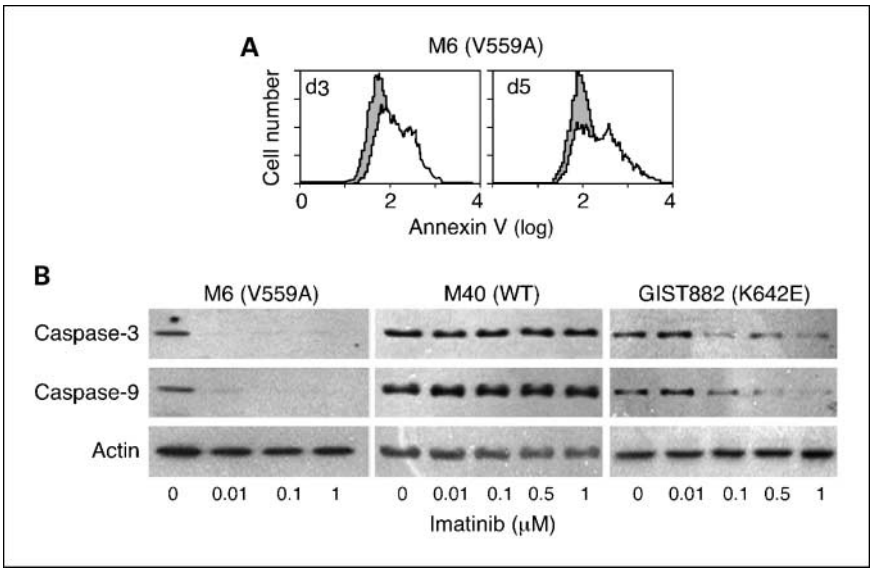

Fig. 3. Compared with cells grown in the absence of drug (shaded areas), Annexin V staining reveals significant cytotoxicity of M6 mucosal melanoma cells cultured in the presence of imatinib mesylate at 3 d (39% versus 14% no drug) and 5 d (51% versus 17% no drug; *A*). Imatinib mesylate-induced apoptosis in M6 and GIST 882 cells but not in M40 cells revealed by loss of caspase-3 and caspase-9 expression as a function of drug concentration exposure (*B*).

survivin in both M6 and GIST882 cells (Fig. 6). There were no changes in the M40 KIT wild-type cells (data not shown).

Discussion

Although mutant forms of the *BRAF*, *NRAS*, and *KIT* oncogenes might serve as therapeutic targets in melanoma (21), phase II trials of imatinib mesylate in metastatic melanoma patients have proven disappointing (15–17). These clinical results are understandable, given that few cutaneous melanomas contain *KIT* mutations or appear to depend on KIT oncogenic signaling. Nonetheless, we recently reported a patient with metastatic mucosal melanoma harboring an activating *KIT* mutation, who had a near complete metabolic response by fluoro-2-deoxyglucose positron emission tomography/computed tomography and >50% reduction in tumor volume 4 weeks after initiation of treatment with imatinib mesylate (18). The *KIT* mutation identified in that patient's melanoma involved the juxtamembrane domain (exon 11), which is the most frequent site of mutation in GIST and predicts response to imatinib mesylate (9, 22). Correspondingly, the mucosal melanoma cultured cells with a *KIT* exon 11 mutation (patient M6) in the current report was highly sensitive to imatinib mesylate *in vitro*, whereas several *KIT* wild-type melanoma cell cultures were imatinib resistant. These *in vivo* and *in vitro* observations show that at least a subset of melanomas exhibit tyrosine kinase "oncogene addiction," comparable with that in most GISTs (and in many lung cancers, breast cancers, and leukemias) and which serve as drug receptive targets.

KIT and its ligand stem cell factor are essential to melanocyte development (23, 24). In a murine B16F10 melanoma model, imatinib mesylate resulted in tumor growth inhibition (25). Furthermore, imatinib mesylate treatment of human melanoma xenografts in athymic nude mice has been shown to inhibit platelet-derived growth factor receptor phosphorylation albeit without reducing tumor size (26). Because imatinib mesylate targets both KIT and platelet-derived growth factor receptor activities, the relative roles of these two receptor tyrosine kinases in these models have yet to be determined.

Until recently, there were few data on the incidence and clinical relevance of melanoma *KIT* mutations. Of 35 primarily cutaneous melanomas expressing the KIT protein, high-resolution melting curve analysis revealed three genomic mutations (8.6%; refs. 27, 28). Recently, a particularly high (11-21%) *KIT* mutation frequency was shown in melanomas of the mucosa, acral skin, and chronically sun-damaged skin

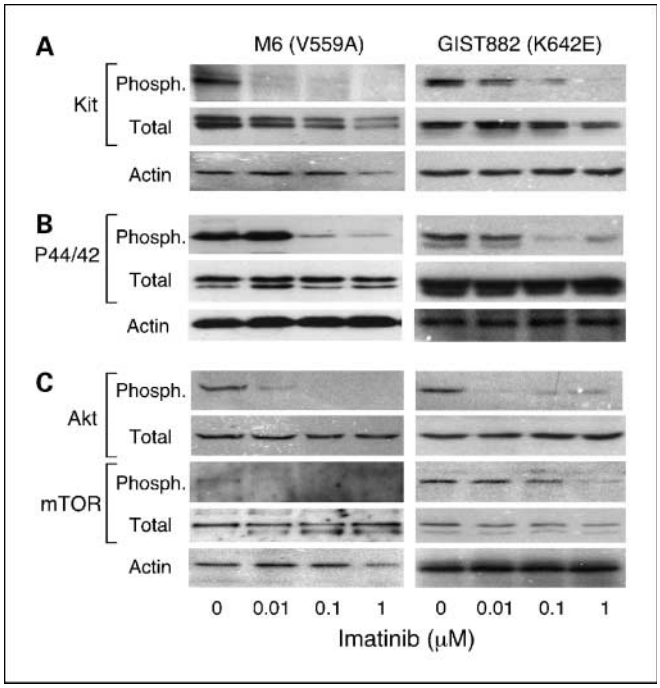

Fig. 4. Imatinib mesylate inhibits KIT phosphorylation in M6 mucosal melanoma cells. Differing concentrations of imatinib mesylate (0, 0.01, 0.1, and 1 μmol/L) were exposed to M6 cells for 2 h. Phospho-KIT and total KIT expression are assessed by Western blot (*A*). Imatinib mesylate also inhibits the mitogen-activated protein kinase pathway as exhibited by decreased phospho-p44/42 with no change in total p44/42 in M6 and GIST882 cells (*B*). Imatinib inhibits the phosphatidylinositol 3-kinase-AKT/protein kinase B-mTOR pathway. Western blots for phospho-AKT, total AKT, phospho-mTOR, and total mTOR as a function of the concentration of drug exposure for M6 and GIST 882 cells (*C*).

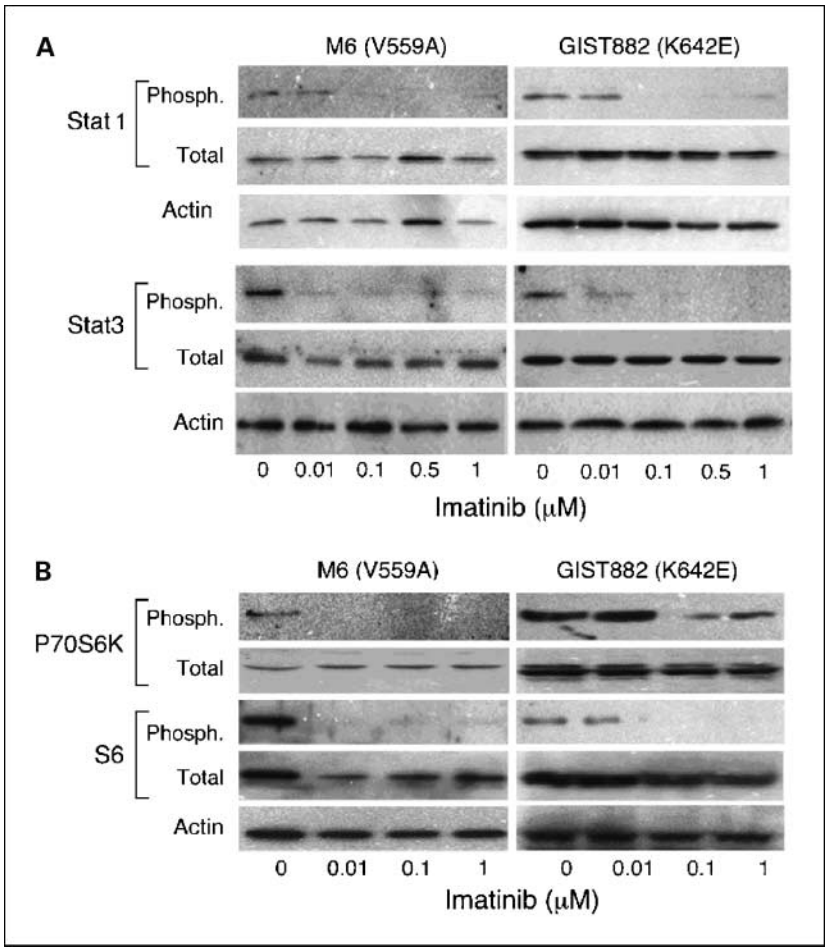

Fig. 5. Imatinib mesylate inhibits STAT phosphorylation. Phospho-STAT1 and STAT3 are reduced in M6 and GIST882 cells as a function of imatinib mesylate concentration at 2 and 72 h, respectively (*A*). Imatinib mesylate inhibits phosphorylation of p70S6K, showing cumulative effects from several upstream pathways. Phospho-S6, a key translational regulator, is also reduced in imatinib mesylate-treated M6 and GIST882 cells following 2 h exposure (*B*).

(3, 5). *KIT* amplification has also been reported in some melanomas with and without intragenic mutations (3, 5). The M6 mucosal melanoma culture described herein has striking *KIT* amplification, suggesting positive selection for the mutant allele. It remains to be determined whether *KIT* amplification correlates with imatinib mesylate sensitivity independent of mutational status. Mutant KIT oncoproteins (particularly those with exon 11-encoded juxtamembrane region mutations) are hypersensitive to imatinib, whereas native KIT is less sensitive. Therefore, melanomas with genomic amplification of wild-type *KIT* might not be targeted as effectively by imatinib compared with melanomas with *KIT* exon 11 oncogenic mutations. Other KIT kinase inhibitors with enhanced potency against native KIT will need to be tested when treating patients whose melanomas containing amplified wild-type *KIT*, although additional unknown variables exist, such as the manner in which these drugs are taken up and/or metabolized by melanoma cells.

Three important pathways initiated by KIT activation are those mediated by RAF/mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT, and STAT signaling (29, 30). Each of these pathways appeared to be significantly affected by imatinib treatment in the M6 KIT-mutant melanoma described here. Although the precise contribution of each pathway to survival or proliferation will require additional study, these are plausible regulators of tumor apoptosis and may participate in clinically relevant cell death mechanisms in melanoma. In addition, these data identify potential biomarkers of *in vivo* drug efficacy within the melanoma cell population. Targeting mutations relatively upstream may prove clinically beneficial, as it appears to affect multiple downstream pathways. Similar patterns have been observed in other diseases, such as epidermal growth factor receptor mutations in non-small cell lung cancer.

The genetic complexity of melanoma is highlighted by the many genomic alterations that occur during progression from primary to metastatic melanoma (31). Nonetheless, our data

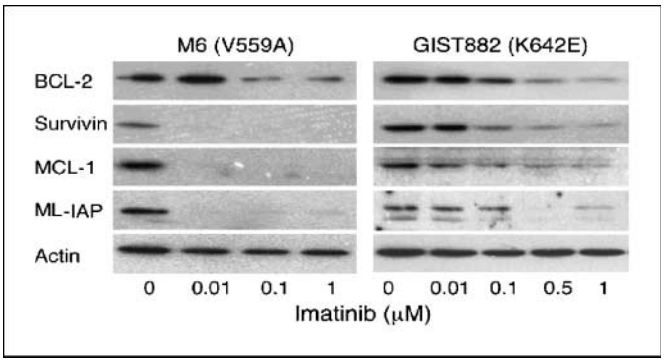

Fig. 6. BCL-2 family members BCL-2 and MCL-1 and inhibitors of apoptosis proteins ML-IAP and survivin are reduced as a function of imatinib mesylate concentration for M6 and GIST882 cells following 72 h exposure.

suggest that the transformed state of certain melanomas depends on mutation-mediated constitutive KIT activation. Previous studies of stable Ba/F3 transformants show differential kinase inhibitor drug sensitivity for a recurrent *KIT* mutation found in melanoma (5). Clinical trials to treat melanomas with *KIT* aberrations will be required to determine the most potentially effective kinase inhibitor agents for particular *KIT* mutation or amplification genotypes.

It is striking that imatinib mesylate triggered certain changes in cell death regulators differentially between susceptible melanoma cells and susceptible GIST cells (Fig. 6). In particular, more potent suppression in expression of survivin, ML-IAP, and MCL-1 was observed in treated melanoma cells. BCL-2 expression was also diminished, although it is less clear whether this effect was distinctive for melanoma cells (versus GIST). Expression of these factors could involve lineage-specific mechanisms, perhaps including the transcription factor MITF, which has been shown to target BCL-2 and ML-IAP (32). Because BCL-2 is essential for melanocyte stem cell survival (33), the potential involvement of BCL-2 in imatinib responsiveness also warrants further study.

Melanoma cells are typically resistant to chemotherapy and radiation therapy, and mucosal melanomas are among the poorest prognosis tumors. Successful culture of melanomas arising from mucosal surfaces has traditionally been a challenge, with very few such resources available for study. The demonstration in the current report of a highly sensitive drug response *in vitro* corresponds to our early clinical observations, suggesting an important opportunity for melanoma translational research. It emphasizes the need to routinely test tumors from patients likely to have *KIT* mutations and/or amplification. Further studies are warranted to better understand the relevant repertoire of KIT-targeting strategies in this patient population as well as molecular pathways responsible for kinase inhibitor drug sensitivity and resistance in these tumors.

Disclosure of Potential Conflicts of Interest

F.S. Hodi has received commercial research support from Novartis, Pfizer, and Bristol-Myers and honoraria from Novartis. C.L. Corless has received commercial research grants and honoraria from Novartis and Pfizer. G.D. Demetri has received commercial research grants from Novartis, Pfizer, Infinity, and Bristol-Myers and honoraria from Novartis, Bayer, Pfizer, and Infinity. M.C. Heinrich has received commercial grants from Novartis and Pfizer and honoraria from Novartis and has an ownership interest in Molecular MD. D.E. Fisher has received commercial research support and worked as a consultant for Novartis.

References

1. Patrick RJ, Fenske NA, Messina JL. Primary mucosal melanoma. J Am Acad Dermatol 2007;56:828–34.
2. Barnhill RL. Textbook of dermatopathology. 2nd ed. New York: McGraw-Hill, Health Pub. Division; 2004.
3. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol 2006;24:4340–6.
4. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med 2005;353:2135–47.
5. Antonescu CR, Busam KJ, Francone TD, et al. L576P KIT mutation in anal melanomas correlates with KIT protein expression and is sensitive to specific kinase inhibition. Int J Cancer 2007;121:257–64.
6. Corless CL, Fletcher JA, Heinrich MC. Biology of gastrointestinal stromal tumors. J Clin Oncol 2004;22:3813–25.
7. Hemesath TJ, Price ER, Takemoto C, Badalian T, Fisher DE. MAP kinase links the transcription factor Microphthalmia to c-Kit signalling in melanocytes. Nature 1998;391:298–301.
8. Bauer S, Duensing A, Demetri GD, Fletcher JA. KIT oncogenic signaling mechanisms in imatinib-resistant gastrointestinal stromal tumor: PI3-kinase/AKT is a crucial survival pathway. Oncogene 2007;26:7560–8.
9. Rubin BP, Heinrich MC, Corless CL. Gastrointestinal stromal tumour. Lancet 2007;369:1731–41.
10. Verweij J, Casali PG, Zalcberg J, et al. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. Lancet 2004;364:1127–34.
11. Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 2002;347:472–80.
12. van Oosterom AT, Judson I, Verweij J, et al. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. Lancet 2001;358:1421–3.
13. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001;344:1031–7.
14. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996;2:561–6.
15. Wyman K, Atkins MB, Prieto V, et al. Multicenter phase II trial of high-dose imatinib mesylate in metastatic melanoma: significant toxicity with no clinical efficacy. Cancer 2006;106:2005–11.
16. Eton O, Billings L, Kim K, et al. Phase II trial of imatinib mesylate (STI-571) in metastatic melanoma (MM) [abstract 1528]. J Clin Oncol 2006 ASCO Annu Meet Proc Part I 2004;22:114s.
17. Ugurel S, Hildenbrand R, Zimpfer A, et al. Lack of clinical efficacy of imatinib in metastatic melanoma. Br J Cancer 2005;92:1398–405.
18. Hodi FS, Friedlander P, Corless CL, et al. Major response to imatinib mesylate in KIT-mutated melanoma. J Clin Oncol 2008;26:2046–51.
19. Corless CL, McGreevey L, Haley A, Town A, Heinrich MC. KIT mutations are common in incidental gastrointestinal stromal tumors one centimeter or less in size. Am J Pathol 2002;160:1567–72.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
21. Fecher LA, Cummings SD, Keefe MJ, Alani RM. Toward a molecular classification of melanoma. J Clin Oncol 2007;25:1606–20.
22. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003;21:4342–9.
23. Yoshida H, Kunisada T, Grimm T, Nishimura E, Nishioka E, Nishikawa S. Review: melanocyte migration and survival controlled by SCF/c-kit expression. J Investig Dermatol Symp Proc 2001;6:1–5.
24. Wehrle-Haller B. The role of Kit-ligand in melanocyte development and epidermal homeostasis. Pigment Cell Res 2003;16:287–96.
25. Redondo P, Lloret P, Andreu EJ, Inoges S. Imatinib mesylate in cutaneous melanoma. J Invest Dermatol 2004;123:1208–9.
26. McGary EC, Onn A, Mills L, et al. Imatinib mesylate inhibits platelet-derived growth factor receptor phosphorylation of melanoma cells but does not affect tumorigenicity *in vivo*. J Invest Dermatol 2004;122:400–5.
27. Willmore-Payne C, Holden JA, Tripp S, Layfield LJ. Human malignant melanoma: detection of BRAF- and c-kit-activating mutations by high-resolution amplicon melting analysis. Hum Pathol 2005;36:486–93.
28. Willmore-Payne C, Holden JA, Hirschowitz S, Layfield LJ. BRAF and c-kit gene copy number in mutation-positive malignant melanoma. Hum Pathol 2006;37:520–7.
29. Sattler M, Salgia R. Targeting c-Kit mutations: basic science to novel therapies. Leuk Res 2004;28:S11–20.
30. Reber L, Da Silva CA, Frossard N. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. Eur J Pharmacol 2006;533:327–40.
31. Chin L, Garraway LA, Fisher DE. Malignant melanoma: genetics and therapeutics in the genomic era. Genes Dev 2006;20:2149–82.
32. McGill GG, Horstmann M, Widlund HR, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 2002;109:707–18.
33. Nishimura EK, Granter SR, Fisher DE. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science 2005;307:720–4.

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