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 mutation 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
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.

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 (Transgenic 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 N-RAS mutational analyses were KIT exon 9 forward GTCTAGTTTCCACGACTCT and reverse AATTCGAGTTCCCTCCTCT, KIT exon 11 forward CCAGAGTGCTCTAATGACTG and reverse ACCCAAGAAGGT-GACATGGA, KIT exon 13 forward CATGAGTTTGGCAGTTGCG and reverse ACAGGCTTACCATTTCAAGT, KIT exon 17 forward TGTATTCAACAGACTTGC and reverse GACTTTGACTTGGC and reverse CAGAAGTGGAATATTTCCITGT, BRAF exon 15 forward TGCCCTGCTTGTAGGAAATAG and reverse AGCATCTAGGGCCAAAAAT, NRAS exon 1 forward CACTAGGGTTTCAATTG and reverse TCCITTAATACGAATAATGGTAAAGA, and NRAS exon 2 forward AATTTGACCTCCCTCCTG and reverse TGTTAATCCCTATTTCCCCCA. PCR products were sequenced and analyzed with Sequencher sequence analysis software (Gene Codes). For real-time 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 ghKIFT exon 12 forward ATGTGGAACATCGCAAAGGCTC and reverse GCCATTGAGAAGGAGCACAACCT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT and β-actin forward CTTCCTATCAAGTTGAC and reverse CACAACT.
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 (Invitrogen); total p44/42 mitogen-activated protein kinase (Thr202/Tyr204), 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 patients, and GIST cell line GIST882 (B). Mucosal melanoma cells from patient Mel 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 Styl 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).
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. GSE816411; Fig. 1C) and quantitative genomic PCR (Fig. 1D). Cell cultures from KIT wild-type mucosal melanomas (Mel1 and M40) and the GIST882 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 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 G1 arrest of M6 cells. Cyclin D1 is reduced after 72 h of drug exposure for M6 and GIST882 cells but with little effect on cyclin E levels (C).

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 G1 arrest of M6 cells. Cyclin D1 is reduced after 72 h of drug exposure for M6 and GIST882 cells but with little effect on cyclin E levels (C). 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
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.
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 contain 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...
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. Expression of these mechanisms, perhaps including the transcription factor MITF, which has been shown to target BCL-2 and ML-IAP (32). 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

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*Clin Cancer Res* 2008;14:7726-7732.

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