Pathological activation of KIT in metastatic tumors of acral and mucosal melanomas.

Short title: KIT in acral and mucosal melanomas.

Atsuko Ashida, Minoru Takata*, Hiroshi Murata, Kenji Kido, and Toshiaki Saida

Department of Dermatology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

*Correspondence: Minoru Takata, M.D., Ph.D.
3-1-1 Asahi, Matsumoto 390-8621, Japan
Tel +81-263-37-2644; Fax +81-263-37-2646
e-mail:mtakata@shinshu-u.ac.jp

Key words: melanoma, skin, mucous membrane, oncogene mutation, molecular targets
Abbreviations : SCF, stem cell factor; GIST, gastrointestinal stromal cell tumor.

Category: Cancer cell biology

This study shows
1) pathlogical activation of wild-type KIT in a substantial number of acral and mucosal melanoma metastases *in vivo*, and
2) growth suppressive effects of sunitinib against acral melanoma cells *in vitro.*

Abstract

Recent studies showed *KIT* gene aberrations in a substantial number of melanomas on acral skin and mucosa, suggesting the therapeutic benefit of tyrosine kinase inhibitors, such as imatinib. We therefore examined the expression and mutations of *KIT* in 4 primary and 24 metastatic acral and mucosal melanomas. Immunohistochemistry revealed moderate or strong KIT protein expression in 13 (48%) tumors. Sequence analysis revealed K642E and D820Y mutations in two metastases. Amplification of *KIT* was identified by real-time PCR in 4 tumors, including one that had K642E. Western blot analysis showed phosphorylation of the KIT receptor in 8 (62%) of 13 cryopreserved samples, indicating the frequent pathological activation of the receptor *in vivo*. Phosphorylation of KIT protein was detected in 2 tumors harboring *KIT* mutations, as well as in one tumor with *KIT* gene amplification. Furthermore, 5 tumors without detectable *KIT* gene aberrations showed phosphorylation of the KIT receptor. Expression of stem cell factor (SCF) in melanoma cells as well as stromal cells suggests SCF/KIT autocrine and paracrine activation in these tumors. Finally, we found significant growth suppressive effects of sunitinib in two acral melanoma cell lines; one harboring the D820Y mutation and one showing SCF-dependent KIT activation. These results show pathological activation of KIT in a substantial number of metastatic tumors of acral and mucosal melanomas, and suggest a potential therapeutic benefit of sunitinib for these melanomas.

Introduction

KIT is a transmembrane tyrosine kinase receptor, which is essential for the maintenance of normal hematopoiesis, melanogenesis, gamategenesis, and differentiation of mast cells and interstitial cells of Cajal.[1] The dysregulation of KIT is thought to play a role in certain neoplastic disorders, including systemic mastocytosis, acute myelogenous leukemia, germ cell tumors, and gastrointestinal stromal cell tumors (GIST). [2] In the melanocytic system, KIT plays a crucial role in the normal development and function of melanocytes in embryonic and postnatal stages [3]. While KIT expression is found in normal melanocytes, benign melanocytic nevi and *in situ* melanomas, it appears to be down-regulated in invasive and metastatic melanomas. [4, 5] *In vitro* study also showed that, although KIT is a potent growth regulator of normal human melanocytes, its activity may not be positively associated with malignant transformation.[6] Recently, however, Curtin et al.,[7] who examined 102 primary melanomas excised from various anatomical sites, found mutations and/or copy number increases of the *KIT* in 39% of mucosal melanomas, 36% of acral melanomas and 28% of melanomas on chronically sun-damaged skin, but not in any (0%) melanomas on non-glabrous skin without chronic sun damage. A couple of recent studies also identified oncogenic *KIT* mutations in 3 (15%) of 20 anal melanomas, [8] and 4 (22%) of 18 oral mucosal melanomas. [9] These findings indicate that *KIT* is an important oncogene in melanomas of mucosa, acral skin or skin with chronic sun damage, and that imatinib and other tyrosine kinase inhibitors targeting KIT might be a promising treatment option for melanomas developing at these anatomical sites [7]. Major clinical responses to imatinib have actually been reported in metastatic mucosal melanomas harboring *KIT* mutations.[10, 11] This is particularly important for non-Caucasian populations, such as the Japanese, in which the vast majority of melanomas occur on acral skin, mucous membranes, and skin with chronic sun-damage.[12, 13] We have therefore examined protein expression and gene aberrations of *KIT* in 28 surgical specimens (mostly metastases) of acral and mucosal melanoma. We have also investigated *in vivo* activation of KIT receptor by Western blot using frozen tumor samples. Furthermore, we have evaluated the ability of imatinib and sunitinib to block KIT-mediated responses and growth of acral melanoma cells *in vitro*.

Materials and methods

Patients and tumor tissue samples

We analyzed 28 tumor samples (24 metastases and 4 primary tumors) of acral or mucosal melanoma excised at the Department of Dermatology, Shinshu University Hospital (Table 1). A representative formalin-fixed, paraffin-embedded block was selected and used for immunohistochemistry as well as for the molecular studies. For 13 patients, cryopreserved material from surgical specimens was also available, and used for Western blotting. This study was approved by the medical ethics committee of the Shinshu University School of Medicine, and conducted according to the Declaration of Helsinki Principles. For excisions, patients gave written informed consent.

Cells

Six acral melanoma cell lines, SMYM-PRGP [14], SM2-1, SM3, MMG1, Mel-2, and Mel-18, all established from Japanese patients, were used in this study. Normal melanocytes, and HMC-1.2, which is a mast cell leukemia line expressing two gain of function mutations (V560G and D816V) in the *KIT* gene,[15] were also used as controls. Source and culture conditions for these cells are given in the supplementary Table 1S.

.

Immunohistochemistry

Immunohistochemistry was performed with a DAKO Envision™ System (Dako Cytomation, Kyoto, Japan) and 3-amino-9-ethylcarbazole as a chromogen. Paraffin sections (4 μm) were de-waxed and incubated in 3% H_2O_2 for 5 min at room temperature. Antigen retrieval was carried out by boiling sections in Target Retrieval Solution (Dako Cytomation, Kyoto, Japan) for 20 min. The polyclonal rabbit antihuman KIT antibody (CD117: Dako Cytomation, Kyoto, Japan) at a dilution of 1:500, and rabbit polyclonal anti- stem cell factor (SCF) (K089: Immuno Biological Laboratory, Gunma, Japan) at a dilution of 1:50 were used as primary antibodies. Sections were counter-stained with hematoxylin. Staining of interstitial mast cells and fibroblasts served as a positive control for KIT and SCF, respectively. For negative controls, the primary antibodies were replaced with phosphate- buffered saline (PBS). Immunostaining was graded in accordance with an immunoreactive score (IRS), in which IRS = percentage of positive

cells (0, negative; 1, 1- 20%; 2, 21- 50%; 3, 51-100%) X staining intensity (1, weak; 2, moderate; 3, strong). The final expression was grouped into three levels: high (3+), IRS 9 or 6; intermediate (2+), IRS 4 or 3; and low (1+), IRS 1 or 2. [16] The predominant staining pattern of cells, either membrane or cytoplasmic, was also assessed.

DNA sequencing

Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections using a MagneSil Genomic Fixed Tissue System (Promega, Madison, WI). We amplified exons 11, 13, 17 and 18 of the *KIT* gene by PCR. Primers (each tagged with the M13 forward or reverse primer) and conditions were those described by Curtin et al.[7] To detect *BRAF* and *NRAS* hotspot mutations, we amplified BRAF exon 15 and *NRAS* exon 3, respectively, as described previously.[17] We purified PCR amplicons with QIAquick (Qiagen, Valencia, CA), and directly sequenced using the Big Dye Terminator sequencing chemistry and an ABI automated sequencer (Applied Biosystems, Foster City, CA).

Fluorescent in situ hybridization (FISH) for cyclin D1

FISH for detecting amplification of the *cyclin D1* gene was performed as described previously.[17]

KIT Gene amplification analysis by real-time PCR

Quantitative real-time PCR was performed using Light Cycler Software version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany) to analyze the amplification status at the *KIT* gene locus, using *ribonuclease P (RNase P)* as a control gene. Primer sequences were as follows: *KIT*, 5'-GAGTAGCTTACCAGAAGCTTCAATAG-3' (forward) and 5'-CATAGGGACTGATGCCTTCC-3' (reverse); *RNase P*, 5'-GGCCATCAGAAGGAGATGAA-3' (forward) and 5'-TTTCAGCCCTCACACTTGG-3' (reverse). PCR was carried out for 45 cycles at 95℃ for 10 seconds and 60℃ for 30 seconds, according to the TaqMan Universal PCR Protocol (Roche Diagnostics, Mannheim, Germany). The DNA extracted from the tissue samples was adjusted to a concentration of 2 ng/μl. For each experiment, we used 5 μl (10 ng) of DNA. For the evaluation of copy numbers of the *KIT* gene, standard curves for both *KIT* and *RNase P* genes were generated with the threshold cycle of five serial dilutions of a normal human blood genomic DNA template. Gene copy numbers were evaluated as a relative value assuming that the gene ratio in normal human blood DNA was one. All

samples were analyzed in triplicate. A ratio of more than 2.0 was regarded as positive for gene amplification. Although a ratio of less than 0.5 might be compatible with gene deletion, this aspect was not considered in the present study.

Western blot analysis

Proteins extracted from tissue samples and cultured cells were subjected to Western blot, as described previously.[14 17] Antibodies used were rabbit polyclonal anti-KIT (CD117) (Dako Cytomation, Kyoto, Japan) and anti-phospho-KIT (Y719) (Cell Signaling, Cambridge, MA) antibodies, and mouse monoclonal antibody to β -actin (Abcam Inc., Cambridge, MA). Normal tissue of the lymph node was used as a negative control. The lysate obtained from HMC-1.2 cells and normal melanocyte cultures stimulated with 10 ng/ml of recombinant human SCF (Relia Tech, Braunschweig, Germany) was used as a positive control of receptor expression levels.

.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction, cDNA synthesis and RT-PCR were performed as described previously.[14] The primers used for amplification of *KIT* and *SCF* were as follows: *KIT*, 5′-TTCTTACCAGGTGGCAAAGGGCATGGCTTTC-3′ (forward) and 5′-GCCCTGAACACGCACCTGCTGAAATGTATGAC- 3′ (reverse); *SCF*, 5′-GTGATTGTGTGGTTTCTTC-3′ (forward) and 5′-CCTTCCTATTACTGCTACT-3′ (reverse). The reaction profile was 38 cycles of one minute at 95ºC, one minute at 54ºC and one minute at 72ºC with a final extension for 7 minutes at 72ºC. PCR products were separated on a 1.5% agarose gel.

In vitro drug testing

Growth inhibitory effects of imatinib and sunitinib (kindly provided by Novartis Pharma, Basel, Switzerland, and Pfizer Inc., New York, NY, respectively) were tested in KIT-expressing acral melanoma cells, SMYM-PRGP and SM3, by cell viability assay. SM2-1 cells that did not express KIT were used as a negative control. Suspended cells were washed with MCDB153 medium containing 0.1% of bovine serum albumin (Sigma-Aldrich Japan, Tokyo, Japan), followed by washing with plain MCDB153 medium. Four thousand of SMYM-PRGP and SM2-1 cell or 10,000 of SM3 cells in 50 μl of MCDB153 medium supplemented with 0.1 % FBS were seeded into each well in Nunclon-coated 96-well

multi-well plates (Nalge Nunc International, Rochester, NY). After 24 hours of incubation in a 5 % O_2/ 5% CO_2 atmosphere at 37℃, 50 μl of MCDB153 medium containing 4% FBS with or without twice the desired concentration of imatinib and sunitinib were added to each well. Right after this treatment, SMYM-PRGP cells were stimulated with 100 ng/ml of recombinant human SCF (Relia Tech, Braunschweig, Germany). Cultures were incubated for an additional 48 hours in the 5 % O_2/ 5% CO_2 atmosphere at 37°C. Then, 10 μl of WST-8 solution (Cell Count Reagent SF ™: Nakarai Tesuque, Kyoto, Japan) was added to each well. After additional 4 hours of incubation at 37°C, optical absorption at 450 nm was measured with a wavelength of 650 nm as the reference.

Statistical analysis

Results of cell proliferation assays and cell viability assays were expressed as the mean ± standard deviation (SD). Cell viability assays were analyzed with a one way analysis of variance test followed by Fisher's PLSD using SPSS for Windows version 14 (SPSS Inc, Chicago, IL)

Results

KIT expression and gene aberration in acral and mucosal melanomas in vivo

By immunohistochemistry using a polyclonal CD117 antibody, we detected KIT expression in 20 (74%) of the 27 melanoma tumors (Table 1). Six tumors showed diffuse membrane staining, whereas 14 showed predominantly cytoplasmic staining. Among the positive cases, while 7 (26%) tumors showed a low level of KIT expression, the expression was moderate to strong in the remaining 13 (48%) samples. Membrane staining was observed exclusively in tumors that showed strong KIT expression (Figure 1A).

Next, we investigated mutations and copy numbers of the *KIT* gene in 19 tumors that mostly showed KIT protein expression. Previous studies in cutaneous and mucosal melanomas showed that genetic aberrations of *KIT* were mainly found in tumors strongly expressing KIT protein [7-9]. We sequenced exons 11, 13, 17 and 18 of the *KIT* gene, in which most of the oncogenic mutations were identified.[18] We found two mutations: K642E and D820Y in a skin and a lymph node metastasis, respectively. No mutation was found in the other 17 tumors. *KIT* gene amplifications, determined by real time PCR assay, were found in 4 tumors, including one that had the K642E mutation (Table 1). Overall, the frequency of *KIT* gene mutation and/or amplification was 19% (5/28) in our cohort, which was somewhat lower than the previous report.[7] The K642E mutation is found in a small number of GIST, and is known to stabilize the juxta-membrane domain of the KIT protein through amino acid interactions.[19] This mutation was already reported in acral and mucosal melanomas.[7, 9] Frequent association of K642E and gene amplification was noted in a previous study, suggesting this mutation to represent weak activation additionally requiring increased gene dosage in order to provide a significant oncogenic signal.[7] The D820Y mutation is within the activation loop in the distal kinase domain, and was reported previously as a secondary mutation in GIST cases that acquired resistance to imatinib treatment,[20] as well as in the germline of one kindred with familial GIST.[21]

As fresh frozen tissues were available in 13 cases, we further investigated the expression and phosphorylation of the KIT receptor by Western blot analysis, using an antibody that specifically recognizes active KIT, phosphorylated at Tyr[719]. We found KIT expression and phosphorylation in 8 (62%) of 13 samples (Table 1, Figure 1B), although the expression levels were variable. Phosphorylation of KIT was detected in all but one (case 1) sample showing strong

staining for KIT by immunohistochemistry. Discrepant results between immunohistochemistry and Western blot in case 1 may be due to the heterogeneity of KIT expression within the same tumor. Phosphorylation of KIT protein was detected in 2 tumors harboring *KIT* mutations (cases 5 and 8), as well as in one tumor with *KIT* gene amplification (case 13). Furthermore, 5 tumors without detectable *KIT* gene aberrations showed the pathological activation of the KIT receptor, as revealed by tyrosine phosphorylation. All of these tumors showed strong SCF expression as revealed by immunohistochemistry (Table 1). Strong SCF expression was also found in the other 5 tumors that showed strong immunostaining for KIT (Figure 1C). SCF expression was observed not only in melanoma cells, but also in fibroblasts and endothelial cells. Such coexpression of KIT and SCF in tumor tissues suggests SCF/KIT autocrine as well as paracrine activation to result in enhanced KIT signaling.

We also examined the *BRAF, NRAS* and *cyclin D1* genes, all of which were implicated in melanoma development.[22] Consistent with previous reports,[17, 22] mutations in the *BRAF* and *NRAS* genes were infrequent in acral and mucosal melanomas. Only 3 (11%) of 28 tumors harbored the $BRAF^{V600E}$ mutation, and none showed *NRAS* mutation. Amplification of the cyclin D1 gene was detected in 3 (12%) of the 25 samples. Notably, two tumors harboring *KIT* amplification (cases 13 and 20) were associated either with *BRAF* mutation or *cyclin D1* amplification (Table 1).

Growth suppressive effects of imatinib and sunitinib in acral melanoma cells in vitro

Among the 6 acral melanoma cell lines, RT-PCR revealed *KIT* mRNA expression in 4 cells (Mel-2, MMG1, SMYM-PRGP, and SM3), while *SCF* was expressed in all 6 cell lines (Figure 2A). Sequence analysis of the KIT gene in the cells expressing KIT revealed that the SM3 cells harbored the D820Y mutation in exon 17, the same missense mutation identified in case 8. Although this mutation was reported in the germline of one kindred with familial GIST,[21] germline of the patient, from whom SM3 was established, showed wild-type. Amplification of the *KIT* gene was not detected in any cell line by real-time PCR. Western blot analysis confirmed the KIT protein expression in 3 of the 4 cell line, expression being weak in Mel-2 cells (Figure 2B). MMG1 did not express the protein, probably due to the post-transcriptional modification. Although no or very weak phosphorylation of the KIT protein was detected in Mel-2 and SMYM-PRGP cells, stimulation with 100 ng/ml of SCF induced weak or strong phosphorylation, respectively. Constitutive phosphorylation of KIT was observed in SM3 cells without SCF stimulation (Figure 2C).

Finally, we examined the growth suppressive effects of two tyrosine kinase inhibitors, imatinib and sunitinib, that are now widely used in the clinic for the treatment of advanced GIST harboring KIT mutations, [18, 23] against acral melanoma cells (Figure 3). We used SM3 harboring the D820Y mutation, and SMYM-PRGP with wild-type KIT that was strongly activated by SCF. SM2-1, which did not express KIT, was used as a control. As expected, imatinib and sunitinib had no effects on the proliferation of SM2-1 cells, although 10 μM of sunitinib had a weak suppressive effect. This may be due to the inhibition of receptor tyrosine kinases other than KIT, such as platelet-derived growth factor receptor (PDGFR), FMS-related tyrosine kinase-3, fibroblast growth factor receptor 1, colony stimulating factor-1 receptor and RET [24]. While imatinib did not affect the proliferation of SM3 cells, sunitinib showed marked growth inhibition against this cell harboring the D820Y mutation (Figure 3A). Both imatinib and sunitinib potently inhibited SCF-induced cell proliferation and phosphorylation in SMYM-PRGP cells. The two drugs caused dose-dependent inhibition of KIT phosphorylation in SCF-stimulated SMYM-PRGP cells. Sunitinib had a more potent effect than imatinib (Figure 3B).

Discussion

Because *KIT* is a target of several small molecule inhibitors, such as imatinib and sunitinib, which are already used in the clinic,[23] the finding of frequent genetic aberrations affecting *KIT* in acral and mucosal melanomas suggests potential therapeutic benefits of these KIT inhibitors.[7] Curtin et al.,[7] identified *KIT*-activating mutations in 11% and 21% of primary acral and mucosal melanomas, respectively. Oncogenic *KIT* mutations were also found in 15% of anal melanomas,[8] and 22% of oral mucosal melanomas.[9] Importantly, the majority of mutations found in these studies, mainly examining primary mucosal melanomas, were predicted to affect the juxta-membrane domain.[7-9] As other cancer types with mutations in the juxta-membrane domain are responsive to imatinib, a substantial number of mucosal melanoma patients may also benefit from imatinib treatment. However, the frequency of *KIT* mutations in our cohort, which mainly consisted of metastatic acral melanomas, was lower (7 %). This difference may be because: (a) although cells harboring activating *KIT* mutations may be selected for in the early stages of melanoma development, metastatic tumors may lose KIT expression,[25] (b) *KIT* gene mutations are less common in acral melanomas than in mucosal melanomas; or (c) the different geographic patient populations may have a diverse KIT expression status in acral and mucosal melanomas. More cases need to be tested to distinguish between these possibilities.

Both imatinib and sunitinib are tyrosine kinase inhibitors that target KIT with proven benefit in GIST.[23] Imatinib is effective in the treatment of a significant proportion of GIST with mutations in the juxta-membrane domain.[26] Thus, melanoma patients with *KIT* mutations affecting the juxta-membrane region, such as K642 and L576, which were found by this and other studies,[7-9] may benefit from imatinib treatment. However, mutations affecting other domains of *KIT* may confer resistance to imatinib.[23] In this study, we identified a rare D820Y mutation that affects the activation loop of KIT protein in one acral melanoma metastasis. Interestingly, an acral melanoma cell line, SM3, established in our laboratory from the primary acral melanoma tumor harbored the same mutation. Whether this particular type of mutation is common in Japanese melanoma patients needs further investigation. While tumors harboring mutations causing amino acid substitutions at residue 820 are reported to be resistant to imatinib,[20] sunitinib may be effective against some tumors that are refractory to imatinib. [27] We actually demonstrated a growth suppressive effect of sunitinib against SM3 cells harboring the D820Y mutation, while imatinib showed no effect. To the best of our knowledge, this is the first study to show the effectiveness of sunitinib against cells bearing this particular *KIT* mutation, and suggests sunitinib to be a useful therapeutic agent to treat *KIT* mutated melanomas, which would be

refractory to imatinib.

While aberrant KIT signaling may be activated by mutation and gene dosage effects, Curtin et al.[7] observed increased expression of the KIT protein in 33% of melanomas without detectable *KIT* mutations or copy number increases. This suggests that another mechanism may cause the constitutive activation of KIT. We have revealed, by Western blotting using fresh frozen surgical specimens, that a significant proportion of tumors with no genetic aberrations showed phosphorylation and activation of the KIT receptor. Since most of these tumors strongly expressed SCF in melanoma cells as well as in stromal cells, it is speculated that the aberrant KIT signaling is induced by a SCF/KIT autocrine and paracrine loop. While a number of *in vivo* tumors show distinct phosphorylation of the KIT receptor, exogenous SCF was required to induce phosphorylation in SMYM-PRGP cells *in vitro*. Thus, paracrine effects involving stroma or other host cells may be more important for this autoactivation. Similar autoactivation of KIT without genetic aberrations has been suggested in other cancers and sarcomas.[16, 28-30] Thus, it is of great clinical importance whether protein kinase inhibitors targeting KIT, such as imatinib and sunitinib, are efficacious in melanomas that are driven by autocrine/paracrine KIT activation. By using the KIT-expressing SMYM-PRGP cell line, we demonstrated that both imatinib and sunitinib inhibited SCF-dependent KIT phosphorylation and cell proliferation. Small cell lung cancers also showed the pathological activation of wild-type KIT by an autocrine loop, [28] and were sensitive to both imatinib and sunitinib *in vitro.* [31, 32] However, the *in vivo* evaluation by treating mice bearing small cell lung cancer cells expressing KIT showed different effect between imatinib and sunitinib: sunitinib treatment resulted in significant suppression of tumor growth, whereas the inhibition by imatinib treatment was less extensive.[31] The stronger growth suppressive effect of sunitinib *in vivo* was explained by the fact that sunitinib efficiently targets not only KIT but also PDGFR and the vascular endothelial growth factor receptor, thus suppressing tumor stroma and angiogenesis. [31] Consistent with the results in animal experiments, imatinib did not have antitumor activity against small cell lung cancer in a clinical trial, even with KIT protein present in tumor specimens.[33] Thus, future investigations should aim at evaluating sunitinib, not imatinib, as a potential therapeutic agent for treating melanomas showing wild-type KIT activation.

Two phase II clinical trials testing imatinib for metastatic melanoma revealed no objective response.[34, 35] Based on these disappointing clinical results, it was concluded that imatinib has no therapeutic effect against metastatic melanoma; however, these clinical trials did not consider either the type of melanoma or the *KIT* genotype of individual

tumors for eligibility criteria. A recent preliminary report of a clinical trial of imatinib in 21 metastatic melanomas showed one complete response in a patient with acral melanoma.[7] Major clinical responses to imatinib were also reported in patients with *KIT*-mutated mucosal melanoma.[10, 11] Thus, a clinical trial investigating imatinib and sunitinib in metastatic melanoma originating from primary tumors of acral skin, mucosa, or chronically sun-damaged skin has been proposed, correlating the treatment outcome with the mutation status of *KIT*.[36] Given the lack of effective treatments for advanced melanoma, clinical trials of new targeted therapy based on the selection of patients with appropriate biomarkers are urgent.[37] As shown in this study, KIT could be a promising target in a subset of patients with acral and mucosal melanomas, if the patients were selected properly according to the molecular profile of their tumors.

Acknowledgements

We thank Ryo Ogawa and Aya Uchiyama for their excellent technical assistance. We also thank Drs. Y. Kawakami (Institute for Advanced Medical Research, Keio University), A. Yamamoto (Saitama Medical University International Medical Center), E. Morii (Department of Pathology, Osaka University) and M. Shiohara (Department of Pediatrics, Shinshu University) for providing cell lines. This work was supported by a Grant-in-Aid for Cancer Research (19-7) from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (20591318).

The authors have no conflict of interests.

Supplementary information

Supplementary Table 1S: Sources and culture conditions of the cell lines used.

References

1. Ashman LK. The biology of stem cell factor and its receptor C-kit. Int J Biochem Cell Biol 1999;31:1037-51

2. Patnaik MM, Tefferi A, Pardanani A. Kit: molecule of interest for the diagnosis and treatment of mastocytosis and other neoplastic disorders. Curr Cancer Drug Targets 2007;7:492-503

3. Wehrle-Haller B. The role of Kit-ligand in melanocyte development and epidermal homeostasis. Pigment Cell Res 2003;16:287-96

4. Montone KT, van Belle P, Elenitsas R, Elder DE. Proto-oncogene c-kit expression in malignant melanoma: protein loss with tumor progression. Mod Pathol 1997;10:939-44

5. Natali PG, Nicotra MR, Winkler AB, Cavaliere R, Bigotti A, Ullrich A. Progression of human cutaneous melanoma is associated with loss of expression of c-kit proto-oncogene receptor. Int J Cancer 1992;52:197-201

6. Funasaka Y, Boulton T, Cobb M, Yarden Y, Fan B, Lyman SD, Williams DE, Anderson DM, Zakut R, Mishima Y, et al. c-Kit-kinase induces a cascade of protein tyrosine phosphorylation in normal human melanocytes in response to mast cell growth factor and stimulates mitogen-activated protein kinase but is down-regulated in melanomas. Mol Biol Cell 1992;3:197-209

7. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol 2006;24:4340-6

8. Antonescu CR, Busam KJ, Francone TD, Wong GC, Guo T, Agaram NP, Besmer P, Jungbluth A, Gimbel M, Chen CT, Veach D, Clarkson BD, 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

9. Rivera RS, Nagatsuka H, Gunduz M, Cengiz B, Gunduz E, Siar CH, Tsujigiwa H, Tamamura R, Han KN, Nagai N. C-kit protein expression correlated with activating mutations in KIT gene in oral mucosal melanoma. Virchows Arch 2008;452:27-32

10. Lutzky J, Bauer J, Bastian BC. Dose-dependent, complete response to imatinib of a metastatic mucosal melanoma with a K642E KIT mutation. Pigment Cell Melanoma Res 2008

11. Hodi FS, Friedlander P, Corless CL, Heinrich MC, Mac Rae S, Kruse A, Jagannathan J, Van den Abbeele AD, Velazquez EF, Demetri GD, Fisher DE. Major response to imatinib mesylate in KIT-mutated melanoma. J Clin Oncol 2008;26:2046-51

12. Ishihara K, Saida T, Otsuka F, Yamazaki N. Statistical profiles of malignant melanoma and other skin cancers in Japan: 2007 update. Int J Clin Oncol 2008;13:33-41

13. Johnson DS, Yamane S, Morita S, Yonehara C, Wong JH. Malignant melanoma in non-Caucasians: experience from Hawaii. Surg Clin North Am 2003;83:275-82

14. Murata H, Ashida A, Takata M, Yamaura M, Bastian BC, Saida T. Establishment of a novel melanoma cell line SMYM-PRGP showing cytogenetic and biological characteristics of the radial growth phase of acral melanomas. Cancer Sci 2007;98:958-63

15. Kanakura Y, Furitsu T, Tsujimura T, Butterfield JH, Ashman LK, Ikeda H, Kitayama H, Kanayama Y, Matsuzawa Y, Kitamura Y. Activating mutations of the c-kit proto-oncogene in a human mast cell leukemia cell line. Leukemia 1994;8 Suppl 1:S18-22

16. Sheu LF, Lee WC, Lee HS, Kao WY, Chen A. Co-expression of c-kit and stem cell factor in primary and metastatic nasopharyngeal carcinomas and nasopharyngeal epithelium. J Pathol 2005;207:216-23

17. Takata M, Goto Y, Ichii N, Yamaura M, Murata H, Koga H, Fujimoto A, Saida T. Constitutive activation of the mitogen-activated protein kinase signaling pathway in acral melanomas. J Invest Dermatol 2005;125:318-22

18. Roskoski R, Jr. Structure and regulation of Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun 2005;338:1307-15

19. Tarn C, Merkel E, Canutescu AA, Shen W, Skorobogatko Y, Heslin MJ, Eisenberg B, Birbe R, Patchefsky A, Dunbrack R, Arnoletti JP, von Mehren M, et al. Analysis of KIT mutations in sporadic and familial gastrointestinal stromal tumors: therapeutic implications through protein modeling. Clin Cancer Res 2005;11:3668-77

20. Antonescu CR, Besmer P, Guo T, Arkun K, Hom G, Koryotowski B, Leversha MA, Jeffrey PD, Desantis D, Singer S, Brennan MF, Maki RG, et al. Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. Clin Cancer Res 2005;11:4182-90

21. O'Riain C, Corless CL, Heinrich MC, Keegan D, Vioreanu M, Maguire D, Sheahan K. Gastrointestinal stromal tumors: insights from a new familial GIST kindred with unusual genetic and pathologic features. Am J Surg Pathol 2005;29:1680-3

22. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D, Bastian BC. Distinct sets of genetic alterations in melanoma. N Engl J Med 2005;353:2135-47

23. Sleijfer S, Wiemer E, Verweij J. Drug Insight: gastrointestinal stromal tumors (GIST)--the solid tumor model for cancer-specific treatment. Nat Clin Pract Oncol 2008;5:102-11

24. Chow LQ, Eckhardt SG. Sunitinib: from rational design to clinical efficacy. J Clin Oncol 2007;25:884-96

25. Shen SS, Zhang PS, Eton O, Prieto VG. Analysis of protein tyrosine kinase expression in melanocytic lesions by tissue array. J Cutan Pathol 2003;30:539-47

26. Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele AD, Druker BJ, Kiese B, Eisenberg B, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003;21:4342-9

27. Prenen H, Cools J, Mentens N, Folens C, Sciot R, Schoffski P, Van Oosterom A, Marynen P, Debiec-Rychter M. Efficacy of the kinase inhibitor SU11248 against gastrointestinal stromal tumor mutants refractory to imatinib mesylate. Clin Cancer Res 2006;12:2622-7

28. Tamborini E, Bonadiman L, Negri T, Greco A, Staurengo S, Bidoli P, Pastorino U, Pierotti MA, Pilotti S. Detection of overexpressed and phosphorylated wild-type kit receptor in surgical specimens of small cell lung cancer. Clin Cancer Res 2004;10:8214-9

29. Tamborini E, Miselli F, Negri T, Lagonigro MS, Staurengo S, Dagrada GP, Stacchiotti S, Pastore E, Gronchi A, Perrone F, Carbone A, Pierotti MA, et al. Molecular and biochemical analyses of platelet-derived growth factor receptor (PDGFR) B, PDGFRA, and KIT receptors in chordomas. Clin Cancer Res 2006;12:6920-8

30. Lagonigro MS, Tamborini E, Negri T, Staurengo S, Dagrada GP, Miselli F, Gabanti E, Greco A, Casali PG, Carbone A, Pierotti MA, Pilotti S. PDGFRalpha, PDGFRbeta and KIT expression/activation in conventional chondrosarcoma. J Pathol 2006;208:615-23

31. Abrams TJ, Lee LB, Murray LJ, Pryer NK, Cherrington JM. SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer. Mol Cancer Ther 2003;2:471-8

32. Krystal GW, Honsawek S, Litz J, Buchdunger E. The selective tyrosine kinase inhibitor STI571 inhibits small cell lung cancer growth. Clin Cancer Res 2000;6:3319-26

33. Krug LM, Crapanzano JP, Azzoli CG, Miller VA, Rizvi N, Gomez J, Kris MG, Pizzo B, Tyson L, Dunne M, Heelan RT. Imatinib mesylate lacks activity in small cell lung carcinoma expressing c-kit protein: a phase II clinical trial. Cancer 2005;103:2128-31

34. Ugurel S, Hildenbrand R, Zimpfer A, La Rosee P, Paschka P, Sucker A, Keikavoussi P, Becker JC, Rittgen W, Hochhaus A, Schadendorf D. Lack of clinical efficacy of imatinib in metastatic melanoma. Br J Cancer 2005;92:1398-405

35. Wyman K, Atkins MB, Prieto V, Eton O, McDermott DF, Hubbard F, Byrnes C, Sanders K, Sosman JA. Multicenter Phase II trial of high-dose imatinib mesylate in metastatic melanoma: significant toxicity with no clinical efficacy. Cancer 2006;106:2005-11

36. Becker JC, Brocker EB, Schadendorf D, Ugurel S. Imatinib in melanoma: a selective treatment option based on KIT mutation status? J Clin Oncol 2007;25:e9

37. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007;445:851-7

Figure legends

Figure 1. (A) Immunohistochemistry for KIT showing membrane staining in a tumor with wild-type *KIT* amplification (case 20). (B) The Western blot showing KIT receptor expression and phosphorylation in a substantial number of surgical specimens of acral and mucosal melanomas. N, normal lymph node as a negative control; HMC-1, human mast cell leukemia line; MC, normal melanocytes stimulated with 10 ng/ml of SCF. (C) Immunohistochemistry showing SCF expression in melanoma cells (case 7). Dark brown pigments are melanin.

Figure 2. (A) RT-PCR showing mRNA expression of *KIT* and *SCF* in acral melanoma cell lines. (B) The Western blot shows weak KIT expression in Mel-2 cells, and strong KIT expression in SMYM-PRGP and SM3 cells. (C) The Western blot shows weak and strong phosphorylation of KIT in Mel-2 and SMYM-PRGP cells, respectively, after the stimulation with 100 ng/ml of SCF. SM2-1 cells that lack KIT expression are used as a control. SM3 harboring a D820Y mutation shows the constitutive phosphorylation of KIT without SCF stimulation.

Figure 3. (A) *In vitro* drug testing shows dose-dependent inhibition of cell proliferation by sunitinib in SM3 cells, whereas imatinib has no effect (left panel). Both imatinib and sunitinib show dose-dependent inhibition of SCF-induced cell proliferation in SMYM-PRGP cells (middle panel). There is little or no growth suppression by imatinib and sunitinib in SM2-1 cells that lack KIT expression (right panel). Columns are mean values for quadruplicate experiments. Bars, SD. *, $p<0.01$. (B) Both imatinib and sunitinib show dose-dependent inhibition of SCF-stimulated KIT phosphorylation in SMYM-PRGP cells. Sunitinib shows more potent inhibition than imatinib.