

Emergence of CTNNB1 mutation at acquired resistance to KIT inhibitor in metastatic melanoma

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Abstract

Purpose The KIT inhibitor, imatinib, has shown promising efficacy in patients with KIT-mutated melanoma; however, acquisition of resistance to imatinib occurs rapidly in the majority of patients. The mechanisms of acquired resistance to imatinib in melanoma remain unclear.

Methods We analyzed biopsy samples from paired baseline and post-treatment tumor lesions in one patient with KIT-mutated melanoma who had had an initial objective tumor regression in response to imatinib treatment followed by disease progression 8 months later.

Results Targeted deep sequencing from post-treatment biopsy samples detected an additional mutation in CTNNB1 (S33C) with original KIT L576P mutation. We examined the functional role of the additional CTNNB1

S33C mutation in resistance to imatinib indirectly using the Ba/F3 cell model. Ba/F3 cell lines transfected with both the L576P KIT mutation and the CTNNB1 S33C mutation demonstrated no growth inhibition despite imatinib treatment, whereas growth inhibition was observed in the Ba/F3 cell line transfected with the L576 KIT mutation alone.

Conclusions We report the first identification of the emergence of a CTNNB1 mutation that can confer acquired resistance to imatinib. Further investigation into the causes of acquired resistance to imatinib will be essential to improve the prognosis for patients with KIT-mutated melanoma.

Keywords KIT · Melanoma · Imatinib · Acquired resistance · CTNNB1

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Introduction

KIT is a transmembrane receptor tyrosine kinase normally expressed on hematopoietic progenitor cells, primordial germ cells, the interstitial cells of Cajal, and melanocytes [1]. KIT has an essential role in cell growth and survival through activation of downstream mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways [2, 3]. The importance of oncogenic KIT mutations as therapeutic targets has been demonstrated in several malignancies, including gastrointestinal stromal tumors (GIST), which frequently harbor activating KIT mutations [4, 5]. Treatment with imatinib, a selective inhibitor targeting KIT, ABL, and platelet-derived growth factor receptor (PDGFR), results in overall response rates of approximately 50% in patients with GIST, with durable responses

and a median progression-free survival of 18 months [6]. Somatic mutation of KIT has been reported in approximately 10–20% of melanomas arising in mucosal membranes, acral skin, and skin with chronic sun-induced damage [7, 8]. Phase II studies testing imatinib in patients with metastatic melanoma with mutated or amplified KIT have reported overall response rates of 20–30% and disease control rates of 35–55% [9, 10]. Furthermore, treatment with imatinib resulted in a trend toward improved response in patients with KIT-mutated melanoma, with response rates of approximately 50%. However, patients receiving imatinib eventually developed resistance to the drug and demonstrated tumor progression within 4 months [11].

Imatinib resistance in patients with GIST is primarily due to acquisition of additional mutations in KIT or PDGFR, which decrease their binding affinity for imatinib [12], and/or activation of pathways other than those mediated by KIT and PDGFR, thereby bypassing the inhibitory effects of KIT/PDGFR-targeted small molecule treatment. In patients with KIT-mutated melanoma, the activation of the MAPK, PI3K signaling pathway due to additional mutations in KIT and the recently described oncogenic N-RASQ^{61k} mutation are associated with secondary resistance to KIT inhibitors [13–15]. However, the mechanisms of acquired resistance to imatinib in malignant melanoma remain unclear. In this study, we assessed the effects of imatinib on the genomic evolution of melanoma and confirmed an association between genetic changes and resistance to imatinib using the Ba/f3 cell model, in an effort to determine genetic mechanisms of acquired resistance to KIT inhibition therapy in melanoma patients.

Materials and methods

Patient selection

The study was approved by the institutional review board of the Samsung Medical Center. We collected and reviewed the medical records of patients with KIT-mutated metastatic melanoma who were treated with imatinib between 2014 and 2016 at a single center in Korea. For inclusion in this analysis, patients were required to meet three selection criteria: first, they must have exhibited an objective tumor response during treatment with imatinib; second, they must have acquired resistance to treatment, defined as systemic progression of disease while receiving continuous treatment with imatinib, after more than 1 month of tumor response; third, biopsy specimens adequate for targeted deep sequencing must have been obtained at two time points (before the initiation of imatinib therapy and after disease progression). Tumor response was assessed by physical examination and

imaging every 6–8 weeks, using the Response Evaluation Criteria in Solid Tumors version 1.1 [16].

Targeted sequencing

Genomic DNA was extracted, and a SureSelect customized kit (Agilent Technologies, Santa Clara, CA, USA) was used to capture 381 cancer-related genes. An Illumina HiSeq 2500 was used for sequencing with 100-bp paired-end reads. The sequencing reads were aligned to the human genome reference sequence (hg19) using BWA (v0.7.5) with the “MEM” algorithm. We used SAMTOOLS (v0.1.18) and Picard (v1.93) for sorting SAM/BAM files and duplicate marking, respectively. Local realignment and base recalibration by GATK (v3.1.1) were carried out based on dbSNP137, Mills indels, HapMap, and Omni. Single-nucleotide variations (SNVs) and insertions/deletions (InDels) were identified using Mutect (v1.1.4) and Pindel (v0.2.4), respectively. ANNOVAR was used to annotate the detected variants. Only variants with >1% allele frequency were included in the results.

Expression vector construction and PCR-based site-directed mutagenesis

For construction of mammalian expression vectors for use in Ba/F3 cell assays (see “Cell lines and transfections”, below) to determine the effects of somatic mutations identified in patient tumor samples, total cellular RNA was isolated from U87MG cells and cDNA generated by reverse transcription using standard protocols. Inserts in wild-type constructs were amplified by PCR using the primers listed in Table S1. To create mutant sequences, PCR-based site-directed mutagenesis was performed. Briefly, cDNA was first amplified by PCR to produce two fragments with a 20–25 bp overlap containing the site to be mutated. Then, another round of PCR using nested primers was performed to fuse the fragments into a single molecule and introduce the mutation. The wild-type and mutant sequences of *KIT* and *CTNNB1* were cloned into the Not I and BamH I sites of p3xFlag-CMV (Promega) using DH5 α competent *Escherichia coli* (Life technologies). All construct sequences were verified by Sanger sequencing. The primer sequences used are described in supplementary table S1.

Cell lines and transfections

Ba/F3 cells were obtained from RIKEN BRC Cell Bank (Ibaraki, Japan), and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1 ng/ml of recombinant IL3 (R&D systems, Minneapolis, USA). Cells (5×10^6) were electroporated with 1.5 μ g of pcDNA3.1/KIT L576P

Table 1 Patient baseline characteristics

Patient	Age	Sex	Primary site	Stage	Metastatic lesions	KIT mutation	Best response ^a	Progression-free survival ^a
1	52	Male	Acral melanoma	IV (M1b)	Multiple lung metastases	Exon 11 (L576P)	Partial response	8 months

^a With imatinib treatment

constructs using the Nucleofector system (Lonza, Basel, Switzerland). Successfully transfected cells were selected in medium containing G418 for 2 weeks. After confirmation of the expression of the full-length proteins by immunoblotting, cells were electroporated with 1.5 µg of the mutant construct, CTNNB1 S33C (p3xFlag-CMV backbone).

Cell growth and cell viability assays

Ba/F3 cells (5×10^3 per well) were plated in quadruplicate in 96-well plates, in 0.1 ng/ml of IL3. Then 1 µM of imatinib was treated per well to detect cell viability. Cell viability was determined using CellTiter-Glo (Promega, Wisconsin, USA) according to the manufacturer's protocol every 24 h for 3 days to determine cell growth rate, and for a further 72 h after imatinib treatment.

Immunoblot analysis

Total proteins were extracted from Ba/F3 cells using Complete Lysis M buffer containing protease-inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase-inhibitor cocktail (Roche). Protein concentrations were determined using the Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Proteins (30 µg) were subjected to 8% SDS PAGE, and electrotransferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dried milk in TBS containing 0.1% v/v Tween 20 and probed overnight at 4 °C with specific antibodies against Flag (1:5000; Invitrogen, Waltham, MA, USA) and beta actin (1:5000; Sigma Aldrich). Horseradish peroxidase-conjugated anti-rabbit and mouse IgGs (Vector, Burlingame, CA, USA) were used as secondary antibodies. Immune complexes were visualized by enhanced chemiluminescence using ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

Statistical analysis

Statistical analyses were performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Cell viability test were analyzed by Tukey's honest significant difference (HSD) test, which is a single step multiple comparison statistical test.

Results

Clinical course of the patient

Of the seven patients with KIT-mutated metastatic melanoma who were treated in our hospital with imatinib between 2014 and 2016, one patient met all three selection criteria for inclusion in this study. This 52-year-old male patient had acral melanoma, which had disseminated to the lung and had progressed after two lines of conventional cytotoxic chemotherapy (dacarbazine-based and paclitaxel-based chemotherapy). The patient harbored a *KIT* mutation in exon 11, encoding a L576P substitution, and had no mutations in *BRAF* at diagnosis. After 8 weeks of imatinib treatment, the patient experienced a continued partial response, with a decrease in size of lung metastases. The lung metastases began to progress after 6 months of tumor response to imatinib treatment was discontinued. The baseline characteristics of the patient are described in Table 1 and the changes in lung metastases during the course of imatinib treatment are illustrated in Fig. 1.

Genetic changes after treatment with KIT inhibitor

A baseline biopsy sample was obtained before an early course of therapy with dacarbazine-based chemotherapy and the post-treatment biopsy sample was obtained immediately following disease progression. Both biopsy samples were obtained from the lung metastases.

To identify mutations that might confer acquired resistance, we extracted DNA from the bulk-tumor biopsy samples and performed targeted deep sequencing to compare baseline and post-treatment tumor tissues. A *KIT* mutation in exon 11 (c.1727T > C), encoding the L576P substitution, which was sensitive to imatinib, was originally identified in the baseline biopsy sample and the post-treatment biopsy sample also contained the same mutation. We screened SNVs and CNVs using 80-gene panel, and *KIT* was the only aberration detected in baseline biopsy. In the post-treatment biopsy sample, we also identified an additional mutation in *CTNNB1* exon 3 (c.98C > G), encoding S33C, which leads to the loss of a phosphorylation site in the β-catenin protein.

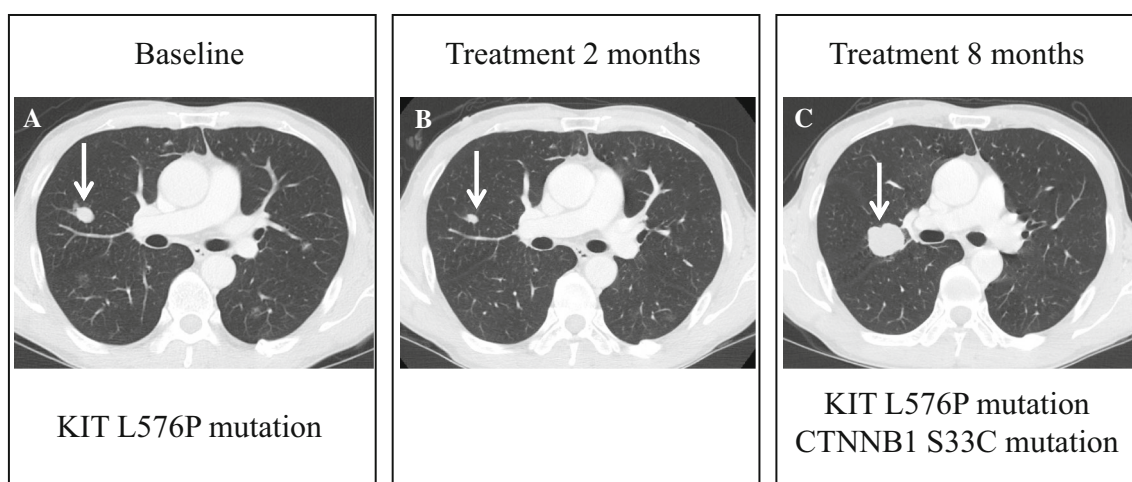


Fig. 1 The changes in lung metastasis during imatinib treatment and results of mutation analysis from baseline and post-treatment biopsy samples

Use of Ba/F3 cells to determine whether the CTNNB1 mutation can confer resistance to imatinib

Ba/F3, a murine interleukin 3 (IL3)-dependent pro-B cell line, is increasingly popular as a model system for assessing the ability of small molecule kinase inhibitors to block kinase activity and for predicting resistance to small molecule kinase inhibitors elicited by point mutations [17].

To determine the relationship between the acquired CTNNB1 and original KIT mutations and the acquisition of resistance to imatinib, the murine Ba/F3 cell line was either transfected with a construct encoding KIT L576P, or co-transfected with both KIT L576P and CTNNB1 S33C constructs. Both transfected Ba/F3 cell lines exhibited sustained growth in the IL3-minimal medium, whereas control Ba/F3 cells did not grow (Fig. 2a). To examine the effect of the additional CTNNB1 mutation on the antitumor effect of imatinib, we performed cell viability analysis after 72 h of imatinib treatment. As shown in Fig. 2b, Ba/F3 cell lines expressing mutated KIT L576P showed enhanced sensitivity to imatinib, with greater inhibition of cell proliferation than Ba/F3 cells expressing both KIT L576P and CTNNB1 S33C mutations or controls. A high IC₅₀ value of 7.592 $\mu\text{M}/\text{mL}$ for imatinib was determined in Ba/F3 cell lines carrying both KIT L576P and CTNNB1 S33C mutations, compared with an IC₅₀ value of 3.258 $\mu\text{M}/\text{mL}$ in the Ba/F3 cell line carrying the KIT L576P mutation alone. This finding suggests that the additional mutation of CTNNB1 could have an important role in the mechanism of tumor resistance to KIT inhibition.

Discussion

Despite the recent development of novel therapeutic agents, including BRAF inhibitors, MEK inhibitors, and immune checkpoint inhibitors, treatment of patients with metastatic melanoma remains challenging owing to the development of acquired resistance to the new therapeutic drugs. The KIT inhibitor, imatinib, has shown promising efficacy in patients with KIT-mutated melanoma in phase II studies; however, acquisition of resistance occurs rapidly in the majority of patients [9, 10]. Recent studies demonstrated that additional A829P or T670I KIT mutations, along with activation of the NRAS, MAPK, and PI3K signaling pathways, were associated with resistance to KIT inhibition [13–15]; however, the cause of resistance to imatinib treatment is likely to be multifactorial and remains unclear.

The activation of WNT/ β -catenin signaling through mutation of CTNNB1 could be one mechanism of resistance to KIT inhibition treatment in malignant melanoma. In our study, we identified a patient with malignant melanoma who originally harbored a L576P mutation in KIT and achieved an initial partial response to treatment with imatinib. The patient progressed 8 months after starting imatinib treatment, with an additional S33C mutation in CTNNB1. We examined the functional role of the additional CTNNB1 S33C mutation in resistance to imatinib indirectly using the Ba/F3 cell model. Ba/F3 cell lines harboring both the L576P KIT mutation and the CTNNB1 S33C mutation demonstrated no growth inhibition despite imatinib treatment, whereas growth inhibition was observed in the Ba/F3 cell line transfected with the L576P KIT mutation alone.

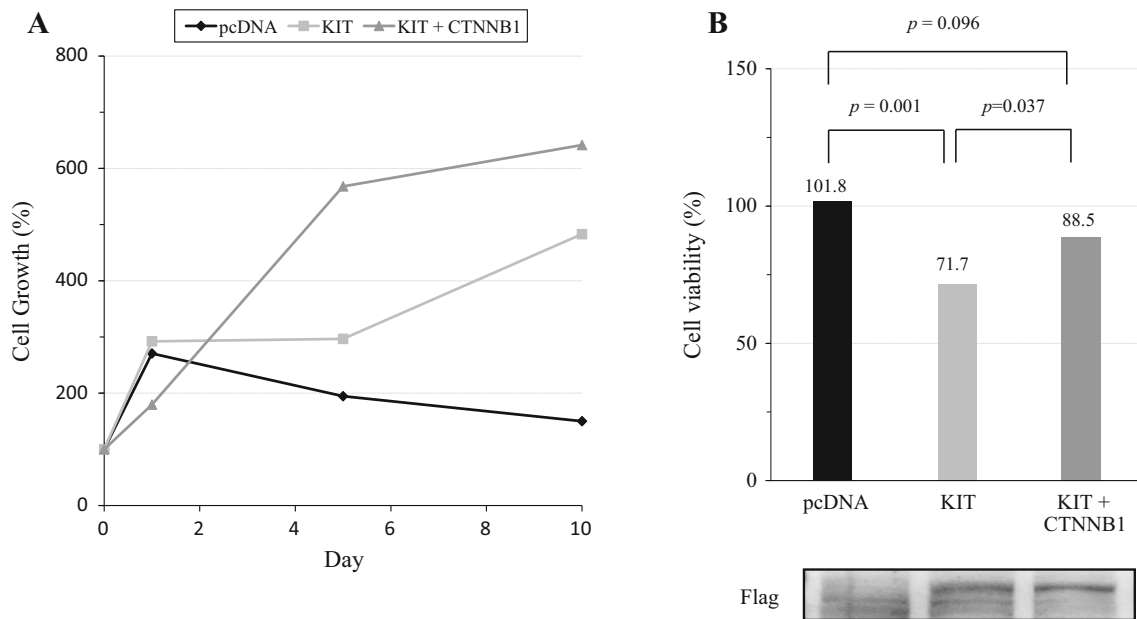


Fig. 2 Analysis of functional role of the additional CTNNB1 S33C mutation on original KIT L576P mutation in resistance to imatinib treatment using the Ba/F3 cell models. **a** Graphs of cell growth rate in 0.1 ng/ml of IL3. Ba/F3 cell lines either transfected with a construct encoding KIT L576P, or co-transfected with both KIT L576P and CTNNB1 S33C constructs exhibited sustained growth in the IL3-

minimal medium, whereas control Ba/F3 cells did not grow. **b** Cell viability test after 72 h of imatinib treatment. Ba/F3 cell lines expressing mutated KIT L576P showed enhanced sensitivity to imatinib, with greater inhibition of cell proliferation than Ba/F3 cells expressing both KIT L576P and CTNNB1 S33C mutations or controls

The *CTNNB1* gene encodes β -catenin, a subunit of the cadherin protein complex, which acts as an intracellular signal transducer in the WNT signaling pathway, which regulates the coordination of cell to cell adhesion and gene transcription [18]. The WNT/ β -catenin pathway inhibits the degradation of β -catenin, leading to its accumulation in the nucleus and consequent regulation of target gene expression. β -catenin is first phosphorylated by casein kinase (CK1) at Ser45, followed by glycogen synthase kinase 3 β (GSK3 β)-mediated phosphorylation at Ser33, Ser37, and Thr41 within the β -catenin destruction complex, which contains the proteins adenomatous polyposis coli (APC), GSK3 β , and Axin [19]. Activating point mutations at these amino acid residues prevent β -catenin from being phosphorylated, thereby blocking its subsequent ubiquitination and proteasomal degradation [20]. Activating mutations in β -catenin (CTNNB1) occur in a high proportion of uterine and colorectal cancers, medulloblastomas, and hepatocellular carcinomas [21]. In several cancers, including colorectal cancer, high levels of nuclear β -catenin, which are normally interpreted as a sign of increased WNT/ β -catenin signaling activity, are correlated with poor prognosis [22].

WNT/ β -catenin signaling is necessary and sufficient to drive neural crest cells toward a melanocyte cell fate, primarily through direct regulation of the transcriptional target, microphthalmia-associated transcription factor (MITF)

[23]. The WNT/ β -catenin signaling pathway is frequently activated in melanoma; however, the relationship between carcinogenesis and aberrant regulation of the WNT/ β -catenin pathway in melanoma remains controversial [24, 25]. In different studies, β -catenin has been associated with both improved and poorer prognosis in malignant melanomas. Beta-catenin was associated with inhibition of proliferation and migration of murine melanoblasts and human melanoma; however, it has also been shown to promote melanocyte immortalization by overcoming senescence, and increase metastatic potential [23]. Interestingly, constitutively active CTNNB1 also synergizes with the active form of NRAS to induce the generation of melanomas with high penetrance and short latency [26]. Similarly, in lung cancer, overexpression of constitutively active CTNNB1 does not result in the development of lung tumors, whereas co-expression of constitutively active forms of both KRAS and CTNNB1 induces lung tumor formation [27]. Crosstalk between the WNT/ β -catenin signaling pathway and other oncogenes has also been demonstrated in mouse models of cervical cancer and hepatocellular carcinoma [28, 29]. These findings suggest that aberration of the WNT/ β -catenin signaling pathway is not sufficient to drive tumorigenesis; however, aberrant CTNNB1 signaling may cooperate with various other oncogenes and tumor suppressor genes to promote aggressive tumorigenesis. Furthermore, our data from

experiments using the Ba/F3 cell model indicate that newly emerged CTNNB1 mutations can also promote resistance to molecular targeted therapies, including treatment with KIT inhibitors.

In our study, comprehensive mechanistic evaluation to examine how CTNNB1 S33C mutation modulates the resistance to KIT inhibition and in vivo test to validate its role was not performed. In addition, it was not verified that in the patients without progression the mutation in the gene was absent. Because of these limitations, further preclinical in vivo and epidemiological studies are required to confirm the role of CTNNB1 mutation on the resistance to KIT inhibition therapy.

In conclusion, we report the first identification of the emergence of a CTNNB1 mutation which confers acquired resistance to imatinib. These results suggest that the WNT/ β -catenin pathway may have an important role in a mechanism of resistance to imatinib in malignant melanoma. Further investigation into the causes of acquired resistance to imatinib will be essential to improve the prognosis for patients with KIT-mutated melanoma.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the local ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent statement For our retrospective study, formal consent is not required.

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