***KIT* and *BRAF* heterogeneous mutations in gastrointestinal stromal tumors after secondary imatinib resistance**

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Abstract

Background and aims Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of the digestive tract and characterized by expression of KIT protein. Imatinib is the frontline therapy for metastatic and unresectable GIST patients showing clinical responses in 80 % of cases. Despite the often long-lasting clinical benefit seen in most patients treated with imatinib, many will eventually suffer disease progression. The most frequent mechanism of imatinib resistance in GIST is the acquisition of secondary mutations in either *KIT* or *PDGFRA*. There are also some imatinib-resistant GIST patients lacking an identifiable mechanism of treatment failure. Recently, activating *BRAF* mutation was detected in a small percentage of GISTs. In this study, we report a case of GIST with acquired resistance to imatinib during therapy.

Methods Histological, immunohistochemical, Western blot and mutational analyses were performed on GIST tissues before and after imatinib resistance.

Results The imatinib-resistant tumor showed not only heterogeneous mutations of *KIT* and *BRAF* besides the primary mutation, but also transdifferentiation into a rhabdomyosarcoma phenotype. According to Western blot analysis, in imatinib-resistant GIST with both *KIT* V559D and *BRAF* V600E mutations, the inhibition of *KIT* V559D by imatinib caused a strong decrease of AKT phosphorylation, while ERK1/2 phosphorylation was not affected.

Conclusions This finding, in combination with the loss of KIT expression, suggests the possibility of activation of RAS-RAF-MEK-ERK pathways driven by a KIT-independent oncogenic mechanism. Understanding the genetic aberrations beyond *KIT* and *PDGFRA* may lead to the identification of additional therapeutic targets for GISTs.

Keywords Gastrointestinal stromal tumor · KIT · BRAF · Rhabdomyosarcomatous differentiation · Imatinib resistance

Introduction

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors arising in the gastrointestinal tract, mainly in the stomach and the small intestine. They are characterized by mutations in the *KIT* or *PDGFRA* gene [1, 2]. Nevertheless, there are 10–15 % of GISTs without *KIT* or *PDGFRA* mutations [3]. Imatinib mesylate is the first drug to target the KIT and PDGFRA proteins responsible for the cancer directly [4]. It is very effective in GISTs bearing the *KIT* exon 11 mutation. It is also effective in treating GISTs bearing the *KIT* exon 9 mutation, but at a higher dose [5]. GISTs with exon 13 or 17 mutations in *KIT*, with D842V mutations in *PDGFRA* or without mutations in *KIT* or *PDGFRA* are more frequently imatinib-resistant.

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The downstream signaling cascade activated by *KIT* includes the RAS-RAF-MEK-ERK (mitogen-activated protein kinase, MAPK) pathway, the phosphatidylinositol-3-kinase (PI3K)-AKT-mTOR pathway, and the JAK-STAT kinase pathway [6, 7]. According to the type of *KIT* mutation, one of these pathways is activated preferentially [5]. The *BRAF* gene encodes for a serine/threonine-protein kinase [8]. Its function is to control proliferation and differentiation through the RAS-RAF-MEK-ERK pathway. It is mutated in a wide range of cancers, with a high rate in malignant melanoma, thyroid carcinoma, and colorectal cancer with microsatellite instability [9]. Most mutations lie within the kinase domain with a single nucleotide substitution at position 1799 in exon 15, leading to the V600E amino-acid substitution (98 %). Recently, the *BRAF* mutation was detected in three GISTs without *KIT* or *PDGFRA* mutation and in one tumor after imatinib treatment failure [10].

It could be postulated that the dysregulation of the RAS-RAF-MEK-ERK (MAPK) pathway may play a role in GIST pathogenesis and/or progression. Thus, the detection of alterations in the MAPK pathway in GIST would be innovative and could be relevant in tumorigenesis and receptor tyrosine kinase inhibitor resistance. The challenge is not only to find new drugs able to act on GISTs resistant to the available drugs, but also to identify other genomic alterations responsible for this cancer. In this study, we report a case of GIST with acquired resistance to imatinib during therapy, where we found heterogeneous mutations of *KIT* and *BRAF* besides primary mutation and rhabdomyosarcomatous transdifferentiation.

Materials and methods

Patient presentation

A 75-year-old male with stomach GIST was admitted with a 1-month history of gastric pain along with reduced appetite and weight loss. A contrast-enhanced computed tomography (CT) scan confirmed the presence of a distal gastric mass $11 \times 8 \times 5$ cm in size with peripheral contrast enhancement. No liver or lymph node metastases were detected. The tumor was surgically removed and diagnosed as CD117-positive gastric GIST. The patient could not afford imatinib adjuvant therapy because of economic reasons, so no adjuvant therapy was given. The disease recurred with multiple hepatic metastases 11 months later (Fig. 2a) when the patient's condition was severe and unsuitable for operation. Treatment with imatinib (400 mg/day) was therefore started. After 1 month, the hepatic metastases had obviously shrunk, and after 6 months of treatment the patient had achieved a near-complete

response in CT scans. Imatinib was used continuously, but after 18 months a new metastatic tumor was found in the pelvic cavity, although hepatic metastases were still under control. Escalation of imatinib (600 mg/day) was given then, but ascites were still developing in the pelvic cavity implant. Surgical debulking of the metastatic pelvic cavity tumor and biopsy of the hepatic metastases were done.

For the study involving human tumor tissues, informed consent was obtained, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Histopathological analysis

Tumor specimens were fixed in formalin and embedded routinely for histological evaluation. The GIST case was evaluated for the following: tumor cell type, cytological atypia and mitotic rate [expressed as the number of mitotic figures per 50 high power fields (HPFs)]. Risk stratification was performed according to the recent National Comprehensive Cancer Network guidelines [11]. Immunohistochemical stains were performed on 4- μ m sections cut from paraffin blocks, using the primary antibodies CD117, desmin, α -smooth muscle actin (SMA) and myogenin for the pre- and post-treatment tumor specimens. Immunohistochemical staining was classified as strong (+++), moderate (++) , weak (+) or negative. The categorization of histopathological subtype was based on standard and widely accepted criteria [12].

Molecular analysis

For mutational analysis, genomic DNA was extracted from fresh tissue or formalin-fixed paraffin-embedded tumor tissue sections. Mutational analysis of *KIT* exons 9, 11, 13, 14 and 17 and *PDGFRA* exons 12 and 18 was performed by polymerase chain reaction amplification, denaturing high-performance liquid chromatography screening and automated sequencing as previously described [13]. In addition, the tumor was also investigated for *KRAS*, *NRAS*, *PI3K* and *BRAF* mutations as described previously [10, 14, 15]. All mutations were confirmed by a second independent round of PCR and reverse sequencing.

Western blot analysis

For Western blotting, 50 mg of tumor was prepared by grinding in the following: a RIPA buffer (Sigma) with phosphatase inhibitors and with some protease inhibitors (Sigma). Equivalent amounts of protein (50 μ g) from clarified lysates were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes and immunoblotted

sequentially with the following antibodies. Anti-KIT and anti-BRAF antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were from Sigma. Anti-AKT, anti-phospho-AKT, anti-phospho-KIT and anti-GAPDH antibodies were from Cell Signal Technology. The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

Results

Histopathological features of prior treatment tumor samples

The primary gastric tumor resection specimen showed typical spindle cell morphology (Fig. 1a), being composed of uniform elongated cells with palely eosinophilic cytoplasm, ill-defined cell borders and ovoid nuclei. The tumor size was 11 cm. The mitotic count was 4–6/50HPF.

Fig. 1 Primary gastric tumor resection specimen. Typical gastrointestinal stromal tumors showing spindle cell morphology with palely eosinophilic cytoplasm, ill-defined cell borders and ovoid nuclei (a). Strong staining for CD117 (b)

Based on the tumor size and mitotic activity, the tumor corresponded to a high-risk GIST in both the NIH and AFIP risk stratification systems [16]. The immunohistochemistry revealed strong expression of CD117 (Fig. 1b) in most of the tumor cells, with limited focal SMA reactivity, while desmin and myogenin protein were negative.

Histopathological features of imatinib-resistant tumor samples

On resection of the metastatic pelvic cavity tumor, despite a heterogeneous response with some areas of fibrosis, there were microscopic foci of viable and proliferating tumor cells, in keeping with a resistant phenotype. In the imatinib-resistant tumor, the morphological appearances varied between different parts of metastases (marked pleomorphism and epithelioid cell type). Some of the imatinib-resistant tumor cells in the progressing pelvic metastases showed marked pleomorphism, which proved to be rhabdomyoblastic differentiation. The rhabdomyoblastic component was composed of spindle cells with round to oval nuclei, focally prominent nucleoli and an amphophilic to deeply eosinophilic cytoplasm with a bipolar or tadpole configuration resembling embryonal rhabdomyosarcoma (Fig. 2a). The rhabdomyoblastic differentiation tumor cells were completely negative for CD117 (Fig. 2b). They diffusely expressed desmin (Fig. 2c) and SMA. Myogenin was also expressed (Fig. 2d). Some parts of the imatinib-resistant tumor were composed of epithelioid cells with a palely eosinophilic to clear cytoplasm and round nuclei, arranged in sheets (Fig. 3a). Small areas of necrosis were seen. Mitotic activity was 8/50HPF. The epithelioid cells were negative for CD117 (Fig. 3b), desmin, SMA, and myogenin.

Mutational analyses

Molecular analysis was performed on genomic DNA gained from the prior and post-imatinib treatment GIST samples. The primary gastric tumor resection specimen harbored heterozygous *KIT* gene exon 11 mutation Val559Asp (V559D) (Fig. 4a). The recurrent metastases prior to imatinib treatment revealed the same V559D without secondary mutation. The imatinib-resistant GIST specimen was also examined. The primary *KIT* gene exon 11 V559D mutation can still be found in the metastatic lesions. Mutational analysis from DNA obtained from micro-dissection of the rhabdomyoblastic differentiation area showed the presence of a *BRAF* V600E mutation (Fig. 4b) in addition to the primary *KIT* V559D mutation. Mutational analysis of the non-rhabdomyoblastic differentiation tumor cells revealed a secondary mutation in *KIT*

Fig. 2 The imatinib-resistant tumor cells in the progressing pelvic metastases showed marked pleomorphism with rhabdomyoblastic differentiation (**a**). The rhabdomyoblastic differentiation tumor cells were completely negative for CD117 (**b**). Immunostaining demonstrates diffused desmin expression in a GIST metastasis with rhabdomyoblastic differentiation (**c**). Immunostaining shows nuclear myogenin expression in a GIST metastasis with rhabdomyoblastic differentiation (**d**)

exon 13 Val654Ala (V654A) (Fig. 4c) besides the primary *KIT* V559D mutation.

Western blot results

Activation of the MAPK and AKT pathways was investigated in GISTs before and after imatinib treatment. Phosphorylation of KIT, extracellular signal-regulated kinase (ERK)1/2 and AKT were investigated by Western blotting. KIT, ERK1/2, AKT, BRAF and GAPDH protein levels are shown as control. In the primary gastric tumor resection specimen, phosphorylation of ERK1/2 was below the detection level, whereas an increase in KIT and AKT phosphorylation was observed. Imatinib treatment strongly reduced KIT phosphorylation, and this resulted in the abrogation of AKT phosphorylation. Expression of ERK1/2 phosphorylation was observed in the imatinib-resistant GIST specimen with *BRAF* V600E secondary mutation, which was not affected by imatinib treatment. Imatinib still abrogated AKT phosphorylation by reducing KIT phosphorylation. No effect of *BRAF* V600E on AKT activation was observed. However, in the imatinib-resistant GIST

specimen with secondary *KIT* V654A mutation, phosphorylations of ERK1/2, AKT and KIT were all below the detection level (Fig. 5).

Discussion

Therapeutic inhibition of KIT or PDGFRA by the small-molecule inhibitor imatinib gives a good clinical response in the majority of advanced GISTs [17]. However, secondary resistance has been reported after a median of 2 years of treatment because of the presence of secondary mutation [18, 19]. Half of the imatinib-resistant GIST patients lack an identifiable mechanism of resistance, such as a secondary *KIT* mutation or *KIT* gene amplification [20, 21]. Resistance of GIST patients to imatinib is a continuous clinical challenge, so multiple novel therapeutic strategies are under development.

As result of *KIT* and *PDGFRA* mutations, GISTs harbor constitutively activated KIT and/or PDGFRA receptors, which in turn lead to a phosphorylation cascade of the tyrosine residues in multiple downstream signaling

Fig. 3 The imatinib-resistant tumor cells in the progressing pelvic metastases showed epithelioid differentiation with pale eosinophilic cytoplasm and round nuclei (a). The epithelioid differentiation tumor cells were completely negative for CD117 (b)

molecules and activation of signal transduction pathways including RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR signaling networks [22]. PI3K pathway activation by growth factors through RTKs leads to AKT activation resulting in expression of mTOR. A second pathway triggers the activation of RAS and therefore starts the RAS-RAF-MEK-ERK pathway. In addition, BRAF and PI3K are interacting with each other by mutual inhibition. Understanding the genetic aberrations beyond KIT and PDGFRA may lead to the identification of additional therapeutic targets for GISTs.

Recently, a further kind of imatinib resistance has been reported to be the presence of mutations in downstream effectors, such as BRAF and KRAS, in a small percentage of GISTs [10, 14, 23, 24]. Furthermore, a *PIK3CA* mutation (H1047L) simultaneously occurring with a 15-bp deletion in *KIT* exon 11 was detected in a GIST patient [15]. The *BRAF* gene encodes for a serine/threonine-protein kinase [8]. Its function is to control proliferation and

Fig. 4 Molecular analysis showed *KIT* exon 11 V559D mutation in the primary gastric tumor (a) and the presence of secondary *BRAF* V600E mutation in the rhabdomyoblastic differentiation area after imatinib resistance (b). Mutational analysis of the non-rhabdomyoblastic differentiation tumor cells revealed a secondary mutation in *KIT* V654A (c) besides the primary *KIT* V559D mutation

differentiation through the RAS-RAF-MEK-ERK pathway. *BRAF* mutations have been found in a wide range of tumors, but at different levels [9]. They are mainly found in melanoma (50 %), thyroid papillary carcinoma (40 %) and colorectal tumors with microsatellite instability (10 %). Most mutations lie within the kinase domain with a single nucleotide substitution at position 1799 in exon 15, leading to the V600E amino-acid substitution (98 %). This modification mimics the phosphorylation of the kinase activation domain leading to permanent activation of the kinase. The remaining 2 % mutations are located in exon 11. Recently, the *BRAF* mutation was detected in three GISTs without *KIT* or *PDGFRA* mutation and in one tumor after imatinib treatment failure [10].

Some imatinib-resistant GIST patients lack an identifiable mechanism of resistance, such as a secondary *KIT* or *PDGFRA* mutation. In this subset the acquisition of mutations in the downstream signaling pathways, which are not targeted by imatinib, may occur. In this study, our hypothesis was that *BRAF* mutations, commonly involved

Fig. 5 Activation of the MAPK and AKT pathways was investigated in GISTs before and after imatinib treatment by Western blot analysis. Primary gastric GIST before imatinib treatment (a). The hepatic metastases after imatinib treatment (b). The imatinib-resistant GIST with *KIT* V559D and *BRAF* V600E mutation (c). The imatinib-resistant GIST with *KIT* V559D and *KIT* V654A mutation (d)

in other cancer types, may trigger an alternate mechanism of imatinib resistance in the GIST patient. The mechanism through which *BRAF* activating mutations in GISTs may affect imatinib-resistance remains unclear. Similar to other tumor types where *BRAF* mutations are more commonly observed, the mutation in GIST was present in the exon 15 V600E hot spot. No other mutations were identified in the *KRAS*, *NRAS* or *PI3K* in our patient. *BRAF* is a member of the RAF family of serine/threonine protein kinases, which are important effectors of RAS activation and involved in the signaling pathway, which connects extracellular signals to transcriptional regulation. Activated RAF proteins phosphorylate MEK1 and 2 (MAPKKs), which in turn phosphorylate ERK1 and 2 (MAPKs), leading to the activation of several cytoplasmic and nuclear targets, including transcription factors such as ETS11, c-JUN and c-MYC. Thus, the MEK-ERK effector pathway is commonly dysregulated in cancer, often through gain-of-function mutations of either RAS or RAF family members [9]. The RAS-RAF-MEK-ERK pathway is activated by many receptor tyrosine kinases, including KIT. Although untreated tumors show consistent activation of ERK1/2 phosphorylation, there is no effect of imatinib treatment on ERK activation [25]. Thus, *BRAF* mutated GIST patients may be similarly resistant to imatinib inhibition.

Our patient with a high-risk gastric GIST showed expression of KIT protein in the primary tumor and direct sequencing demonstrated a primary *KIT* exon 11 V559D in the absence of *BRAF*, *KRAS*, *NRAS* and *PI3K* mutations. The imatinib-resistant tumor was resected after 18 months of imatinib therapy and showed not only loss of KIT protein expression, but transdifferentiation into a rhabdomyosarcoma phenotype. These foci showed a high proliferation rate and diffuse expression for skeletal muscle markers, such as desmin and myogenin. A similar phenotypic switch or dedifferentiation phenomenon, with loss of KIT expression and acquisition of aberrant lines of differentiation, has been previously reported in rare cases of imatinib-resistant patients, but thus far was not attributed to a particular genotype [26, 27]. According to Martinelli et al. [28], a substantial minority of embryonal rhabdomyosarcoma shows mutations in the RAS-RAF-MEK-ERK pathway. As reported by Shukla et al. [29], dysregulation of RAS-RAF-MEK-ERK signaling is a major event contributing to embryonal rhabdomyosarcoma pathogenesis. Our findings provide evidence that a secondary *BRAF* mutation is perhaps a cause contributing to transdifferentiation into a rhabdomyosarcoma phenotype after imatinib resistance in GIST.

Mutational testing of samples showing heterologous rhabdomyoblastic differentiation shed light on specific molecular mechanisms, which might account for this unusual line of differentiation. Nevertheless, this finding, in combination with loss of KIT expression, suggests the possibility of activation of novel pathways driven by a KIT-independent oncogenic mechanism. According to Western blot analysis, in our imatinib-resistant GIST with both *KIT* V559D and *BRAF* V600E mutations, the inhibition of *KIT* V559D by imatinib caused a strong decrease of AKT phosphorylation, while ERK1/2 phosphorylation was not affected. However, in the imatinib-resistant GIST with a secondary *KIT* V654A mutation, phosphorylations of ERK1/2, AKT and KIT were all below the detection level. These data indicate that in GIST with both *KIT* V559D and *BRAF* V600E mutations, imatinib abrogates the *KIT* V559D-triggered AKT phosphorylation. However, it is not capable of affecting ERK1/2 phosphorylation, which is driven by *BRAF* V600E. According to Miranda et al. [24], in vitro experiments in cell lines coexpressing an imatinib-responding KIT mutant and constitutively activated *BRAF* proteins showed that imatinib treatment was able to switch off KIT and its downstream signaling but not extracellular signal-regulated kinase (ERK)1/2 activation driven by the mutated *BRAF*. These data suggest the activation of the MAPK pathway as a possible novel mechanism of resistance to imatinib in GIST.

In summary, this is the first report detecting heterogeneous *KIT* and *BRAF* secondary mutations in an imatinib-resistant GIST with heterologous rhabdomyoblastic differentiation. This finding delineates a new molecular group

of patients who may benefit from selective BRAF inhibitors as an alternative therapeutic option to imatinib. Furthermore, the acquisition of *BRAF* mutations may play a role in triggering imatinib-resistance in GIST patients and may induce a KIT-negative transdifferentiation phenotype in these tumors. More cases are thus required to confirm that *BRAF* mutated GIST indeed shows a distinct clinico-pathological phenotype.

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Conflict of interest All authors declare that they have no conflicts of interest.

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