**Characterization of S628N A Novel KIT Mutation Found in a Metastatic Melanoma**

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**IMPORTANCE** The KIT receptor is mutated in approximately 15% of acral, mucosal, and chronic, sun-damaged melanomas. The status of KIT mutations is of interest because they usually are mutually exclusive with N-RAS and B-RAF mutations and because of the availability of KIT kinase inhibitors in the clinic. Some recurrent KIT mutations are well characterized; others are poorly described.

**OBSERVATIONS** We describe a novel KIT mutation in a patient with metastatic melanoma. The mutation, located in exon 13, resulted in S628N substitution in the KIT receptor. Using all-atom molecular dynamics simulations, biochemical assays, and cell-based assays, we showed that the mutation is a bona fide gain-of-function oncogenic mutation. Furthermore, we evaluated the sensitivity of the mutant to imatinib and dasatinib.

**CONCLUSIONS AND RELEVANCE** We report a novel KIT gain-of-function mutation with S628N substitution (exon 13) and show that it is sensitive to imatinib in vitro. Therefore, patients with this mutation may be eligible for KIT kinase inhibitor-based therapy. Further studies are needed to evaluate the clinical benefit of such therapy.

**Report of a Case**

**Clinical Description**

The patient was a woman in her 80s, with several comorbidities including chronic renal failure and Parkinson disease. Two years earlier, she underwent surgery for a cutaneous, nodular, malignant melanoma of the right forearm displaying features associated with poor prognosis: 8.5-mm Breslow thickness, ulceration, and high mitotic index. No adjuvant treatment was delivered.

One year later, the woman developed a metastatic relapse located in the bones and lungs. A lesion on the 12th dorsal vertebra was surgically removed because of extension within the spinal medullary canal. Pathologic and immunohistochemical analysis confirmed the diagnosis of a 6-cm melanoma metastasis with numerous mitoses. The resection was microscopically complete. The sample was sent to our institution (Institut Paoli-Calmettes, Marseille, France) for mutational analysis (see below). First-line dacarbazine chemotherapy was started 1 month later. Disease stabilization was

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Among skin cancers, melanoma has the greatest metastatic potential and the worst prognosis. During the past 40 years, limited progress has been made in the treatment of metastatic disease through the use of chemotherapy (dacarbazine), immunotherapy (interleukin 2 and interferon), and biochemotherapy. Advances in molecular oncology recently led to the development of promising new drugs such as vemurafenib,1 an inhibitor of B-RAF tyrosine kinase mutant V600E (observed in 50%-60% of cutaneous melanomas), and ipilimumab, an anti-CTLA4 monoclonal antibody that enhances and prolongs T-cell responses.2 Curative treatment of metastatic melanoma remains elusive, even if such drugs improve the survival results, with 3-year overall survival now more than 20%.1,2

Another therapeutic target is the KIT receptor, a critical regulator of melanocyte development. KIT (OMIM 164920; GenBank NM_000222) is overexpressed and/or mutated in approximately 15% of melanomas from acral, mucosal, and chronically sun-damaged sites.3,4 Although the role of KIT receptor expression and function in melanoma has not been clear for the past 20 years, recent phase 2 clinical trials and case reports indicate that KIT inhibition may be an effective treatment when tumors harbor KIT mutations.5-7 We report what we believe to be a new KIT mutation, with gain-of-function and imatinib mesylate sensitivity features, identified in a patient with metastatic melanoma.
obtained after the first 3 cycles, but lung metastatic progression occurred after the eighth cycle.

A biopsy was performed on a sample of the lung metastasis for pathologic and mutational analyses. Histologic characteristics were similar to those of the bone metastasis. The mutation was not present in blood leukocytes, confirming its somatic nature, and immunohistochemical analysis confirmed the expression of KIT protein by metastatic cells (Figure 1A). The patient was thus referred to our institution for treatment. Her World Health Organization performance status was equal to 1, with a weight loss of 9 kg during the past year. Physical examination results revealed no additional symptoms. Biological blood test results indicated moderate renal failure. A fludeoxyglucose F 18 positron emission tomography/computed tomography (PET/CT) scan showed several hypermetabolic lung metastases. Given the clinical setting and the activating and sensitive nature of the observed KIT mutation (see below), our oncology pluridisciplinary staff recommended second-line imatinib therapy rather than ipilimumab. Because of the patient’s age and comorbidities, imatinib was given at a dosage of 300 mg/d during the first 3 weeks and then at 400 mg/d. Treatment was relatively well tolerated except for the occurrence of grade 2 fatigue and grade 1 nausea and thrombopenia. After 3 months of treatment, a PET/CT scan showed disease progression with a moderate increase in size (+30%) and standardized uptake value of lung metastases. Given the patient’s age and toxic effects, the imatinib dosage was not increased (continued at 400 mg/d). After 3 additional months of imatinib therapy, the patient was hospitalized because of left hemiplegia and deterioration of her performance status. A CT scan showed multiple supratentorial brain metastases with perilesional edema and deviation of median structures, as well as an increase in the size of the lung metastases. The patient died 5 days later.

Identification of a Novel KIT Mutation
A search for B-RAF, N-RAS, and KIT mutations was done on genomic DNA obtained from the bone metastasis localized in the 12th dorsal vertebra. A point mutation was found in KIT exon 13. No mutation was found in B-RAF and N-RAS hotspot exons. The same genotype was confirmed on the lung metastasis from a biopsy specimen obtained a few months later. The KIT mutation was a missense at codon 628, c.1883G>A p.Ser628Asn, resulting in an S628N substitution that, to our knowledge, has not been previously reported (Figure 1B). The mutation was somatic, since it was not found in peripheral blood cells.

Structure and Dynamics of KIT S628N Mutant
Residue 628 is localized near the invariant lysine residue (K623) of the adenosine triphosphate binding pocket in subdomain II of the catalytic domain. To gain insight regarding...
the effect of S to N substitution, we analyzed the kinase domain of KIT S628N by molecular dynamics simulations (Figure 1C). The structural, dynamic, and thermodynamic characteristics of KIT S628N were compared with those found for the native KIT and for the most frequently observed KIT oncogenic mutant, KIT D816V (eMaterials and Methods in the Supplement).

The D816V mutation induces 2 structural effects that are classified as short range when the manifestations involve the close environment (ie, <10 Å) or as long range when the perturbed structure belongs to remote regions in the protein sequence. A short-range effect was evidenced by a partial unfolding in the activation loop (A-loop) at proximity to the mutation site, and a long-range effect was manifested by an important structural reorganization of the juxtamembrane region (JMR), which was distant by more than 15 Å from the point mutation.9

The major structural modifications introduced by the S628N mutation appear to be long-range effects. First, a secondary structure alteration of the JMR, folded as a random coil in the wild type (WT) and a well-shaped antiparallel β-sheet (β1-β2) in the KIT S628N mutant, was accompanied by a global displacement of the juxtamembrane switch fragment of JMR in the axial position respective to the kinase domain (Figure 1C). Second, the A-loop also affected by the S628N mutation shows (1) a destabilization of the short 3α-helix at residues 817-819 and (2) displacement of the β-hairpin of the A-loop from its initial position in WT KIT. These structural changes of the A-loop have a local character in KIT D816V, but in KIT S628N they correspond to long-range effects. We also observed specific effects for KIT S628N. In particular, the Ca-helix is significantly more flexible in KIT S628N than in KIT D816V and shifted from its position in WT KIT (Figure 1C). Taking into account the position of the S628N mutation, this effect can be classified as a short-range or local event. Finally, the solvent-exposed loops in the N-lobe and P-loop are replaced from their positions in WT KIT. In conclusion, we evidenced that the S628N mutation has a greater effect on the KIT structure than does the classic D816V oncogenic mutation, suggesting a significant effect of this substitution on the catalytic domain structure.

Activity of KIT S628N Mutant
The activity of tyrosine kinase receptors can be visualized in cell lysates through their autophosphorylation and the activation of downstream signaling pathways. To evaluate the putative effect of the mutation on KIT receptor function, we introduced the S628N mutation in WT KIT complementary DNA (cDNA) by in vitro mutagenesis. The WT and mutant receptors’ cDNA was then transfected in COS-7 cells to analyze the receptor kinase activity. As seen in Figure 2, unlike WT KIT, KIT S628N is phosphorylated on tyrosine residues in the absence of stem-cell factor (SCF) (Figure 2, lane 5). Furthermore, as with other KIT oncoproteins, the lower migrating immature form of the receptor is the major phosphorylated form. On stimulation with SCF, the cell surface-mature glycosylated form (higher molecular weight form) of both WT and mutant receptors became phosphorylated.

To assess the activity of the mutant receptor more directly, we looked for the phosphorylation of a downstream signaling protein, STAT3.10 The KIT receptor was transfected, and activated STAT3 was detected using a phospho-specific antibody. As expected, STAT3 was phosphorylated downstream of WT KIT on SCF stimulation (Figure 2, lane 4). In the presence of KIT S628N, STAT3 was phosphorylated in the absence of SCF, indicating that S628N mutant is active in the absence of ligand stimulation (Figure 2, lane 5).

In addition, the AKT and ERK1/2 pathways were activated in cells transfected with KIT S628N mutant (lane 3 in eFigure 1 in the Supplement). We concluded that KIT S628N mutant is a novel gain-of-function mutant of KIT.

Transformation Assays Using KIT S628N Mutant
To investigate the functional consequence of the mutation, we stably expressed KIT S628N in Rat2 fibroblasts. These cells are an immortalized, nontumorigenic cell line commonly used to challenge the transforming properties of oncogenes. In particular, these cells are suitable for an essay of anchorage-independent growth in agar.11 Rat2 cells were infected with a control retroviral vector or retroviral vectors carrying either WT or S628N KIT cDNA. Stable populations of infected cells were obtained (Figure 3A and eFigure 2 in the Supplement). As previously described, Rat2 fibroblasts with stable expression of WT KIT did not form colonies in agar in the absence of exogenous ligand. In the same settings, KIT S628N-expressing cells formed large colonies in agar (Figure 3B). We concluded that KIT S628N is a gain-of-function mutant with oncogenic properties.

Sensitivity of KIT S628N to Kinase Inhibitors
Several small chemical inhibitors of KIT kinase are routinely used in the clinical setting. Furthermore, it has been
established that, although some KIT mutations are sensitive, others are resistant to these inhibitors, notably, the exon 17 KIT mutant at codon 816. We tested 2 KIT inhibitors: imatinib and dasatinib. Both inhibitors abolished the autophosphorylation of KIT S628N at a moderate concentration (1μM) (Figure 4). KIT L576P, a recurrent KIT mutant found in melanoma, was used as a positive imatinib-sensitive control, and KIT D816V was used as a resistant control (eFigure 3 in the Supplement). We concluded that KIT S628N mutant is sensitive to imatinib and dasatinib.

Discussion

KIT-activating mutations occur in mastocytosis, gastrointestinal stromal tumors, acute myeloid leukemias, germ-cell tumors, and melanomas. Mutations affect either the catalytic or the regulatory domains of the receptor. Regulatory mutants are generally sensitive to adenosine triphosphate–competitive chemical inhibitors, but some catalytic domain mutants confer resistance to these kinase inhibitors. For instance, although JMR mutants (exon 11 mutations) are sensitive to classic KIT inhibitors, such as imatinib, the recurrent D816 mutant (exon 17) is resistant.

To our knowledge, the exon 13 mutation identified in our patient with metastatic melanoma has not previously been reported. The most frequent KIT mutation identified in melanomas is located in exons 11 (approximately 70%), 13 (approximately 13%), and 17 (approximately 9%). The most common mutations are L576P in exon 11 (34%) and K642E in exon 13 (15%), which confer sensitivity to imatinib. Like the other described exon 13 mutations (K642E and R634W), the KIT S628N mutant is constitutively activated and is sensitive to imatinib in vitro. Unfortunately, we observed a discrepancy between the preclinical and clinical results. Our patient did not respond to imatinib, 400 mg/d; instead, the disease moderately progressed after 3 months of treatment. Multiple reasons might explain this therapeutic failure, including suboptimal imatinib doses that might be due to poor therapeutic adherence and/or drug interactions in a patient in her 80s with several comorbidities and/or abnormal drug metabolism,14 alteration of the cell efflux (glycoprotein P) and/or influx (organic cationic transporter) of imatinib, alteration of the therapeutic target (KIT amplification or absence of protein expression), activation of parallel biological pathways (eg, phosphoinositide 3-kinase and mitogen-activated protein kinase pathways), absence of KIT protein expression, and, of course, resistant mutation. Our immunohistochemical and in vitro analyses eliminated 2 of these reasons: the tumor cells were stained by CD117 antibody, and in vitro pharmacologic data indicated that KIT S628N mutant was sensitive to imatinib and dasatinib. Unfortunately, we could not test the other hypotheses.

Conclusions

We identified a novel gain-of-function mutation in KIT exon 13 (S628N) that is activating and sensitive to imatinib and dasatinib, which suggests that patients with melanoma bearing such a mutation might be candidates for treatment with such drugs. Further studies are warranted to evaluate the clinical benefit of this therapy.
Characterization of S628N: A Novel KIT Mutation

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