Activate and resist: L576P-KIT in GIST

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Abstract

L576P is a rare KIT mutation often reported in cancers other than gastrointestinal stromal tumors (GIST). In GISTs, it correlates with features linked to an aggressive phenotype, eventually resulting in secondary mutations. In vitro findings point out that L576P/KIT is constitutively activated, and shows poor imatinib sensitivity. In this work, histological, immunohistochemical, and biochemical analyses, coupled with mutational-molecular analysis and fluorescence in situ hybridization, were applied to surgical specimens. In parallel, the affinities of wild-type, L576P/KIT, and Δ559/KIT for imatinib were estimated by in silico studies. Despite imatinib treatment and the apparent clinical-imaging response, the detected histological response was very low. KIT resulted, expressed and activated in absence of secondary mutations, BRAF/NRAS mutations, and KIT/PDGFRα gene alterations. Computer modeling proved that L576P/KIT is two times less sensitive than the wild-type counterpart and considerably less affine to imatinib than the sensitive Δ559/KIT. Accordingly, the modeling evidence strongly supports the lack of tumoral regression we observed at the histological level.

Materials and Methods

Both paraffin-embedded and frozen materials were available from the patients treated with imatinib 400 mg/day (discontinued 24 hours before surgery). Written informed consent was obtained from the patients.

GIST Diagnosis and Genotyping

GIST diagnoses were confirmed as previously described (4). Paraffin-embedded samples were analyzed for KIT and PDGFRα mutations according to (4). Both cases showed the presence of KIT L576P exon 11 missense mutation.

Gene Mutation Analysis

The paraffin-embedded samples were analyzed for KIT and PDGFRα secondary mutations. Analysis was done in six tumoral deposits of the intrabdominal recurrence in case 1, and in five samples obtained from the primary tumor in case 2. Additional three cryo-preserved samples were analyzed in case 1. BRAF and NRAS gene analysis was done in one tumoral sample for each case, as previously reported (5).

Fluorescence In situ Hybridization Analysis

Fluorescence in situ hybridization (FISH) analysis was done as described in (4). Patient treatment and data...
collection were done in accordance with the guidelines of an appropriate surveillance committee.

Results

Clinical History: Case 1

A 60-year-old male presented with a peritoneal metastasis from a high risk ileal GIST resected 1 year before. He was started on imatinib 400 mg/day, resulting in a complete remission. One year after, he developed a focal peritoneal progression and was operated on. The progressing lesion was resected, and, unexpectedly, many peritoneal implants (not visible on CT scans) were found, most of which were removed. He was further continued on imatinib 800 mg/day. Sixteen months after surgery he developed a generalized progression, which was also not responsive to sunitinib or to nilotinib. He eventually died of abdominal liver and peritoneal metastases 3 years after imatinib onset.

Clinical History: Case 2

A 70-year-old male presented with a large duodenal GIST. A preoperative treatment was started in order to avoid a major surgical procedure (pancreatic-duodenectomy). He received imatinib 400 mg/day for 10 months, obtaining a radiological response (reduction in tumor volume and density on CT scan). He was then operated on, and a conservative duodenal resection could be done. A postoperative treatment with imatinib 400 mg/day for one more year was planned.

Histological Findings: Case 1

The intrabdominal surgical debulking material consisted of six spindle cell tumoral nodules ranging from 0.5 to 3 cm in diameter. One nodule showed a moderate response, whereas the remaining five exhibited a low response rate (Fig. 1A and B). CD117 immunostaining showed dot-like immunoreactivity (Fig. 1C and D), and PDGFRα revealed a light cytoplasmic staining (data are not shown).

Histological Findings: Case 2

The primary duodenal tumor (6 × 4 × 4 cm) that was removed was completely sampled. In this case also, the pattern of tumoral growth was spindle cells. With the exception of a 1-cm² area in aggregate showing high response, the remaining samples showed a low response rate (Fig. 2A and B). CD117 immunostaining revealed cytoplasmic immunoreactivity (Fig. 2C), and PDGFRα exhibited a very light cytoplasmic staining (data are not shown).

Biochemical Analysis

A highly expressed and phosphorylated-activated KIT was present in the sample of case 1 (Fig. 1E), whereas in the sample of case 2, KIT was not particularly expressed, phosphorylated, and/or activated (Fig. 2D).

Figure 1.

Case 1. Recurrence. Pathological evaluation of the post-treatment surgical tumoral specimens and biochemical findings. On fixed material, the pathological response rate was defined by averaging the residual viable tumoral cellularity, the mitotic index, and the Mib-1 labeling index (for further details see ref. 4). Briefly, a high response rate (<10 to <50% residual viable tumoral cells with no mitosis, no obvious Ki-67 immunostaining), a moderate response rate (>50–90% or >90% tumoral cells, no mitosis, Ki-67 immunostaining in 0 to <10% of cells), and a low response rate (>50–90% or >90% tumoral cells, mitotic index > 10/50 HPF (high power field), Ki-67 immunostaining in 20–30% or >30% of cells) were considered. A, hematoxilin-eosin (H/E) and B, Mib-1 immunodecoration of one of the five intrabdominal nodules showing a low response rate consistent with a residual cellularity >90%; mitotic index >10/50 HPF (70/50 HPF) and a proliferation index >30%. C, dot-like KIT immunostaining. D, insert: higher power field. E, immunoprecipitation and Western blotting to valuate the phosphorylation-activation and expression level of KIT. In particular, 0.5 mg of protein lysates were immunoprecipitated and blotted as previously described (4), in order to verify receptor activation (anti-PTyr antibody) and expression level (anti-KIT antibody). The KIT/Δ559 cell line overexpressing an activated KIT receptor was used as positive control (C + ). The sample used for biochemical analysis was characterized by a high residual cell component (residual cellularity >90%).
Neither patient carried secondary mutations. Wild-type \textit{BRAF} and \textit{NRAS} were found. Both cases showed a disomic pattern for \textit{KIT} and \textit{PDGFRA}.

**Molecular Dynamic Simulations**

The application of the MM-PBSA recipe to the L576P/\textit{KIT} in complex with imatinib resulted in a calculated free energy of binding between inhibitor and kinase, $\Delta G_{\text{bind}}$, of $-7.75$ kcal/mol. For comparison, the estimated affinity of the imatinib sensitive $\Delta 559$ isoform of \textit{KIT} toward the inhibitor was $\Delta G_{\text{bind}} = -9.35$ kcal/mol, whereas the corresponding value for the wild-type \textit{KIT} was $-8.08$ kcal/mol. Figure 3A and B shows the equilibrated molecular dynamics (MD) models of the wild-type and L576P/\textit{KIT} in complex with imatinib, respectively. Structurally, in the L576P/\textit{KIT} the induced fit for imatinib binding is no longer preserved, because of a conformational change that resembles the more active form of \textit{KIT} for which imatinib is known to have lower binding affinity (Fig. 3A and B).

**Discussion**

L576P is a rare mutation located in the JMD of \textit{KIT} described in GISTs (6–10), melanomas (11), and canine GISTs and mastocytomas (12, 13). We observed three L576P mutations among 332 profiled GISTs, of which we report upon two cases here (the third being enrolled into the 62024 EORTC trial). Case 1 represents an intrabdominal recurrence of a high risk GIST arising from the ileum, whereas case 2 is a primary post-treatment high risk tumor of the duodenum. Both cases showed a low histological response score, despite the apparent clinical and imaging response.

The functional significance of L576P substitution and its imatinib sensitivity have been previously investigated on cell lines. In particular, the COS-7 cells expressing the L576P/\textit{KIT} c-DNA revealed spontaneous \textit{KIT} phosphorylation (13), and \textit{in vitro} sensitivity tests highlighted the need of a tenfold higher dose of imatinib to switch off activated L576P/\textit{KIT} with respect to V599D/\textit{KIT} (11).

In terms of \textit{KIT} activation, in case 1 the strong \textit{KIT} expression-activation is consistent with the presence of secondary mutations that we were ultimately unable to detect, despite extensive searches with the available technique (definitely less sensitive than the recently proposed allele specific PCR; ref. 8). In case 2, the expression-activation of \textit{KIT} on primary tumor was not particularly high, a finding otherwise consistent with the high heterogeneity of both \textit{KIT} expression and phosphorylation previously reported in naïve and treated GISTs (6, 14). However, the evidence of post-treatment phosphorylation, along with the low rate of histological regression, support the notion that imatinib 400 mg/day was unable to switch off \textit{KIT}.

In order to find a rationale for these findings, we did “\textit{in silico}” experiments on two mutant KITs along with...
was calculated according to the Molecular Mechanics/Poisson-Boltzmann Surface Area method (MM-PBSA; ref. 20). The binding free energy for each kinase-inhibitor system, ΔGbind, was calculated according to the Molecular Mechanics/Poisson-Boltzmann Surface Area method (MM-PBSA; ref. 20). The crystallographic coordinates of the inactive KIT structure in complex with imatinib (1T46.pdb; ref. 17) were superimposed three-dimensional structures of wild-type (green) and L576P mutated KIT (sand) in complex with imatinib. Water molecules and counterions are omitted for clarity. B, details of the binding site of wild-type (green) and L576P mutated KIT (sand) in complex with imatinib. The most affected residues are depicted as sticks and labeled. Imatinib is portrayed in stick-and-ball representation. Hydrogen atoms, water molecules, and ions are omitted for clarity. In details, the L576P mutation imposes significant energetic constraints on several amino acids of the imatinib binding pocket, among which K623, E640, and C673 are the most affected.

Thus, the modeling evidence gives strong support to the lack of tumoral regression that we observed at a histological level. On the basis of this study, the structural similarity of both sunitinib and nilotinib with imatinib, and the analogous binding mode to KIT of these three small molecule ATP-competitive inhibitors (data are not shown), also account for the failure of their action on L576P mutated KIT. Therefore, relying on structural homology and other direct experimental (15) and modeling evidence, we can hypothesize that dasatinib, for instance, could be an effective inhibitor of L576P KIT mutant in GISTs (in vitro experiments are ongoing).

Figure 3. Computer simulations were carried out by using the AMBER 9 suite of programs (16) on 128 processors of the IBM/BCX cluster of the CINECA supercomputing facility (Bologna, Italy). The crystallographic coordinates of the inactive KIT structure in complex with imatinib (1T46.pdb; ref. 17) were employed as starting geometry. Mutations were introduced into the corresponding wild-type KIT structure, and molecular dynamics simulations were run with explicit water and counterions, according to a well-validated procedure (18, 19). The binding free energy for each kinase-inhibitor system, ΔGbind, was calculated according to the Molecular Mechanics/Poisson-Boltzmann Surface Area method (MM-PBSA; ref. 20).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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