Detection of Mutant Free Circulating Tumor DNA in the Plasma of Patients with Gastrointestinal Stromal Tumor Harboring Activating Mutations of CKIT or PDGFRα

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Abstract

Purpose: In gastrointestinal stromal tumor (GIST), there is no biomarker available that indicates success or failure of therapy. We hypothesized that tumor-specific v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (CKIT)- or platelet-derived growth factor receptor-α (PDGFRα)-mutant DNA fragments can be detected and quantified in plasma samples of patients with GIST.

Experimental Design: We prospectively collected 291 plasma samples from 38 subjects with GIST harboring activating mutations of CKIT or PDGFRα detected in tumor tissue, irrespective of current disease status or treatment. We used allele-specific ligation PCR to detect mutant free circulating DNA (fcDNA).

Results: We were able to detect fcDNA harboring the tumor mutation in 15 of 38 patients. Patients with active disease displayed significantly higher amounts of mutant fcDNA compared with patients in complete remission (CR). The amount of mutant fcDNA correlated with disease course. We observed repeated positive test results or an increase of mutant fcDNA in five patients with progressive disease or relapse. A decline of tumor fcDNA or conversion from positive to negative was seen in five patients responding to treatment. A negative to positive conversion was seen in two patients with relapse and one patient with progression. In two cases, we aimed to identify additional mutations and found four additional exchanges, including mutations not known from sequentially conducted tumor biopsies.

Conclusions: Our results indicate that fcDNA harboring tumor-specific mutations in the plasma of patients with GIST can be used as tumor-specific biomarker. The detection of resistance mutations in plasma samples might allow earlier treatment changes and obviates the need for repeated tumor biopsies. Clin Cancer Res; 19(17); 4854–67. ©2013 AACR.

Introduction

In gastrointestinal stromal tumor (GIST), the main objective of treatment is complete resection. Tyrosine kinase inhibitors (TKI) such as imatinib or sunitinib are used for neoadjuvant, adjuvant, or palliative treatment. Responses to imatinib can be achieved. However, treatment is not curative unless complete resection is possible. In advanced GIST, a partial remission can be attained in 50% of patients treated with imatinib, and there are single cases of complete responses. However, most patients experience disease progression receiving imatinib (1–7). Patients with progression receiving imatinib only transiently respond to sunitinib (8).

The response to therapy is evaluated by diagnostic imaging, which displays limited sensitivity and specificity. Computed tomography (CT) constitutes the gold standard of imaging in GIST (9, 10). However, sensitivity of CT in GIST is insufficient. A randomized trial investigating the interruption of imatinib in patients with advanced GIST was terminated prematurely due to a high rate of progression events in the interruption arm even in patients displaying CT-morphologic complete remission (CR) at the time of randomization (11). Currently, 2[18F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) represents the most sensitive imaging technique for staging and monitoring response in GIST. However, the sensitivity and specificity of FDG-PET in GIST is limited (12–14). In a
Mutant Free Circulating Tumor DNA in GIST

**Translational Relevance**
In gastrointestinal stromal tumor (GIST), response to treatment is assessed by diagnostic imaging techniques, which is associated with limited sensitivity and specificity. There is no blood biomarker available to detect success or failure of therapy. In GIST, activating v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (CKIT) or platelet-derived growth factor receptor-α (PDGFRA) mutations can be detected by DNA sequencing in tumor specimens in 90% of the cases. Mutated genomic DNA fragments are highly specific for the tumor. We here show that tumor-specific mutations in CKIT or PDGFRA can be detected and quantified in free circulating DNA (fcDNA) in plasma samples derived from patients with GIST by quantitative ligation PCR, and that fcDNA levels correlate with the clinical course of the GIST lesions as measured by diagnostic imaging. Thus, tumor-specific fcDNA in plasma can be used as a highly specific biomarker in patients with GIST, and might be used to predict response to treatment and relapse, allowing earlier treatment changes.

**Materials and Methods**
**Trial design**
This study is an open-label, nonrandomized, noninterventional, explorative phase IIIb trial that aims to detect tumor-specific mutations of CKIT or PDGFRA in fc plasma DNA in patients with GIST harboring activating mutations of CKIT or PDGFRA detected in tumor tissue irrespective of current disease status and current or planned treatment. Patients were treated at the Klinikum Rechts der Isar, Technische Universität München (München, Germany). We prospectively collected 291 plasma samples from 38 subjects between October 2007 and May 2012. All patients gave written informed consent. After a monocentric pilot phase, the trial was continued as multicenter study. The study was approved by the Institutional Review Board of the Technische Universität München. This trial is registered under Eudra-CT No. 2011-002544-27 and ClinicalTrials.gov NCT01462994. The present report focuses on the monocentric pilot phase of the trial.

**Patients**
The 38 cases included 18 patients with active disease, which was defined as having at least one GIST lesion that could be measured by imaging at any time after inclusion. Of these, 9 displayed active disease before TKI treatment was instituted (first diagnosis, \( n = 2 \); relapse, \( n = 5 \); after surgical debulking, \( n = 2 \)) and 9 patients had relapse or progression receiving TKI treatment, including 3 patients with response to TKI treatment where no samples were available at progression before TKI. Twenty patients were in CR but with high (\( n = 14 \)), moderate (\( n = 3 \)), or low (\( n = 1 \)) risk of relapse according to Miettinen criteria (33), or were in CR and Miettinen criteria were not applicable because mitotic index was not available (\( n = 1 \)) or treated local peritoneal metastasis (\( n = 1 \); see Table 1). Twenty-nine patients received medical treatment with imatinib, sunitinib, nilotinib, dasatinib, sorafenib, everolimus, or doxorubicin. Nine patients did not receive medical treatment during participation in the trial. Tumor response was measured radiographically with CT, MRI, or FDG-PET/CT.

**Mutation analysis of DNA from tumor tissue**
A tumor-specific mutation site of each subject was determined by Sanger sequencing of genomic DNA extracted from tumor tissue. Formalin-fixed paraffin-embedded (FFPE)
tumor tissue was obtained from each subject. Genomic DNA from FFPE sections was extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer’s instructions. Sequence analysis of CKIT exons 9, 11, 13, 14, 17, and 18 and PDGFRA exon 18 was conducted on a routine basis. Furthermore, in all cases where tumor specimens were available (30 of 38 cases; 79%), we repeated Sanger sequencing for confirmation of the initial mutation status.

Isolation of tumor DNA from plasma

Peripheral blood samples were collected in EDTA tubes (Sarstedt). At the same day, tubes were centrifuged at 800 × g for 10 minutes. The supernatant was transferred to 10-mL tubes and centrifuged at 1,600 × g for 10 minutes to remove debris. Plasma supernatants were transferred in 1.0 mL aliquots to 1.5 mL tubes and stored at −80°C. Total genomic DNA of plasma aliquots was extracted from 1 mL plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s instructions.

Detection and quantification of mutant and LINE1-fcDNA from plasma

Analyses of plasma samples were conducted in a blinded fashion with respect to the clinical information of individual patients. The sequences of the primers and probes used for each test are listed in the Supplementary Data. fcDNA

<table>
<thead>
<tr>
<th>Table 1. Overview of test results in relation to clinical setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant fcDNA</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Active disease</td>
</tr>
<tr>
<td>Postsurgery in CR: risk of relapse (Miettinen)</td>
</tr>
<tr>
<td>High</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Low</td>
</tr>
<tr>
<td>N/A</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>NOTE: Number of cases with detection of mutant fcDNA in plasma samples according to the clinical setting. Positive denotes 0.1% or more mutant allele to wt ratio (except for the K642E assay: 1% or more due to high cross-reactivity). Low-level positive refers to mutant/wt allele ratios of &lt;0.1% (except for the K642E assay: &lt;1% due to high cross-reactivity). Active disease denotes at least one lesion that could be measured and either being pre-TKI treatment or with progression receiving TKI treatment. Postsurgery patients were in CR without evidence of residual disease, and the risk of relapse was calculated according to the Miettinen criteria based on tumor localization, tumor size, and mitotic index. Abbreviation: N/A, not applicable. aIncludes 3 cases with samples available at the time of response only (see Table 2).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Overview of test results in relation to clinical setting for patients with active disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant fcDNA</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Pre TKI</td>
</tr>
<tr>
<td>Relapse</td>
</tr>
<tr>
<td>Postsurgery</td>
</tr>
<tr>
<td>With response receiving TKIa</td>
</tr>
<tr>
<td>In progression receiving TKI</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>NOTE: Shown are the numbers of cases with detection of mutant fcDNA in plasma samples according to the clinical setting in patients with active disease. Active disease denotes at least one lesion that could be measured and either being pre-TKI treatment or with progression receiving TKI treatment. Pre-TKI patients were either at first diagnosis, at relapse without treatment, or after debulking before start of TKI treatment. With response receiving TKI refers to patients displaying response to TKI treatment where no sample was available at the time of relapse before TKI was started, and in progression receiving TKI denotes patients displaying progression while on TKI treatment based on imaging studies. For explanation of positive and low-level positive refer to Table 1. Allocation to patient numbers corresponding to Fig. 2 is given in Supplementary Table S2. aSamples available at the time of response only.</td>
</tr>
</tbody>
</table>
purified from 1 mL plasma was used to quantify mutated and LINE1 GIST DNA. The principle used to quantify point mutations in 

\( \text{CKIT} \) (GenBank acc. no. NM_000222.2) or 
\( \text{PDGFRA} \) (GenBank acc. no. NM_006206.4) gene based on the MLPA technique as previously described (34, 35). To assess the load of total fcDNA, LINE1 was selected as target for quantitative real-time PCR.

**Mutant fcDNA.** For detection of CKIT- or PDGFRA-mutant fcDNA, CKIT- or PDGFRA-mutated sequences were first amplified in a standard PCR reaction (“accumulation PCR”) from total genomic DNA extracted from plasma. The second step involved the stringent (high temperature) hybridization to this PCR product of two probes, A1 and A2, which span the mutation region of interest. Each probe consists of a CKIT or PDGFRA hybridizing sequence flanked by a PCR primer-binding site. One of these probes (A1) carries the mutation at the 5’-end, whereas the 5’-end of the other (A2) corresponds to the neighboring base. A high-temperature ligation reaction serves to join these two probes only when there is no mismatch at the mutation site. Finally, the yield of ligated probe is determined by real-time PCR using primers complementary to the primer-binding sites at the nonligated ends of the two probes. Using universal primers, the parallel L-PCR amplification of mutation-specific CKIT (PDGFRA) and total CKIT (PDGFRA) sequences in the same run ensures comparability. This allows normalization of the mutation-specific cycle threshold \( C_t \) values to those of total CKIT (PDGFRA). The comparative \( \Delta C_t \) method was used \( [2E - (\Delta C_t) \times 100] \) to calculate the relative percentage. \( C_t \) values of mutation and wild-type (wt) reaction were directly compared to determine mutated allele in the wt background (minus cross-reactivity) in percentage. Positive samples underwent a second independent run. The mean of the two runs was used to calculate the mutated/wt ratio. Purified DNA harboring the specific mutation was used as a positive control. Because some L-PCR runs do have a background hybridization of the mutation-specific probes in a nonmutated sample, DNA from healthy subjects was included in each assay as negative control. This cross-reactivity determines the sensitivity of the specific test run and the nonspecific allele level were subtracted from a positive allele level.

**LINE1 quantification.** LINE1 are one of the most abundant sequences in the human genome and therefore correlate with the load of fcDNA. To assess the load of total fcDNA, LINE1 was selected as target for quantitative real-time PCR. In detail, 180 bp were amplified with the primers 5’-CAGAGGCGAATCATGTGGA-3’ (forward) and 5’-CTTCCATTCCATGACATGG-3’ (reverse). The final reaction mixture for quantitative PCR consisted of 0.2 μmol/L forward and reverse primers, 1× SYBR Green/polymerase mix (QuantiTect SYBR Green PCR Kit, Qiagen; #2041445) and as template 2 μL plasma DNA in a total volume of 25 μL PCR reaction. The real-time PCR amplification was conducted with an initial activation of 95°C for 15 minutes, followed by 40 cycles at 94°C (15 seconds), 58°C (30 seconds), and 72°C (35 seconds) using the 7300 Real-Time PCR-system (Applied Biosystems). The LINE1 DNA fragments in each sample were calculated using a LINE1 plasmid standard curve.

**Statistical analyses**

Statistical analysis was conducted using Student unpaired \( t \) test with 95% confidence intervals.

**Results**

**Validation of allele-specific L-PCR application**

The application of the L-PCR showed in a total of 25 assays an interassay coefficient of variation (SD/mean) of 0.2 to 0.5, investigated in five independent runs conducted on different days and calculated from five \( \log_{10} \) dilutions between 10\(^{-4}\) and 10\(^{0}\) CKIT exon 9 insertion 502-503 mutated copies in duplicates. The intraassay coefficients of variations were 0.4 to 0.5 calculated from the means of eight replicates of plasmid dilutions of CKIT exon 9 insertion 502-503 between 10\(^{-4}\) and 10\(^{2}\) copies. Sensitivity has been tested for six cloned key mutations (CKIT exon 9 insertion 502-503; CKIT exon 11 deletion 557-558; CKIT exon 11 V559D; PDGFRA exon 18 D842V; CKIT exon 13 V654A; CKIT exon 13 K642E) and ranged from 0.01% to 0.1% mutated allele/wt dependent on cross-reactivity of hybridizing probes. This results in a dynamic detection range of 4 to 5 \( \log_{10} \). One exception was K642E with a higher cross-reactivity of 1% and a subsequent detection range of 3 \( \log_{10} \).

**Detection of tumor-specific CKIT and PDGFRA mutations in plasma DNA**

We developed and validated 25 different allele-specific L-PCR assays covering CKIT and PDGFRA mutations identified in all patients (Supplementary Table S1). Three mutations appeared in more than 2 patients [CKIT\(_{\text{InsA502-503}}\) (patient 5; 11; 25; 38), CKIT\(_{\text{Del W557-K558}}\) (patient 1; 23; 31; 36), and PDGFRA\(_{\text{D842V}}\) (patient 12; 17; 18; 19; 20; 21; 35)]. Using our L-PCR assays, we examined 291 plasma samples from 38 patients (Fig. 2). Eighteen of 38 patients had active disease, which was defined as having at least one lesion that could be measured at any time after inclusion and being either before institution of TKI treatment \( (n = 9) \) or with relapse or progression receiving TKI treatment \( (n = 9; \text{Tables 1 and 2}). In 9 of these 18 patients, we detected fcDNA harboring the tumor-specific mutation, with mutant to wt allele ratios of more than 0.1% \( (up to 15.6\%) \) in 5 cases and less than 0.1% in 4 cases (Table 1). We identified mutant fcDNA in 4 of 6 patients with progression or relapse receiving TKI (patients 5, 7, 11, and 15; Table 2; Fig. 2; Supplementary Table S2). We did not detect mutant fcDNA in 3 patients who were already responding to TKI treatment at the time of the first plasma sample (patients 2, 4, and 9). In contrast to patients with active disease, none of the patients in CR displayed mutant to wt allele ratios above 0.1%, but were still able to detect low levels of mutant fcDNA \(<0.1\%) \) in 6 of 17 patients with high or intermediate risk of relapse according to Miettinen.
When we analyzed "untreated and progression" versus "CR" patients with positive test results, the former category displayed significantly higher fcDNA ratios (Fig. 1). Thus, in relation to the clinical setting, more patients with active disease had positive test results compared with CR patients.

Quantification of tumor-derived plasma DNA and correlation with radiographic response to treatment

We next examined whether the amount of tumor fcDNA correlated with response. The clinical characteristics and treatments for all 38 patients are shown in Table 3. In 5 of 18 patients with active disease, we found fcDNA harboring the tumor-specific mutation, with mutant to wt allele ratios of more than 0.1% (Table 1 and Fig. 3A–E). We observed repeated positive test results in patients with progression (patients 5, 11, and 15) or relapse (patients 7 and 10; see Fig. 2). In patients 7 and 10, tumor relapse was paralleled by a negative to positive conversion.

Mutated fcDNA test result of patient 5 shortly after surgery on day 0 (0.04%) was low-level positive with the tumor-specific \textit{CKIT} exon 9 insertion AY502-503 mutation (Fig. 3A). After repeatedly negative samples, a negative to positive conversion of mutated fc \textit{CKIT} DNA (day 641) to 3.87% paralleled the progression of liver metastases (day 662, red arrows). Mutant fcDNA decreased to 0.03% shortly before high-frequency thermo-therapy (HFTT). Sunitinib was stopped because of a stroke. Day 737 imaging shows disintegration of two known liver lesions after HFTT (green arrows) but extrahepatic progression (red arrow), and mutant fcDNA levels concomitantly increased to 1.29% and 7.72% at days 737 and 856, respectively. Day 926 imaging showed massive progression, paralleled by a decrease of the known mutant allele, whereas the regression line of logarithmic \textit{LINE1} levels showed a steady increase over time.

Patient 7 with a \textit{CKIT} exon 11 deletion Y553-Q556 mutation displayed one low-level mutated test result on day 57 (0.005%) 2 months after surgery with small residual...
peritoneal nodules and converted to repeated negative samples, whereas peritoneal nodules resolved completely receiving adjuvant imatinib (Fig. 3B). Although being in CR, the patient converted to 0.015% mutant fcDNA on the day 716, paralleled by an increase of LINE1 DNA fragments. Three months later, this patient presented with peritoneal metastases, and further increased with mutant fcDNA to 1.21%, which rapidly returned to 0% after 18 days of sunitinib treatment. CT conducted 2 months later showed response (day 901; see Fig. 3B).

Patient 10 displayed two tumor-specific mutations, a CKIT exon 11 deletion V555-D572 insG known from the primary tumor and an exon 17 D820Y point mutation identified in surgically debulked tumor at month 26 (Fig. 3C and Table 3). Upon hepatic relapse, mutant fcDNA with the CKIT exon 11 deletion/insertion, converted from 0% to 0.19% together with an increase of LINE1 DNA fragments. After 2 months of imatinib, CKIT exon 11 mutant fcDNA converted back to negative with a decrease of LINE1 DNA fragments, and CT scans confirmed tumor regression at days 268 and 517. The additional D820Y mutation remained detectable until day 367, and thus might have contributed to hepatic relapse.

Patient 8 was diagnosed with cardiac GIST and liver metastasis with a CKIT exon 11 K530-K538 deletion. We observed a positive to negative conversion from 14.1% to 0% mutant plasma fcDNA after only 14 days of treatment with imatinib (Fig. 3D). Of note, CT scans starting from day 94 showed an ongoing response of liver lesions (red arrows) with persistently negative tumor fcDNA with a follow-up of 29 months and a corresponding steady decline of LINE1 DNA fragments over time.

In accordance with the rapid positive to negative conversion seen in patients 7 and 8, we did not detect fcDNA in plasma samples from 3 additional patients who responded to TKI treatment where pretreatment samples were not available (see Table 2, category “With response receiving TKI”; patients 2, 4, and 9 in Table 2).

In 2 patients, sequentially conducted molecular analyses of surgically debulked peritoneal tumor revealed secondary resistance mutations in addition to the primary mutation (CKIT exon 13 V654A in patient 11, Fig. 3E, red arrows). In plasma samples of this patient, fcDNA containing the initial mutation (CKIT exon 11 V559D) decreased despite the rapid progression (Fig. 3E). In parallel, a secondary CKIT exon 13 V654A mutation identified from a mesenteric lesion after debulking on day 0 (Fig. 3E, black arrow) emerged in fcDNA during progression on day 328 together with a steady increase of LINE1 DNA fragments over time. We therefore suspected polyclonal disease and suspected additional secondary mutations. Indeed, we were able to detect CKIT exon 9 insertion AY502-503 in 4 samples (0.01%–0.05%), and exon 13 K642E in 2 samples (0.11%–0.24%; data not shown).

Next, we correlated our findings with the global load of fcDNA as measured by LINE1 DNA fragments using quantitative real-time PCR. Healthy volunteers were compared with GIST patients in CR or progression/relapse. Ten healthy donors showed plasma LINE1 DNA fragments in the range of 1 × 10² to 4 × 10⁵ with a median of 3 × 10⁵. In comparison, 55 consecutive samples of patients with GIST in CR had a higher median of 9 × 10⁵ (range, 1 × 10⁵ to 5 × 10⁷) plasma LINE1 DNA fragments, and 41 patients with progression or relapse displayed an even higher median of 18 × 10⁵ (range, 1 × 10⁵ to 1×10⁷; Supplementary Fig. S1) but the difference was not statistically significant. However, the regression curve of serial LINE1 levels generally seemed to follow the clinical course, with a decrease in patients responding (patients 8 and 10; Fig. 3C and D) and increase in patients with relapse or progression (patients 5, 7, and 11; Fig. 3A, B, and E).

Discussion

Methods for evaluating response in GIST are confined to imaging. Because the specificity and sensitivity of imaging techniques are limited (11, 15, 16), there is a need to develop improved and specific methods for monitoring disease activity. Most established biomarkers suffer from low sensitivity and specificity, and provide clinically valuable information only in specific settings. In theory, tumor-derived fcDNA might offer advantages. In leukemia, DNA- or RNA-based assays are routinely used for monitoring response and minimal residual disease, and for example in chronic myelogenous leukemia (CML) determine progression-free and overall survival (37, 38). Cancer cells release nucleic acids into the circulation (26, 39). The amount of tumor-derived fcDNA might be determined by tumor size, vascularization, and cell turnover. FcDNA thus might well reflect disease activity and could be used for monitoring cancer. It has been shown that mutant fcDNA might have clinical use for monitoring in colorectal cancer (mutations of APC, KRAS, PIK3CA, and TP53; ref. 32) or melanoma (BRAFV600E; ref. 40). GIST tumors in 90% of the cases carry mutations in CKIT or PDGFR. Thus, mutant DNA in any compartment indicates viable tumor cells. The individual mutation is known from routine sequencing of the tumor specimen. Allele-specific PCR allows specific amplification and quantification of the mutated CKIT and PDGFR DNA fragments resulting in high specificity and sensitivity.

In this study, we could show that mutant fcDNA specifically can be detected and quantified in the plasma of patients with GIST. Our data indicate that the amount of mutant fcDNA correlates with response. We observed repeatedly positive results and increasing mutant fcDNA in patients with progression, negative to positive conversions in patients with relapse, and positive to negative conversions in patients responding to TKI treatment.
Table 3. Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Time from first diagnosis, mo</th>
<th>Initial site</th>
<th>Treatment initial site, time</th>
<th>Relapse site, time</th>
<th>Treatment relapse site, time</th>
<th>Mutation status primary</th>
<th>Mutation status follow-up (site and time; type)</th>
<th>Ligation probes used</th>
<th>Clinical setting at inclusion</th>
<th>Change of clinical setting to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Gastric</td>
<td>Resection, mo 1; Ima adjuvant, mo 3–16</td>
<td>Peritoneal, mo 28</td>
<td>Debuking peritoneal, mo 29; Ima, since mo 30</td>
<td>CKIT delW557-K558</td>
<td>Peritoneum mo 29; CKIT delW557-K558</td>
<td>CR-MR</td>
<td>A, A-R, CR</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Gastric</td>
<td>Resection; mo 1</td>
<td>Liver, mo 34</td>
<td>Ima, since mo 34</td>
<td>CKIT insP573(57bp)</td>
<td>NE</td>
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<td>CR-MR</td>
<td>A-R</td>
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<td>3</td>
<td>6</td>
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<td>Resection, mo 6; Ima adjuvant, mo 6 to 31</td>
<td>Peritoneal, mo 42</td>
<td>Debuking peritoneal, mo 43; Ima, since mo 44</td>
<td>CKIT V560D</td>
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<td>Ima, since mo 1</td>
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<td>5</td>
<td>7</td>
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<td>Liver, mo 8; mesenterium, mo 32</td>
<td>Sun, mo 9–24; HFTT, mo 24; Das, mo 32–33</td>
<td>CKIT insA502-Y503</td>
<td>Liver mo 9; CKIT insA502-Y503</td>
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<td>Peritoneal, mo 1</td>
<td>Ima, since mo 1; Debuking peritoneal, mo 10</td>
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<td>Peritoneum mo 1; CKIT delY553-K558</td>
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<td>A, A-R, CR</td>
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<td>Sun, since mo 27</td>
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<td>Resection, mo 1</td>
<td>Liver, mo 2</td>
<td>Ima, since mo 2; Hemihepatectomy, mo 3</td>
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<td>Peritoneal, mo 26; Liver, mo 8</td>
<td>Ima, mo 8–26; debuking peritoneal, mo 26; Das, mo 40</td>
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<td>Resection, mo 63</td>
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<td>A, A-R, A-PD</td>
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<td>Resection, mo 13</td>
<td>Liver, mesenterial, mo 9</td>
<td>Ima, since mo 9</td>
<td>CKIT delV559-N566</td>
<td>NE</td>
<td>CKIT delV559-N566</td>
<td>CR</td>
<td>A, A-R</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
<td>Gastric</td>
<td>Resection, mo 45</td>
<td>Liver, mo 32</td>
<td>Ima, since mo 11</td>
<td>CKIT insL589(45bp)</td>
<td>NE</td>
<td>CKIT insL589(45bp)</td>
<td>A-R</td>
<td>A-PD</td>
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(Continued on the following page)
Table 3. Clinical characteristics of the patients (Cont’d)

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Time from first diagnosis, mo</th>
<th>Initial site</th>
<th>Treatment initial site, time</th>
<th>Relapse site, time</th>
<th>Treatment relapse site, time</th>
<th>Mutation status primary</th>
<th>Mutation status follow-up (site and type)</th>
<th>Ligation probes used</th>
<th>Clinical setting at inclusion</th>
<th>Change of clinical setting to</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>132 Ileum</td>
<td>Resection, mo –132;</td>
<td>Mesenterial, mo –22</td>
<td>CKIT V556I + L576P</td>
<td>Mesenterial mo 1; CKIT V556I + L576P; Mesenterial mo 6; CKIT V556I + L576P + PDGFRA P567R</td>
<td>NE</td>
<td>CKIT</td>
<td>NE</td>
<td>A-PD</td>
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<tr>
<td>16</td>
<td>85 Small bowel, peritoneal, liver</td>
<td>Debulking, mo –85;</td>
<td>—</td>
<td>—</td>
<td>Liver mo 4; CKIT delE554-I571</td>
<td>CKIT</td>
<td>CR-HR</td>
<td>—</td>
<td>A-PD</td>
<td>—</td>
</tr>
<tr>
<td>17</td>
<td>1 Colon, peritoneal</td>
<td>Debulking, mo –1;</td>
<td>Surgery</td>
<td>PDGFRA D842V</td>
<td>NE</td>
<td>PDGFRA D842V</td>
<td>A</td>
<td>A-PD</td>
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</tr>
<tr>
<td>18</td>
<td>1 Peritoneal</td>
<td>Debulking, mo –1;</td>
<td>—</td>
<td>—</td>
<td>PDGFRA D842V</td>
<td>NA</td>
<td>PDGFRA D842V</td>
<td>CR-HR</td>
<td>—</td>
<td>—</td>
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<tr>
<td>19</td>
<td>14 Gastric</td>
<td>Resection, mo –14;</td>
<td>—</td>
<td>—</td>
<td>PDGFRA D842V</td>
<td>NA</td>
<td>PDGFRA D842V</td>
<td>CR-HR</td>
<td>—</td>
<td>—</td>
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<tr>
<td>20</td>
<td>7 Gastric</td>
<td>Resection, mo –5;</td>
<td>PDGFRA D842V</td>
<td>NA</td>
<td>PDGFRA D842V</td>
<td>CR-HR</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>21</td>
<td>5 Gastric</td>
<td>Resection, mo –5;</td>
<td>PDGFRA D842V</td>
<td>NE</td>
<td>Rectum mo –34; CKIT delE533-D572</td>
<td>CR</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>22</td>
<td>53 Rectum</td>
<td>Resection, mo –53</td>
<td>Rectum, mo –34</td>
<td>NE</td>
<td>Rectum mo –34; CKIT delE533-D572</td>
<td>CR</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>23</td>
<td>1 Gastric</td>
<td>Resection, mo –1;</td>
<td>—</td>
<td>—</td>
<td>CKIT delE557-K598</td>
<td>NA</td>
<td>CKIT delE557-K598</td>
<td>CR-MR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>1 Gastric</td>
<td>Resection, mo –1</td>
<td>—</td>
<td>—</td>
<td>CKIT delE557-K598</td>
<td>NA</td>
<td>CKIT delE557-K598</td>
<td>CR-MR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>25</td>
<td>1 Ileum</td>
<td>Resection, mo –1;</td>
<td>—</td>
<td>—</td>
<td>CKIT insA502-Y503</td>
<td>NA</td>
<td>CKIT insA502-Y503</td>
<td>CR-HR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>26</td>
<td>31 Jejunum</td>
<td>Resection, mo –31;</td>
<td>-</td>
<td>-</td>
<td>CKIT K642E</td>
<td>NA</td>
<td>CKIT K642E</td>
<td>CR-HR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>27</td>
<td>43 Esophagus</td>
<td>Imatinib</td>
<td>—</td>
<td>—</td>
<td>CKIT V560G</td>
<td>NA</td>
<td>CKIT V560G</td>
<td>CR-MR</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Table 3. Clinical characteristics of the patients (Cont’d)

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Initial site</th>
<th>Treatment initial site, time</th>
<th>Relapse site, time</th>
<th>Treatment relapse site, time</th>
<th>Mutation status primary</th>
<th>Mutation status follow-up (site and time; type)</th>
<th>Ligation probes used</th>
<th>Clinical setting at inclusion</th>
<th>Change of clinical setting to</th>
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<tbody>
<tr>
<td>28</td>
<td>Small bowel, peritoneal</td>
<td>Resection, thermoablation, mo -1; Imatinib, since mo 2</td>
<td>—</td>
<td>—</td>
<td>CKIT delD579</td>
<td>NA</td>
<td>CKIT delD579</td>
<td>CR</td>
<td>—</td>
</tr>
<tr>
<td>29</td>
<td>Jejunum</td>
<td>Resection, mo -1; Imatinib, since mo 2</td>
<td>—</td>
<td>—</td>
<td>CKIT delV560-Y578</td>
<td>NA</td>
<td>CKIT delV560-Y578</td>
<td>CR-HR</td>
<td>—</td>
</tr>
<tr>
<td>30</td>
<td>Gastric</td>
<td>Resection, mo -94; FDG-PET uptake pancreas, mo -72</td>
<td>Lma, since mo -69</td>
<td>CKIT delM552-V559</td>
<td>NE</td>
<td>Liver mo -20; CKIT delW557-K558</td>
<td>CR</td>
<td>—</td>
<td></td>
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<tr>
<td>31</td>
<td>Small bowel</td>
<td>Resection, mo -42</td>
<td>Liver, mo -24 and mo -20</td>
<td>Resection, mo -24 and mo -20; Lma, mo -20 to -5 and since mo 4</td>
<td>NE</td>
<td>CKIT delW557-K558</td>
<td>CR</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Gastric</td>
<td>Resection, mo -1</td>
<td>—</td>
<td>—</td>
<td>CKIT V560D</td>
<td>NA</td>
<td>CKIT V560D</td>
<td>CR-LR</td>
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</tr>
<tr>
<td>33</td>
<td>Jejunum</td>
<td>Resection, mo -1; Lma adjuvant, since mo 3</td>
<td>—</td>
<td>—</td>
<td>CKIT K558N, delV569</td>
<td>NA</td>
<td>CKIT K558N, delV569</td>
<td>CR-HR</td>
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</tr>
<tr>
<td>34</td>
<td>Gastric</td>
<td>Resection, mo -1; Lma adjuvant, since mo 2</td>
<td>—</td>
<td>—</td>
<td>CKIT insP585(42bp)</td>
<td>NA</td>
<td>CKIT insP585(42bp)</td>
<td>CR-HR</td>
<td>—</td>
</tr>
<tr>
<td>35</td>
<td>Gastric</td>
<td>Resection, mo -1</td>
<td>—</td>
<td>—</td>
<td>PDGFRA D842V</td>
<td>NA</td>
<td>PDGFRA D842V</td>
<td>CR-HR</td>
<td>—</td>
</tr>
<tr>
<td>36</td>
<td>Cardia, jejunum</td>
<td>Resection, mo -2</td>
<td>—</td>
<td>—</td>
<td>CKIT delW557-K558</td>
<td>NA</td>
<td>CKIT delW557-K558</td>
<td>CR-HR</td>
<td>—</td>
</tr>
<tr>
<td>37</td>
<td>Gastric</td>
<td>Resection, mo -1; Lma adjuvant, since mo 2</td>
<td>—</td>
<td>—</td>
<td>CKIT V560G</td>
<td>NA</td>
<td>CKIT V560G</td>
<td>CR-HR</td>
<td>—</td>
</tr>
<tr>
<td>38</td>
<td>Duodenum</td>
<td>Resection, mo -59</td>
<td>Liver, mo -4</td>
<td>Resection, mo -4; Lma, since mo -2</td>
<td>NE</td>
<td>Liver mo -4; CKIT insA502-Y503</td>
<td>CR</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Shown are the sites of disease, course of treatment, molecular characteristics of the tumor, and the L-PCR probes used. Patients 1 to 18 displayed active disease, defined as having at least one lesion, at least at one time point. Patients 19 to 38 were in CR. For patients in CR, the risk of relapse was calculated according to the Miettinen criteria based on tumor localization, tumor size, and mitotic index, where applicable. CR-HR, CR-MR, and CR-LR denote CR with high, moderate, and low risk of relapse, respectively. Abbreviations: A, active disease; A-R, active disease with response to treatment; A-PD, active disease with disease progression; NA, not applicable; NE, not evaluated.
Figure 3. A–E, LINE1 and mutant fcDNA in correlation with clinical response in individual patients over time. LINE1 (long interspersed nuclear elements; left y-axis) reflects total fcDNA; mutant allele in percentage wt indicates the amount of mutant fcDNA (right y-axis). Treatments and results of imaging (MRI, CT, and FDG-PET/CT) are shown below the time line (x-axis). Patient numbers correspond to Fig. 2. A, patient 5 (age, 76 years; female), CKIT exon 9 insertion AY502-503 mutation (BSC, best supportive care). B, patient 7 (age, 75 years; female), CKIT exon 11 deletion Y553-Q556 mutation.
Figure 3. (Continued) C, patient 10 (age, 51 years; female), *CKIT* exon 11 deletion V555-D572 insG and exon 17 D820Y mutation. D, patient 8 (age, 49 years; female), *CKIT* exon 11 deletion K550-K558 mutation.
Our finding of very rapid (within 2 weeks) and durable positive to negative conversion in 2 patients responding, and a negative to positive conversion preceding radiographic relapse by 3 months suggest that detection of fcDNA might be used as tumor-specific biomarker to predict response early after initiation of treatment, and to predict relapse. We were able to detect mutant fcDNA in 9 of these 18 cases with active disease. There are several possible explanations for the negative cases. First, 3 negative cases fulfilled the prespecified criteria for active disease (at least one lesion that could be measured) but, however, no samples were available at the time of progression, and samples at the time of response to TKI were negative (Table 2). In addition, biologic properties inherent to the tumor such as vascularization, cell turn over, apoptosis rate, active secretion of DNA and/or RNA, and localization might impact the release of nucleic acids into the blood stream (26, 39). Furthermore, 3 of 9 negative patients with active disease carried large duplications that can hamper detection caused by ineffective probe hybridization (43–57 base pairs; patient 2, 4, and 14; Supplementary Table S1). Sensitivities of our assays ranged between 0.01% and 0.1% mutation/wt allele. The K642E assay was an exception with 0.8% cross-reactivity reducing the sensitivity to 1%, due to a high GC content of the hybridization probe. More sensitive mutation detection assays might reduce false-negative results. In addition, polyclonal disease and clonal competition may hamper the detection of individual mutations in plasma, especially when clones become dominant that do not harbor the initial mutation. Imatinib resistance in GIST lesions is associated with acquisition of secondary CKIT or PDGFRA mutations that mediate inhibitor resistance (40–43). Importantly, specific lesions may carry different mutations, indicating polyclonal resistance (40, 43). However, repeated biopsies of different lesions are not feasible. In 2 patients, we were able to separately track back and forth distinct clones harboring individual mutations, including mutations not known from biopsies, indicating clonal heterogeneity and competition during treatment. In the future, alternative technologies such as targeted massive parallel sequencing or multiplex assays might simultaneously identify and quantitate secondary disease subclones, allowing unbiased detection of sequence changes and thus might mirror clonal heterogeneity. This includes secondary mutations mediating resistance to treatment, allowing earlier treatment changes without repeated tumor biopsies. We are currently expanding our analysis of secondary resistance mutations in plasma samples of patients with progressive disease and low or decreasing amounts of the initial mutation.
In 6 of 20 patients in CR, we were able to detect mutant fcDNA in plasma at low levels, indicating that viable tumor remains in a proportion of patients after removal of the primary tumor. We were not able to establish a correlation of Miettinen risk to fcDNA positivity due to low number of patients, and possibly also due to the fact that all high-risk patients received adjuvant imatinib. However, with longer follow-up, it will be important to correlate test positivity with relapse especially in high-risk patients, as it is currently not known which subgroup of patients benefit most from adjuvant treatment, and which patients are at risk of relapse. Together, our results indicate that mutant fcDNA specifically can be detected and quantified in the plasma of patients with GIST using quantitative L-PCR. The amount of mutant, tumor-derived fcDNA correlated with response, and our approach detected secondary mutations that are known to confer treatment resistance. Thus, detection of tumor fcDNA might be used as tumor-specific biomarker to predict both tumor response and relapse in patients with GIST.

Disclosure of Potential Conflicts of Interest

T. Lange has commercial research grant from Novartis, Bristol-Myers Squibb, Ariad, and Pfizer, and is a consultant/advisory board member of Novartis, Pfizer, Ariad, and Bristol-Myers Squibb. N. von Bubnoff has honoraria from Speakers Bureau of Novartis Oncology, and has not been presented or submitted for publication elsewhere.

References

Detection of Mutant Free Circulating Tumor DNA in the Plasma of Patients with Gastrointestinal Stromal Tumor Harboring Activating Mutations of CKIT or PDGFRA

Jacqueline Maier, Thoralf Lange, Irina Kerle, et al.