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Consultations in Molecular Diagnostics

A Case of *FIP1L1-PDGFRA*-Positive Chronic Eosinophilic Leukemia with a Rare *FIP1L1* Breakpoint

Frédéric Lambert,* Pierre Heimann,† Christian Herens,* Alain Chariot,‡ and Vincent Bours*

From the Departments of Human Genetics and Medical Chemistry,‡ Groupe Interdisciplinaire de Genoproteomique Appliquee, Centre Hospitalier Universitaire de Liège, University of Liège, Liège; and the Department of Human Genetics,† Free University of Brussels, Brussels, Belgium*

The idiopathic hypereosinophilic syndrome (HES) has remained for a long time a diagnosis of exclusion. Differential diagnosis between the HES and the related chronic eosinophilic leukemia (CEL) relied on the identification of signs of clonality that allowed, when present, the reclassification of patients as CEL. Recently, a new acquired mutation was described in approximately 50% of the HES/CEL patients: a cryptic deletion on chromosome band 4q12 generating a *FIP1L1-PDGFRA* fusion gene. According to the World Health Organization classification, this clonal abnormality has been proposed as a new surrogate marker for chronic eosinophilic leukemia diagnosis. Fluorescence *in situ* hybridization and reverse transcriptase-polymerase chain reaction protocols were developed for an accurate del(4)(q12q12) and *FIP1L1-PDGFRA* fusion gene detection. Here, we report a patient with a rare *FIP1L1* intron 16 breakpoint located outside of the reported *FIP1L1* breakpoint region (ie, from *FIP1L1* introns 9 to 13). This case illustrates the risk of false-negative results with diagnostic procedures that do not take into account the occurrence of rare *FIP1L1* breakpoints. As targeted therapy with tyrosine kinase inhibitors has dramatically changed the prognosis of *FIP1L1-PDGFRA* (+) CEL, false-negative results could hamper accurate diagnosis and treatment. ***(J Mol Diagn 2007, 9:414–419; DOI: 10.2353/jmoldx.2007.060196)***

The idiopathic hypereosinophilic syndrome (HES) represents a heterogeneous group of diseases characterized by sustained hypereosinophilia (over 1500 eosinophils/μl), lasting for more than 6 months, and associated with end organ damages in the absence of any identifiable reactive conditions.[1] According to the World Health Organization guidelines, the identification of a clonal origin of the disease allows the reclassification of these cases as chronic eosinophilic leukemia (CEL).[1] Recently, empirical trials of imatinib mesylate (Gleevec; Novartis, Basel, Switzerland) targeted therapy allowed the discovery of an 800-kb submicroscopic deletion on chromosome 4q12 in some responsive HES/CEL patients.[2] The cysteine-rich hydrophobic domain 2 (*CHIC2*) deletion (del*CHIC2*), a surrogate marker for the del(4)(q12q12), and the FIP1-like-1 (*FIP1L1*)-platelet-derived growth factor receptor α (*PDGFRA*) fusion gene, resulting from the subsequent fusion of the 5′ part of *FIP1L1* to the 3′ part of *PDGFRA*, represent powerful clonality markers for the direct diagnosis of CEL.[3–5] The FIP1L1-PDGFRα transforming properties have been described in *vitro* as well as *in vivo*.[2,6] As a consequence, powerful tools are mandatory for an accurate detection of these abnormalities, which offer new opportunities in HES/CEL differential diagnosis and therapy.[3,5] The del(4)(q12q12) cannot be detected by conventional cytogenetics, and consequently, the majority of HES/CEL patients harbor a normal karyotype.[2,5] The diagnosis therefore relies on fluorescence *in situ* hybridization (FISH) and on reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of the del(4)(q12q12) and the *FIP1L1-PDGFRA* fusion gene, respectively.[2,4,7,8] Both tools are sensitive, but false-negative results can occur. For these reasons, a combination of both FISH and RT-PCR has been advised for appropriate diagnosis.[9]

Previously reported patients presented a breakpoint invariably located in exon 12 of *PDGFRA*, whereas *FIP1L1* breakpoints scattered over a 40-kb region encompassing

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Address reprint requests to Dr. F. Lambert, Department of Medical Genetics, CHU B35, University of Liège, Sart-Tilman, 4000 Liège, Belgium. E-mail: flambert@chu.ulg.ac.be.

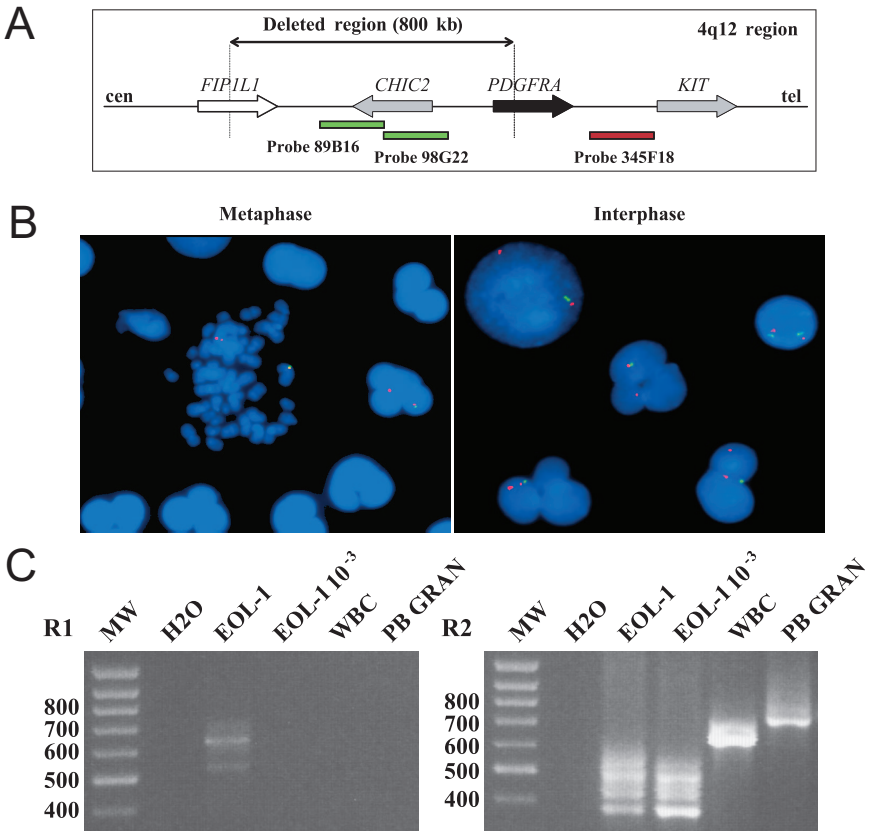

Figure 1. FISH and *FIP1L1-PDGFRA* RT-PCR. **A:** Schematic representation of the 4q12 region. The 800-kb submicroscopic deletion demonstrated in CEL involves a portion of chromosome 4 that includes the *CHIC2* locus and generates a *FIP1L1-PDGFRA* fusion gene. Two overlapped probes, 89B16 and 98G22, were tagged with Spectrum Green and used to map the *CHIC2* locus, whereas one Spectrum Orange-tagged probe, 345F18, mapped a sequence between the *PDGFRA* and the *KIT* loci. Normal chromosome 4 should demonstrate a red-yellow-green fusion signal generated by the juxtaposition of the green- and red-labeled probes, whereas the del*CHIC2* would generate an isolated red signal. **B:** An abnormal metaphase demonstrating a normal chromosome 4 and a 4q12 deletion characterized by an isolated red spot (left). The same pattern of hybridization is observed on interphase with three polylobulated nuclei bearing the del*CHIC2* characterized by the loss of one green signal, whereas a round nucleus is not deleted (right). **C:** Nested RT-PCR performed on RNA extracted from white blood cell (WBC) and peripheral blood granulocytes (PB GRAN) collected at diagnosis. EOL-1 cells were used as a positive control. The *FIP1L1-PDGFRA* fusion gene was present in both fractions but required two rounds of PCR to be detected [left, first round (R1); right, second round (R2)] MW, molecular weight.

introns 7 to 10, as numbered in Cools et al[2,5] nomenclature, or introns 8 to 13, according to the LeukemiaNet exon-intron numbering.[2,5,10,11] Here, we report a CEL patient with a breakpoint located in intron 16 of *FIP1L1*, thus outside of the reported *FIP1L1* breakpoint cluster region. This case illustrates the sensitivity of the previously published RT-PCR technique and highlights the risk of false-negative results with real-time quantitative (RQ) RT-PCR approaches that do not take into account rare *FIP1L1* breakpoints downstream of *FIP1L1* exon 13.[10]

Case Reports

A 41-year-old man was referred for primary hypereosinophilia differential diagnosis. Secondary etiologies associated with reactive hypereosinophilia were extensively excluded. A peripheral blood cell count disclosed hyperleukocytosis with an absolute eosinophil count of 27.3×10^9/L. Serum vitamin B12 was >2000 pg/ml, and tryptase was >75 μg/L (N <11.5 μg/L). A bone marrow aspiration disclosed 38% of eosinophilic granulocytes, and trephine biopsy showed hypercellularity with marked granulocytic hyperplasia and eosinophilia. Conventional cytogenetic analysis revealed a 46,XY karyotype. However, combining a FISH analysis as described by Pardanani et al[4] and the RT-PCR procedure described by Cools et al,[2] a del(4)(q12q12) and a *FIP1L1-PDGFRA* fusion gene were demonstrated, respectively (Figure 1). According to the World Health Organization guidelines, a diagnosis of CEL was therefore established.[1] A specific treatment with the tyrosine kinase inhibitor imatinib mesylate was initiated and led to a dramatic improvement of the symptoms and a rapid normalization of the white blood cell count (see below).

Materials and Methods

FISH Analysis

FISH assay was performed on fresh peripheral blood and fixed bone marrow cells. Deletion of the 4q12 region was assessed by a two-color FISH strategy. The *CHIC2* locus is located telomeric to *FIP1L1* but centromeric to *PDGFRA* and thus represents a surrogate marker for the *FIP1L1-PDGFRA* fusion gene, resulting from the 800-kb del(4)(q12q12) involving the region between *FIP1L1* and *PDGFRA*. The *CHIC2* status was evaluated both on metaphase and interphase nuclei. We used two overlapped bacterial artificial chromosomes (BACs), clones 89B16 (accession no. AC105384) and 98G22 (accession no. AQ317591), labeled with Spectrum Green that mapped the *CHIC2* locus and a Spectrum Orange (Vysis, Downers Grove, IL) labeled probe, BAC clone 345F18 (accession no. AC110611) that mapped a sequence between the *PDGFRA* and the *KIT* loci. Glycerol stocks of the BAC clones were obtained from P. Dejong's library (*http://bacpac.chori.org/*). The map position of each BAC clone was determined according to the University of California Santa Cruz Human Genome Project (*http://genome.ucsc.edu*). Nick translation labeling was per-

formed using the Vysis Nick Translation Kit. Hybridization signals were visualized using an Olympus BX51 fluorescence microscope equipped with a cooled charge-coupled camera and were digitally recorded (Cytovision; Applied Imaging Ltd., Newcastle upon Tyne, UK). Hybridization of the three labeled probes on each chromosome results in two red-yellow-green fusion signal on normal 4q12 regions, whereas a chromosome 4 with a *CHIC2* deletion would have lost its green signal.

Three thousand interphase nuclei from six normal individuals were scored in a blinded manner by three investigators for the presence or absence of the green (BACs 89B16 and 98G22) and red (BAC 345F18) hybridization signals. The normal cut-off value was evaluated from six healthy controls and 3000 nuclei observations, which yielded two signals in 96.3% of observed nuclei. The threshold value was established at 91.8% with a 95% confidence interval. At least 100 interphase nuclei of the studied patients were scored for the presence of the red and the absence of the green signals.

RT-PCR and Sequencing

RT-PCR detection of the *FIP1L1-PDGFRA* fusion was performed as described by Cools et al.[2] In brief, total RNA was purified from blood and bone marrow using the RNeasy columns (Qiagen, Santa Clarita, CA). One μg of RNA was reverse transcribed using random hexamers and the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) in a total volume of 20 μl, according to the manufacturer's instructions. A first round of PCR amplification was performed starting from 1/10th of the cDNA in a total volume of 50 μl containing 15 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 20 pmol of each primer, and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). The primer pairs were FIP1L1-F4 (5′-ACCTGGTGCTGATCTTTCTGAT-3′) and PDGFRA-R1 (5′-TGAGAGCTTGTTTTTCACTGGA-3′) for the first PCR. One μl of the first amplification was subsequently subjected to a second round of PCR with primers FIP1L1-F5 (5′-AAAGAGGATACGAATGGGACTTGTG-3′) and PDGFRA-R2 (5′-GGGACCGGCTTAATCCATAG-3′). PCR conditions were as follows: 94°C, 10 minutes, followed by 35 cycles of 94°C, 1 minute; 61°C, 30 seconds; 72°C, 45 seconds, and a final elongation at 72°C for 10 minutes. The EOL-1 cell line (DSMZ, Berlin, Germany) bearing a *FIP1L1-PDGFRA* fusion gene was used as a positive control. The RNA quality as well as the cDNA synthesis efficacy were evaluated with an RQ RT-PCR amplifying the housekeeping gene β-glucuronidase (*GUS*) as described.[12] Amplified PCR products were purified using QIAquick PCR purification columns (Qiagen) and sequenced on both strands using the BigDye Terminator Sequencing protocol on an automated ABI 3100 sequencer. Sequences were analyzed with Sequence Analysis software V.5.1.1 (Applera, Norwalk, CT). After comparison with the published sequences (Ensembl software, *http://www.ensembl.org/index.html*), specific patient's forward (FIP1L1S15: 5′-TGGACATTCCTCTGGTTATGA-3′) and reverse (PDGFRAAS13: 5′-TGTTCCTTCAACCACCTTCC-5′) primers were designed with the Primer3 freeware *(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi*). RNA (50-ng equivalent) was amplified using the following PCR conditions: 95°C, 10 minutes, followed by 35 cycles of 94°C, 30 seconds; 60°C, 30 seconds; 72°C, 45 seconds, and a final elongation at 72°C for 5 minutes.

For RQ RT-PCR, Primer Express 2.0 software (Applied Biosystems) was used to design a *FIP1L1* exon 16 forward primer, FIP1L1E16T (5′-CCATCTTCCTGGTTCTGCTC-3′), a carboxyfluorescein dye-labeled TaqMan probe, P-PDGF12 (5′-TGACTCAAGATGGGAGTTTCCAAGAGATGG-3′), and a reverse primer, AS-PDGF12 (5′-CAAGACCCGACCAAGCACTAG-3′), located downstream to the *PDGFRA* exon 12 breakpoint. The *GUS* gene amplification was used as normalization control.[12] One microgram of total RNA was isolated from blood and reverse transcribed as described for the qualitative RT-PCR. One-twentieth of the cDNA was then used for the RQ RT-PCR reaction on an ABI Prism 7000 Sequence Detection system (Applied Biosystems). PCR conditions and cycling parameters were in accordance with the default parameters of the ABI SDS 7000 apparatus (40 cycles of annealing/extension for 1 minute at 65°C). PCR products were detected using the TaqMan chemistry (Applied Biosystems).

Results

We report a case of HES with biochemical parameters compatible with a primary hematological process.[8] Clonality of the eosinophils was assessed by molecular assays. A FISH analysis was first performed, with the aim of identifying the 4q12 deletion. One hundred interphase nuclei were examined, and 83% of them showed the *CHIC2* locus deletion, a surrogate marker for the *FIP1L1-PDGFRA* fusion gene. The majority of the 17% of normal cells were round and mononucleated and were most likely lymphocytes (Figure 1, A and B). A *FIP1L1-PDGFRA* RT-PCR confirmed the presence of the fusion gene. Of note, a nested PCR was necessary to clearly identify the patient's fusion gene (Figure 1C). According to the World Health Organization rules, these results demonstrated the clonal origin of the disease, and a definitive diagnosis of a CEL was thus made.[1,5] The identification of the *FIP1L1-PDGFRA* fusion gene is known to assess clonality in around 50% of hypereosinophilic syndrome.[1,2]

To initiate a molecular follow-up of our patient, we sequenced the *FIP1L1-PDGFRA* PCR product. Whereas all of the previously reported patients had *FIP1L1* breakpoints scattered between introns 7 to 10 (or 9 to 13 following the recently proposed LeukemiaNet nomenclature) (Figure 2A),[11] we surprisingly observed a *FIP1L1* breakpoint in intron 16 (Figure 2B). This broader dispersion of the *FIP1L1* breakpoints could probably explain some false-negative *FIP1L1-PDGFRA* RT-PCR. Moreover, direct sequencing of the RT-PCR products revealed several splicing variants that joined exons 13, 15, or 16 of *FIP1L1* to *PDGFRA* exon 12, generating PCR products

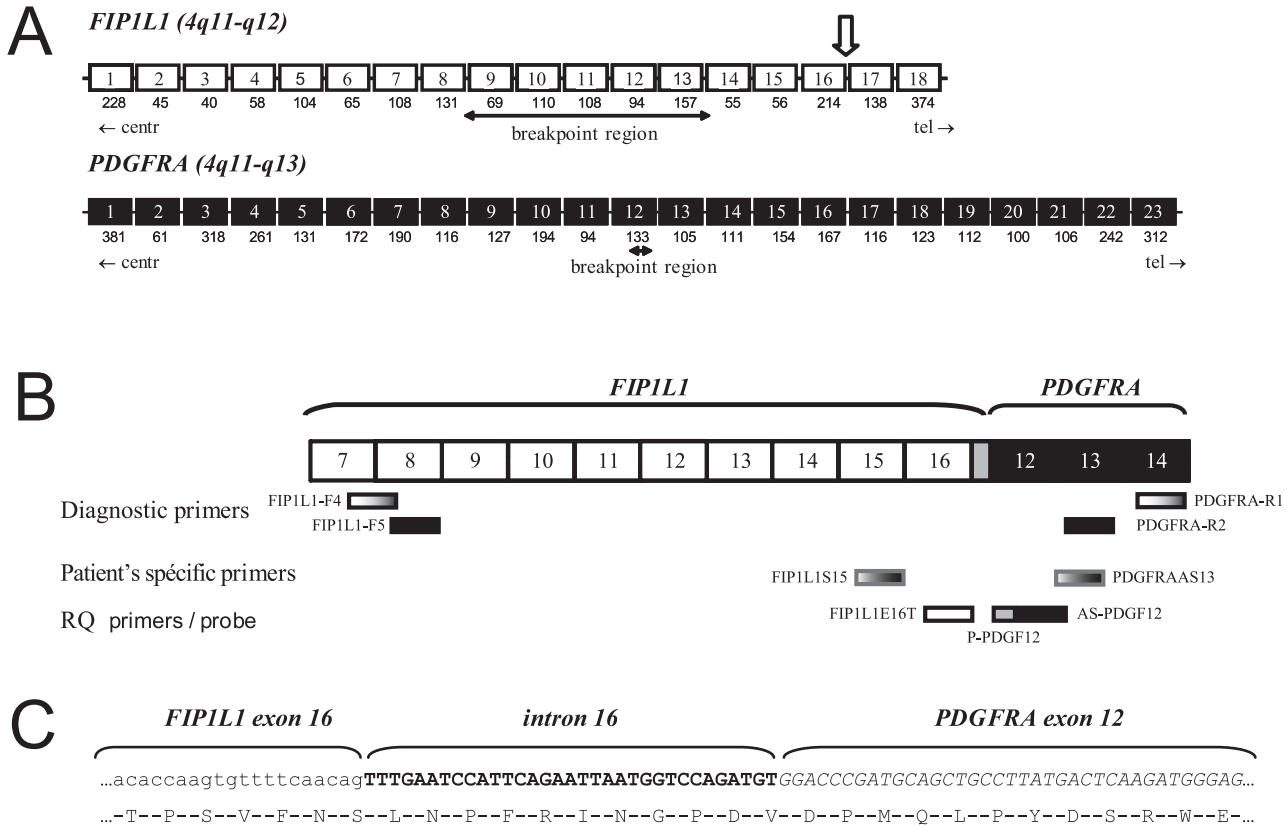

Figure 2. Molecular analysis of the patient's *FIP1L1-PDGFRA* fusion gene. **A:** Maps of *FIP1L1* and *PDGFRA* genes. Boxes represent exons according to the LeukemiaNet exons numbering (*FIP1L1*, Ensembl Gene ID: ENSG00000145216; *PDGFRA*, ENSG00000134853). Numbers below the boxes indicate exon sizes in bp. The previously described *FIP1L1* breakpoint region is indicated, encompassing introns 8 to 13, whereas *PDGFRA* breakpoint is invariably located in exon 12. The **white arrow** indicates our patient's *FIP1L1* intron 16-peculiar breakpoint. **B:** Patient's specific *FIP1L1-PDGFRA* fusion gene juxtaposing a short portion of *FIP1L1* intron 16 (33 bp) to *PDGFRA* exon 12. Primers and probes used in our assay are indicated. The diagnosis qualitative RT-PCR was performed with the primer pairs FIP1L1-F4 and PDGFRA-R1 for the first round and FIP1L1-F5 combined with PDGFRA-R2 for the second round (Diagnostic primers). Patient's specific primers used for the qualitative follow-up were FIP1L1S15 and PDGFRAAS13 (Patient's specific primers). For the quantitative RQ RT-PCR, exon 16 forward primer FIP1L1E16T was used in conjunction with a carboxyfluorescein dye-labeled TaqMan probe, P-PDGF12, and a reverse primer, AS-PDGF12 (RQ primers/probe). **C:** One of the patient's specific *FIP1L1-PDGFRA* sequences represented at the mRNA and protein levels. The fusion gene conserved an open reading frame.

from 629 to 738 bp long. Sequence analysis revealed an open reading frame only for one transcript. The resulting predicted sequence of the fusion protein is illustrated in Figure 2C.

Long-term follow-up of *FIP1L1-PDGFRA*(+) patients treated with imatinib mesylate has not been well documented so far. For this purpose, we built two PCR assays. First, a nonquantitative RT-PCR was designed, with a forward primer located in exon 15 of *FIP1L1*, and the fusion gene load was followed with this qualitative RT-PCR using the primers described in Figure 2B. The *FIP1L1-PDGFRA* transcript level dropped below the sensitivity level of this assay at day 55 of treatment (Figure 3A). We further analyzed the fusion gene kinetics by RQ RT-PCR. Patient's specific primers and probes were designed after direct sequencing of the PCR product. We built a common reverse primer, AS-PDGF12, and a carboxyfluorescein dye-labeled TaqMan probe, P-PDGF12, located downstream the *PDGFRA* exon 12 breakpoints reported in the literature. The forward primer, FIP1L1E16T, was located in exon 16 of *FIP1L1* (Figure 2B). With this assay, the FIP1L1-PDGFRA transcript dropped below the level of quanti-

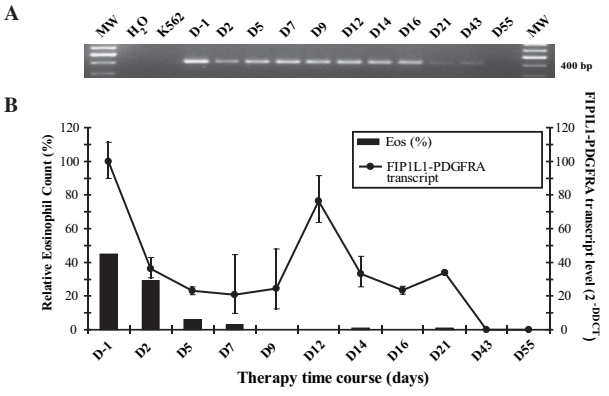

Figure 3. Molecular follow-up during imatinib mesylate treatment. **A:** Qualitative RT-PCR during targeted therapy with the tyrosine kinase inhibitor imatinib mesylate. The chronic myelogenous leukemia K562 cell line was used as negative control. Molecular remission was achieved at day 55. MW, molecular weight. **B:** Quantitative monitoring of the relative eosinophilia as well as the fusion gene load during imatinib mesylate treatment. *FIP1L1-PDGFRA* mRNA expression was calculated relative to the housekeeping gene *GUS* using the comparative Ct method.[16] Relative eosinophil count is indicated by black columns. The patient's *FIP1L1-PDGFRA* transcript load rapidly decreased after initiation of molecular targeted therapy with imatinib and fell under the limit of detection at day 43. The patient remained in molecular remission 1 year after treatment initiation.

fication at day 43 and remained undetectable thereafter (Figure 3B).

Discussion

An accurate CEL diagnosis is essential as these patients greatly benefit from a specific and well-tolerated treatment with imatinib mesylate.[2,5,8] Our report supports the use of both the FISH and RT-PCR approaches to avoid false-negative results and subsequent inadequate treatments for some patients. Indeed, the FISH technique is robust but not as sensitive as the RT-PCR. For instance, a HES patient previously treated with interferon-α was addressed to our lab for *FIP1L1-PDGFRA* detection. FISH failed to detect the del*CHIC2*, whereas the *FIP1L1-PDGFRA* fusion gene was easily demonstrated by RT-PCR (data not shown). On the other hand, the *FIP1L1-PDGFRA* RT-PCR could be tricky, because it often generates multiple bands (Figure 1C). In theory, a *FIP1L1* breakpoint localized downstream of exon 13 should generate, with the primers designed by Cools et al,[2] an RT-PCR product longer than 1000 bp. Such a long PCR product could be difficult to amplify or detect. However, in our case, the skipping of exon 11 or part of exon 13 resulted in amplicon sizes of 629, 709, and 738 bp shorter than theoretically expected, which were easily visualized on agarose gel electrophoresis after two rounds of PCR (Figure 1C). Such a rare breakpoint had already been reported in a single patient by Chung et al.[13] Moreover, we observed the present case and another one with a *FIP1L1* exon 14 breakpoint out of six HES/CEL patients (data not shown). Therefore, although such events are rare, they should be considered when a new assay is proposed. For instance, Jovanovic et al[10] recently proposed a new diagnostic approach based on real-time PCR. These RQ-PCR assays were designed for *FIP1L1* breakpoints leading to fusion of exons 9 to 13[7–10] of *FIP1L1* to exon 12 of *PDGFRA*. Although this recent strategy is not the "gold" standard for *FIP1L1-PDGFRA* fusion gene detection at diagnosis, its easy procedure could encourage laboratories to choose it as a tool for screening purposes in HES. In another type of leukemia, we have recently described three false-negative *BCR-ABL1* detection using a RQ RT-PCR screening approach in the process of chronic myelogenous leukemia diagnosis, as a consequence of complex genetic rearrangements.[14] Our observation thus indicates a risk of false-negative results associated with simplified RQ RT-PCR strategies, whereas the technique described by Cools et al appropriately identified the fusion transcript.

Moreover, *FIP1L1-PDGFRA*-"negative" patients who respond to imatinib have been described.[15] Our results illustrate that breakpoints in *FIP1L1* may vary and that infrequent *FIP1L1-PDGFRA* fusion gene variants should be thoroughly excluded as a possible source of false-negative results in such cases. In conclusion, because of the therapeutic repercussions of a *FIP1L1-PDGFRA* (+) CEL diagnosis, it is essential to use the most appropriate diagnostic procedure to avoid false-negative results linked to unusual breakpoint.

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