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## A Tyrosine Kinase Created by Fusion of the PDGFRA and FIP1L1 Genes as a Therapeutic Target of Imatinib in Idiopathic Hypereosinophilic Syndrome

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### ABSTRACT

#### BACKGROUND

Idiopathic hypereosinophilic syndrome involves a prolonged state of eosinophilia associated with organ dysfunction. It is of unknown cause. Recent reports of responses to imatinib in patients with the syndrome suggested that an activated kinase such as ABL, platelet-derived growth factor receptor (PDGFR), or KIT, all of which are inhibited by imatinib, might be the cause.

#### METHODS

We treated 11 patients with the hypereosinophilic syndrome with imatinib and identified the molecular basis for the response.

#### RESULTS

Nine of the 11 patients treated with imatinib had responses lasting more than three months in which the eosinophil count returned to normal. One such patient had a complex chromosomal abnormality, leading to the identification of a fusion of the Fip1-like 1 (FIP1L1) gene to the PDGFR $\alpha$  (PDGFRA) gene generated by an interstitial deletion on chromosome 4q12. FIP1L1-PDGFR $\alpha$  is a constitutively activated tyrosine kinase that transforms hematopoietic cells and is inhibited by imatinib (50 percent inhibitory concentration, 3.2 nM). The FIP1L1-PDGFR $\alpha$  fusion gene was subsequently detected in 9 of 16 patients with the syndrome and in 5 of the 9 patients with responses to imatinib that lasted more than three months. Relapse in one patient correlated with the appearance of a T674I mutation in PDGFRA that confers resistance to imatinib.

#### CONCLUSIONS

The hypereosinophilic syndrome may result from a novel fusion tyrosine kinase — FIP1L1-PDGFR $\alpha$  — that is a consequence of an interstitial chromosomal deletion. The acquisition of a T674I resistance mutation at the time of relapse demonstrates that FIP1L1-PDGFR $\alpha$  is the target of imatinib. Our data indicate that the deletion of genetic material may result in gain-of-function fusion proteins.

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**T**HE HYPEREOSINOPHILIC SYNDROME is a rare hematologic disorder with sustained overproduction of eosinophils in the bone marrow, eosinophilia, tissue infiltration, and organ damage. The diagnosis is based on the criteria of Chusid et al.<sup>1</sup>: sustained eosinophilia (more than 1500 eosinophils per cubic millimeter) for more than six months; the absence of other causes of eosinophilia, including parasitic infections and allergies; and signs and symptoms of organ involvement, most frequently the heart, the central and peripheral nervous system, the lungs, and the skin. The syndrome is more common in men than women (ratio, 9:1) and occurs predominantly between the ages of 20 and 50 years. Total leukocyte counts are usually less than 25,000 per cubic millimeter, with 30 to 70 percent eosinophils. Bone marrow eosinophils are increased (30 to 60 percent), but myeloblasts are usually not.<sup>2</sup> It has been difficult to assess the clonality of the hypereosinophilic syndrome, but some cases are clonally derived, as demonstrated by clonal karyotypic abnormalities and X-inactivation assays.<sup>3,4</sup>

Treatment of the hypereosinophilic syndrome attempts to limit organ damage by controlling the eosinophil count and includes prednisone, hydroxyurea, interferon alfa, and cytotoxic chemotherapy. In most cases, however, the disorder is fatal. Recently, it was reported that four of five cases responded to imatinib mesylate (Gleevec, Novartis).<sup>5</sup> Imatinib, a 2-phenylaminopyrimidine-based tyrosine kinase inhibitor,<sup>6</sup> has been approved for the treatment of BCR-ABL-positive chronic myeloid leukemia (CML) and acute lymphoblastic leukemia.<sup>7,8</sup> Besides the ABL tyrosine kinase, imatinib also inhibits the type III transmembrane receptors KIT and platelet-derived growth factor receptor (PDGFR)  $\beta$ .<sup>6,9,10</sup> Hence, imatinib is also a promising new treatment for gastrointestinal stromal tumors, which frequently harbor activating mutations in the KIT gene,<sup>11,12</sup> and chronic myeloproliferative diseases with rearrangements of the gene for PDGFR $\beta$  (PDGFRB).<sup>13</sup> The clinical response to imatinib suggests that the hypereosinophilic syndrome may also be associated with constitutive activation of ABL, KIT, PDGFR, or an as yet unidentified target. Therefore, we evaluated the response to imatinib in patients with the hypereosinophilic syndrome and the molecular basis of the response.

## METHODS

### PATIENTS AND TREATMENT

The study was conducted from June 2001 to October 2002. We studied 16 patients who had received a diagnosis of the hypereosinophilic syndrome and 1 who had acute myelogenous leukemia with marrow fibrosis that developed from a rapidly progressive hypereosinophilic myeloproliferative disorder (Table 1). Only Patients 1 through 11, who had symptomatic disease, were treated with imatinib, at a dose of 100 to 400 mg per day. Patients 12 through 17 were not treated with imatinib. No patient concurrently received cytotoxic therapy.

Written informed consent was obtained for the prospective accrual of clinical data and the collection of biologic specimens for analysis of genes known to be inhibited by imatinib. Complete hematologic remission was defined by a white-cell count of less than 10,000 per cubic millimeter, a platelet count of more than 100,000 per cubic millimeter, the presence of fewer than 5 percent eosinophils in the peripheral blood and bone marrow, the absence of blasts and promyelocytes in the peripheral blood, and the absence of extramedullary involvement.

### FLUORESCENCE IN SITU HYBRIDIZATION

Fluorescence in situ hybridization was performed as described previously.<sup>14</sup> Probes were obtained from the Roswell Park Cancer Institute libraries RPC16 and RPC111 (<http://www.chori.org/BACPAC>).

### RAPID AMPLIFICATION OF COMPLEMENTARY DNA ENDS

Trizol (Invitrogen) was used to extract RNA from white cells. DNA was extracted with use of the QIAmp DNA blood Maxi Kit (Qiagen). First-strand complementary DNA (cDNA) was synthesized from 2  $\mu$ g of total RNA with the use of the Superscript first-strand synthesis system (Invitrogen) with a gene-specific primer or random primers. Rapid amplification of cDNA ends was performed as previously described<sup>15</sup> with primers PDGFRA-R1 for cDNA synthesis and PDGFRA-R2 and PDGFRA-R3 for a nested polymerase chain reaction (PCR).

### PCR ASSAY

Fusion of the Fip1-like 1 (FIP1L1) gene to the PDGFR $\alpha$  (PDGFRA) gene was confirmed on cDNA by nested PCR with the use of primer pairs FIP1L1-

F4 and PDGFRA-R1 and FIP1L1-F5 and PDGFRA-R2. The reciprocal fusion was detected with the use of primer pairs PDGFRA-F5 and FIP1L1-R1 and PDGFRA-F3 and FIP1L1-R2. Amplification of the fusion gene at the DNA level was performed with use of the Expand Long template PCR system (Roche). For mutation analysis of the *FIP1L1-PDGFR*A fusion gene in Patient 5, the gene was amplified with primers FIP1L1-F5 and PDGFRA-R12. Exon 15 of PDGFRA was amplified with primers PDGFRA-F14 and PDGFRA-R15. PCR products were cloned in a pGEM-T-easy plasmid (Promega) and sequenced with use of an ABI sequencer (Perkin Elmer). Long-distance inverse PCR was performed as previously described,<sup>16</sup> with use of an *AseI* digest (New England Biolabs) and primer pairs LDI1 and PDGFRA-R2 in the first PCR and LDI2 and PDGFRA-R3 in the nested PCR.

#### CONSTRUCTS

The open reading frame of the *FIP1L1-PDGFR*A fusion gene was amplified by PCR from cDNA from Patient 1 with use of the proofreading enzyme HF-2 (Clontech) and primers FIP1L1-Fc and PDGFRA-Rc. This PCR product was cloned in the retroviral vector MSCV-EGFP (kindly provided by W. Pear, University of Pennsylvania). Constructs with point mutations, constructs with deletion mutations, and epitope-tagged constructs were obtained by PCR from the *FIP1L1-PDGFR*A clone and cloned into MSCV-EGFP. Similarity searches were performed with use of the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>).

#### CELL CULTURE

293T cells were grown in Dulbecco's modified Eagle's medium with 10 percent fetal-calf serum, and Ba/F3 cells were grown in RPMI medium with 10 percent fetal-calf serum and 1 ng of mouse interleukin-3 per milliliter. Production of retroviral supernatant and transduction have been described previously.<sup>17</sup> Interleukin-3-independent growth was assessed by plating transduced Ba/F3 cells in interleukin-3-free medium, after the cells were washed three times in phosphate-buffered saline. Imatinib was stored as a 10 mM stock solution in water and diluted in RPMI medium for use. For Western blotting, Ba/F3 cells were incubated in the presence of imatinib for 90 minutes before lysis. For dose-response curves, Ba/F3 cells were incubated for 24 hours in the presence of imatinib, and the

number of viable cells at the start and at the end was determined with the Celltiter96A<sub>Queous</sub> One solution proliferation assay (Promega). Dose-response curves were fitted with use of OriginPro 6.1 software (OriginLab).

#### WESTERN BLOTTING

Immunoprecipitation was performed with use of anti-MYC antibody (Cell Signaling) and protein G agarose (Roche). Each procedure involved 6 million Ba/F3 cells with stable expression of MYC-tagged wild-type *FIP1L1-PDGFR* $\alpha$  fusion protein or the T674I mutant. Cells were lysed in lysis buffer (Cell Signaling) containing 1 mM sodium orthovanadate, 20  $\mu$ M phenylarsine oxide (Calbiochem), and protease inhibitors (complete tablets, Roche). For Western blotting, Ba/F3 cells were collected, lysed in loading buffer (Cell Signaling), separated by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis, and transferred to membranes. The following antibodies were used: anti-phospho-ERK1/2, anti-phospho-STAT5 and antiphosphotyrosine (P-Tyr-100/102, Cell Signaling), anti-PDGFR $\alpha$  (Upstate), anti-STAT5b (Santa-Cruz), antimouse peroxidase, and antirabbit peroxidase (Amersham Pharmacia Biotech). Detection was performed with use of the Western Lightning system (Perkin Elmer).

#### PRIMERS

The following primers were used: PDGFRA-F3, 5'gagcagagaaccgagagctc; PDGFRA-F5, 5'caaagtggaggagaccatcg; PDGFRA-F14, 5'gaacattgtaaacctgctggg; PDGFRA-R1, 5'tgagagcttgttttctactgga; PDGFRA-R2, 5'gggaccggcttaatccatag; PDGFRA-R3, 5'ggctgttcttcaaccacct; PDGFRA-R12, 5'catagctcctgctttca; PDGFRA-R15, 5'catagctcctgctgttca; PDGFRA-Rc, 5'cgaattcccagttacaggaagctgtct; FIP1L1-Fc, 5'tggatcccggccatgctgccggcgag; FIP1L1-F4, 5'acctggtgctgatctttctgat; FIP1L1-F5, 5'aaaggatagcgaatgggacttg; FIP1L1-R1, 5'agtgctgacagtcggaggag; FIP1L1-R2, 5'gagctcctggagggaataaac; LDI1, 5'gaacgtacgatgtttggtttcca; LDI2, 5'gtctctaaccatccaggttgc; GAPDH-F, 5'tggaatcccatcaccatct; and GAPDH-R, 5'gtcttctgggtggcagtgat.

## RESULTS

#### RESPONSE TO IMATINIB IN PATIENTS WITH HYPEREOSINOPHILIC SYNDROME

The median age of the 11 patients (9 men and 2 women) who received imatinib was 46 years

**Table 1. Characteristics of 17 Patients with the Hypereosinophilic Syndrome (HES).\***

Patient No. and Diagnosis	Age (yr)/ Sex	Date of Diagnosis	Dominant Clinical Findings	Prior Therapy	Cytogenetic Findings	Start of Imatinib Therapy	Best Response and Time to Response	Duration of Response	FIP1L1-PDGFR $\alpha$ Present	Base-Line Bone Marrow Eosinophils	Base-Line Absolute Eosinophil Count $\times 10^{-3}/mm^3$
Treated with imatinib											
1, HES	43/M	12/2001	Constitutional symptoms, pulmonary infiltrates	Prednisone, hydroxyurea	t(1;4) (q44;q12)†	2/2002	Complete, 2 wk	>9 mo	Yes	Increased	19.3
2, HES	28/M	11/2000	Cauda equina syndrome	Prednisone, hydroxyurea, interferon alfa	46,XY	8/2001	Complete, 1 mo	>16 mo	No	Increased	14.2
3, HES	53/M	11/2001	Splenomegaly	Prednisone, hydroxyurea	46,XY	2/2002	Complete, 6 wk	>11 mo	No	40% eosinophils	28.9
4, HES	53/M	11/1995	Loeffler's endocarditis, hepatosplenomegaly, GI eosinophil infiltrates	Prednisone, hydroxyurea, vincristine, interferon alfa	46,XY	4/2002	Complete, 2 mo	>7 mo	Yes	Increased	49.4
5, eosinophilia, leukemia	39/M	11/2000	Cranial-nerve palsies, paraspinal masses, splenomegaly, pleural effusions	Hydroxyurea, dexamethasone, cytarabine	Trisomy 8 and 19, add2q, del6q	1/2002	Complete, 2 wk	>5 mo	Yes	Increased, myeloblasts present	0.14
6, HES	61/M	2/2001	Deep venous thrombosis, pulmonary embolism	Prednisone, hydroxyurea	46,XY	3/2002	Complete, 1 wk	>8 mo	Yes	Increased	1.7
7, HES	58/M	1/2002	Adult-onset asthma	Inhaled corticosteroids	46,XY	9/2002	Complete, 1 wk	>3 mo	Not tested	10%	1.8
8, HES	40/F	7/1995	Constitutional symptoms, pruritus, anasarca	Hydroxyurea, interferon alfa, cyclosporine	46,XX	5/2002	Transient complete, 2 wk	NA	No	22%	3.6

Table 1. (Continued.)

Patient No. and Diagnosis	Age (yr)/ Sex	Date of Diagnosis	Dominant Clinical Findings	Prior Therapy	Cytogenetic Findings	Start of Imatinib Therapy	Best Response and Time to Response	Duration of Response	<i>FIP1L1-PDGFR</i> A Present	Base-Line Bone Marrow Eosinophils	Base-Line Absolute Eosinophil Count $\times 10^{-3}/mm^3$
9, HES	52/M	7/1994	Constitutional symptoms, pruritic rash, splenomegaly	Hydroxyurea, cytarabine, cladribine (2-chlorodeoxyadenosine)	46,XY	11/2001	Complete, 3 wk	3†	Yes	44%	6.6
10, HES	41/M	2/1994	Constitutional symptoms, ventricular thrombus, transient ischemic attack	Prednisone, hydroxyurea, interferon alfa	46,XY	8/2002	Complete, 2 wk	>3	No	10%	1.2
11, HES	43/F	8/1999	Abdominal pain, fatigue, erythematous rash	Prednisone, hydroxyurea, interferon alfa	46,XX	4/2002	None	NA	No	17%	1.9
Not treated with imatinib											
12, HES	51/M	10/2001	Myalgias, allergic rhinitis, erythematous rash, CNS involvement	Prednisone, hydroxyurea	46,XY	NA	NA	NA	Yes	Increased	15.2
13, HES	61/F	1/1997	Fatigue	Hydroxyurea, interferon alfa	46,XX	NA	NA	NA	Yes	31%	2.96
14, HES	50/M	11/2001	Fatigue	Prednisone, hydroxyurea, interferon alfa	46,XY	NA	NA	NA	Yes	40%	6.6
15, HES, with Langerhans'-cell histiocytosis	48/M	11/1997	Fatigue, Langerhans'-cell histiocytosis	Etoposide, vincristine, prednisone, daunorubicin, cytarabine	46,XY	NA	NA	NA	No	Increased	5.9
16, HES	28/F	10/1998	Constitutional symptoms, endomyocardial eosinophilic infiltrates	Prednisone, interferon alfa	46,XX	NA	NA	NA	No	45%	10.0
17, HES	55/M	5/2000	Fatigue, Claude-Bernard-Horner syndrome, Loeffler's endocarditis	Hydroxyurea, interferon alfa	46,XY	NA	NA	NA	Yes	22%	5.7

\* GI denotes gastrointestinal, NA not applicable, and CNS central nervous system.  
 † The patient's karyotype was revised after fluorescence in situ hybridization to t(1;4)(q44;q12)ish.del(4)(q12q12) (RPC16-200D9-).  
 ‡ The patient died while in complete remission.

(range, 28 to 61) (Table 1). Prior therapies included corticosteroids in 9, hydroxyurea in 10, interferon alfa in 5, cytotoxic chemotherapy in 2, cyclosporine in 1, and radiotherapy for extramedullary disease in 2. Patients had one or more of the following: endomyocardial fibrosis or restrictive cardiomyopathy, gastrointestinal involvement, central nervous system or paraspinal disease, pulmonary involvement, skin involvement, hepatosplenomegaly, and thrombosis. The median eosinophil count at presentation was 14,500 per cubic millimeter (range, 4960 to 53,000). Nine patients had a normal karyotype; one patient had t(1;4)(q44;q12), and the patient with leukemia had trisomy 8, trisomy 19, add2q, and del6q. All patients were BCR-ABL-negative on cytogenetic analysis or fluorescence in situ hybridization. Imatinib was initiated at doses ranging from 100 to 400 mg daily. A complete hematologic remission was achieved in 10 of 11 patients after a median of 4 weeks (range, 1 to 12), although 1 of the 10 had only a transient response, which lasted several weeks, and had no response to an increased dose of imatinib. The response lasted more than 3 months in the other nine patients (median duration, 7 months; range, 3 to 15). Analysis of a bone marrow aspirate and biopsy specimen obtained before therapy from Patient 5, who had a response, showed hypereosinophilia, with the large hypolobated eosinophils characteristic of the hypereosinophilic syndrome. After therapy, there was extensive necrosis, Charcot-Leyden crystals, and patchy, normal hematopoiesis. This patient relapsed at five months, with recurrent cytogenetic abnormalities.

#### CLONING OF *FIP1L1-PDGFR $\alpha$*

Patient DNA was analyzed for activating mutations in known targets of imatinib: PDGFRA, PDGFRB, and KIT.<sup>6,9,10</sup> No mutations were found in exons encoding the activation loops or juxtamembrane domains (data not shown). One patient (Patient 1) had t(1;4)(q44;q12). The combination of this patient's response to imatinib and translocation at 4q12, a region where PDGFRA and KIT are located, prompted investigation of their involvement. Fluorescence in situ hybridization showed that a probe spanning the KIT locus (586A2) was translocated to the der(1) chromosome, indicating that the break point was centromeric to KIT. A probe at the *CHIC2* locus (200D9), centromeric to PDGFRA,<sup>18</sup> was deleted (Fig. 1B). Taken together, these results indicated the

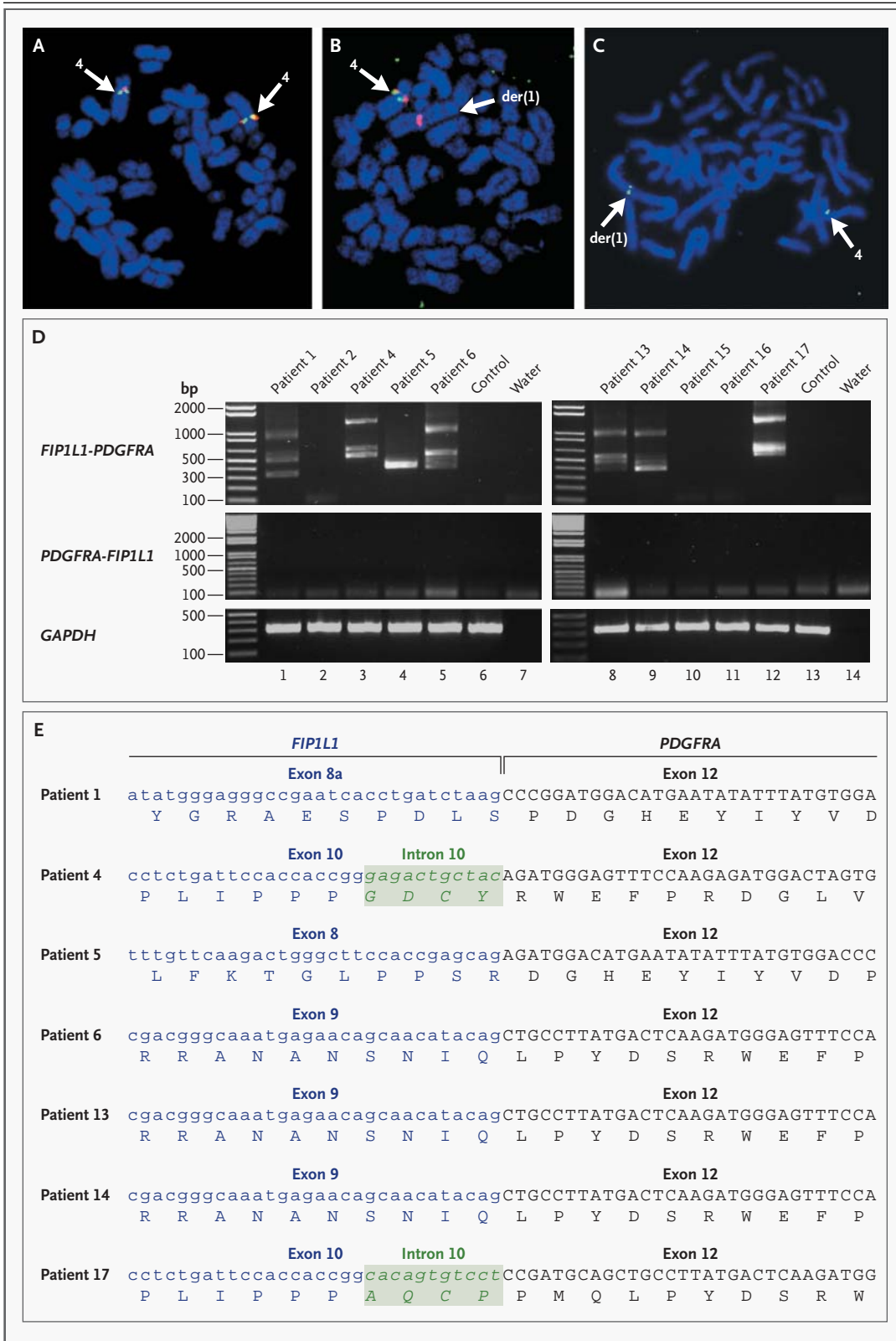
presence of a translocation associated with a deletion on 4q12 with a break point near PDGFRA.

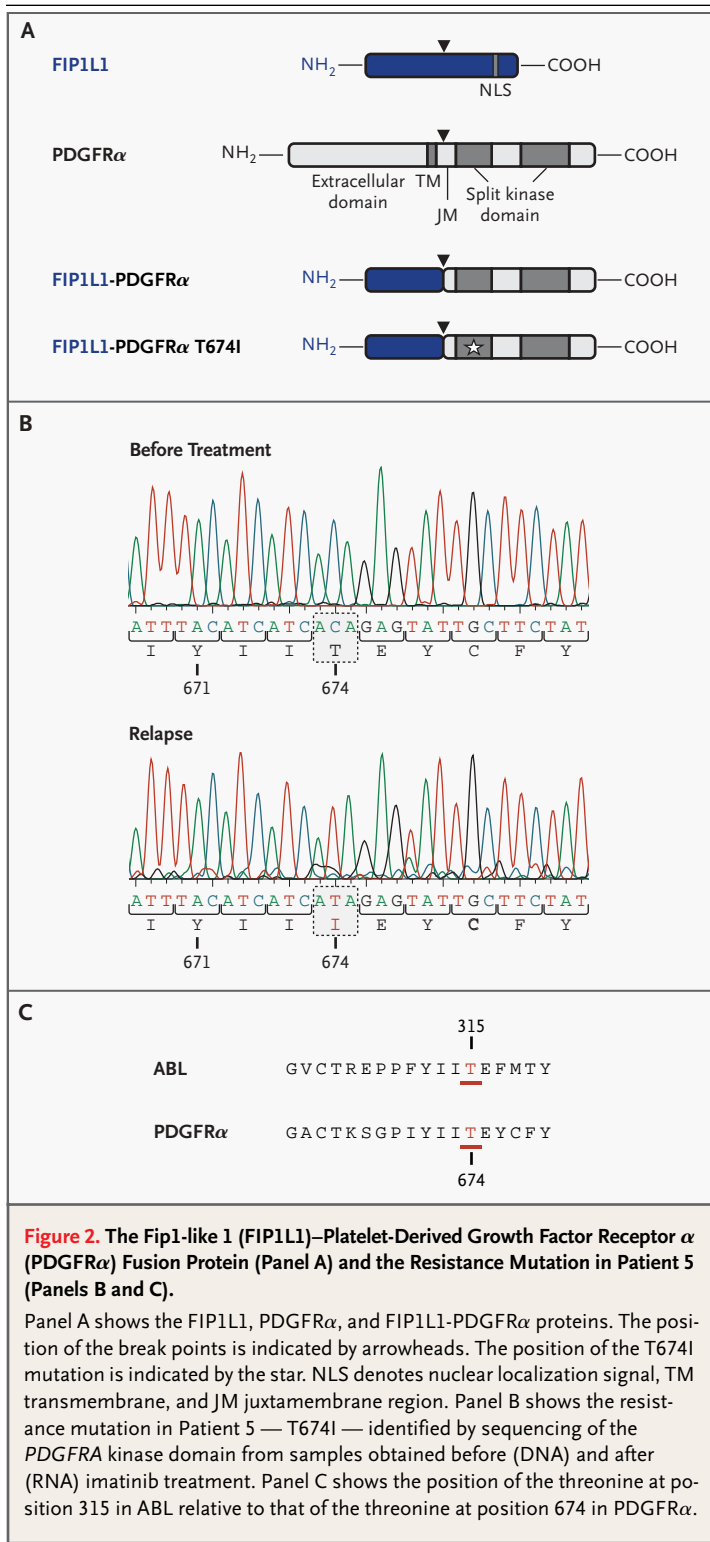
To determine whether a chimeric PDGFRA transcript was present, we performed 5' rapid amplification of cDNA ends on the sequence, encoding the kinase domain of PDGFRA.<sup>19</sup> Sequence analysis of the resultant products revealed that the kinase domain of PDGFRA was fused to an uncharacterized gene (GenBank accession number NM\_030917), encoding a putative 520-amino-acid protein that most closely resembled Fip1, an essential component of the *Saccharomyces cerevisiae* polyadenylation machinery.<sup>20</sup> Therefore, we named the human gene *FIP1L1* (Fip1-like 1). According to data from Expressed Sequence Tags (<http://www.ncbi.nlm.nih.gov/dbEST>) and Ensembl (<http://www.ensembl.org>), *FIP1L1* is widely expressed and undergoes alternative splicing. The *FIP1L1-PDGFR $\alpha$*  fusion gene was in-frame and fused the first 233 amino acids of *FIP1L1* to the last 523 amino acids of PDGFRA (Fig. 1 and 2).

Surprisingly, *FIP1L1* was not located on chromosome 1, as might have been expected owing to a reciprocal translocation, but was approximately

#### Figure 1 (facing page). Detection of the Fusion Gene Formed by the Genes for Fip1-like 1 (*FIP1L1*) and Platelet-Derived Growth Factor Receptor $\alpha$ (*PDGFRA*).

Panels A and B show the results of fluorescence in situ hybridization with a probe at the *KIT* locus (586A2, red signal) and a probe at the *CHIC2* locus (200D9, green signal). In normal cells in metaphase (Panel A), these probes colocalize on chromosome 4q12. In cells in metaphase from Patient 1 (Panel B), colocalization is observed on the normal chromosome 4, but only the red signal (*KIT*) is detected on the der(1) chromosome. No green signal (*CHIC2*) was observed on the der(4) or any other chromosome, indicating that this chromosomal region was deleted. Panel C shows the results of fluorescence in situ hybridization with probe 120K16, located directly centromeric to *FIP1L1*. The presence of this probe on the der(1) (green signal) confirms that the translocation break point is separate from the deletion break points. Panel D shows the results of reverse-transcriptase-polymerase chain reaction indicating a fusion of *FIP1L1* to *PDGFRA* in RNA from Patients 1, 4, 5, 6, 13, 14, and 17. The different bands represent splice variants. GAPDH was used as a control. Panel E shows the sequence of one in-frame splice variant for each patient with the fusion gene. *FIP1L1* sequences are shown in lowercase and in blue or green, and *PDGFRA* sequences are shown in uppercase and in black. Sequences shown in green are derived from introns of *FIP1L1*.





800 kb upstream of PDGFRA on 4q12 (NCBI contig NT\_022853) (Fig. 3). Taken together, this information indicated that the fusion gene was created by either del(4)(q12) or t(4;4)(q12;q12) rather than t(1;4)(q44;q12). The fusion of FIP1L1 and PDGFRA was confirmed by reverse-transcriptase–PCR on RNA and PCR on DNA from Patient 1 (Fig. 1D and 4A). The reciprocal PDGFRA-FIP1L1 fusion gene was not detected in RNA or DNA, strongly suggesting that the fusion was the consequence of an interstitial deletion (Fig. 1D and 4A).

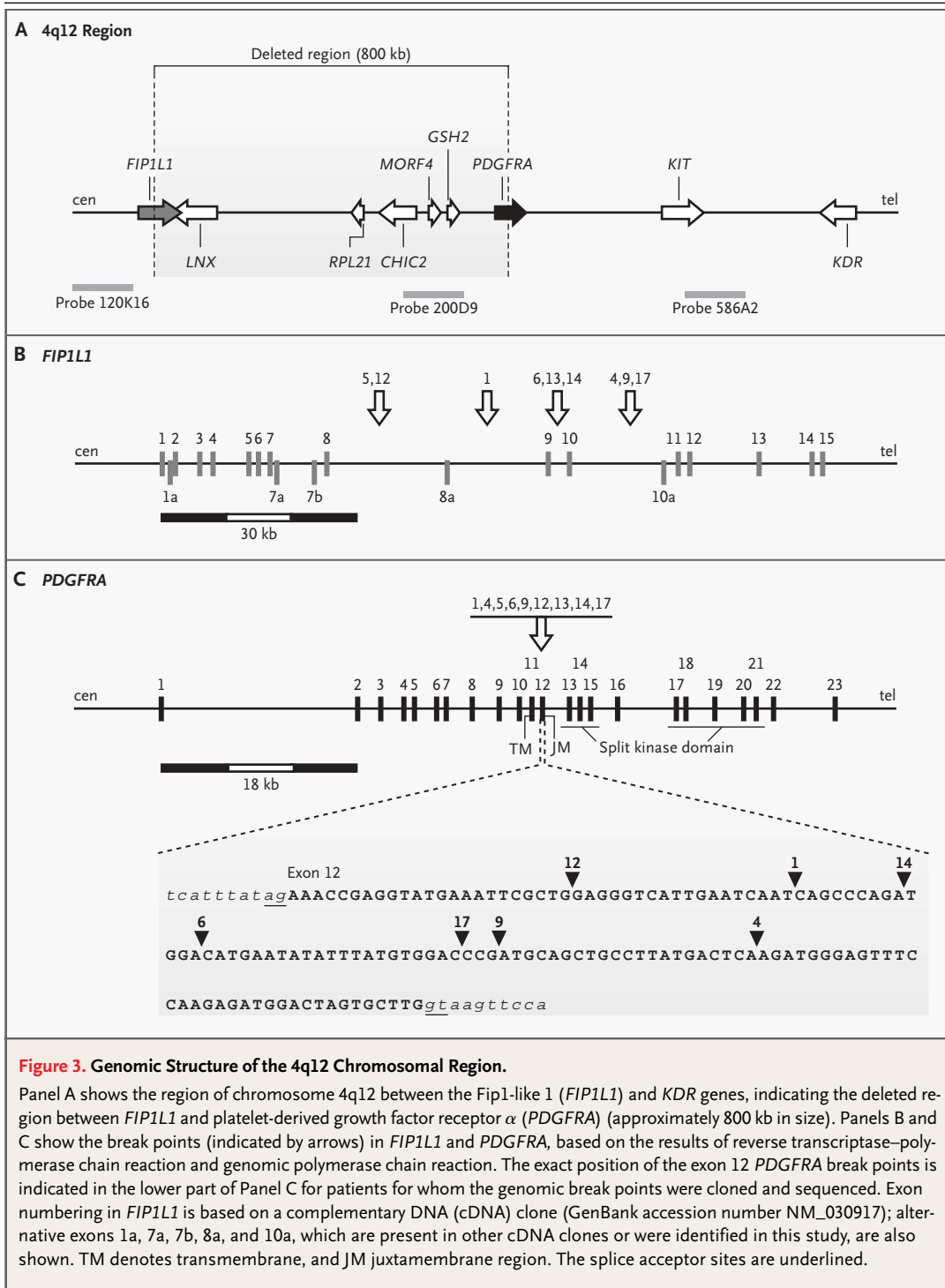
**INCIDENCE OF FIP1L1-PDGFR $\alpha$  IN THE HYPEREOSINOPHILIC SYNDROME**

An additional four of nine patients for whom pre-treatment RNA or DNA was available had the FIP1L1-PDGFR $\alpha$  fusion gene. Four of six patients with the hyper eosinophilic syndrome who did not receive imatinib were also found to have the gene on the basis of RNA or DNA analysis (Fig. 1 and 4). Thus, the FIP1L1-PDGFR $\alpha$  fusion gene occurred in 9 of our 16 patients (56 percent).

Sequence analysis of DNA from peripheral-blood samples from seven of nine patients with the fusion gene confirmed that all break points in PDGFR $\alpha$  occurred in exon 12 and that cryptic splice sites were used within introns of FIP1L1 or within exon 12 of PDGFR $\alpha$ , to allow splicing between exons of FIP1L1 and the interrupted exon 12 of PDGFR $\alpha$  (Fig. 4). Attempts to amplify the reciprocal fusion genes on RNA and DNA were unsuccessful, indicating that all fusions were the result of a deletion on 4q12 and not of t(4;4) (Fig. 1 and 4).

**RELAPSE DURING IMATINIB TREATMENT ASSOCIATED WITH AN ACQUIRED MUTATION IN PDGFR $\alpha$**

Patient 5, who relapsed during imatinib treatment, harbored the FIP1L1-PDGFR $\alpha$  fusion gene initially and at the time of relapse. We hypothesized that relapse might be attributable to mutations in the PDGFR $\alpha$  moiety that conferred resistance to imatinib. Sequence analysis of the PDGFR $\alpha$  kinase domain at the time of relapse showed that the fusion protein had acquired a T674I mutation (Fig. 2B). This mutation occurred in the ATP-binding region of PDGFR $\alpha$  at the same position as the T315I mutation in BCR-ABL<sup>21,22</sup> (Fig. 2C), which is known to confer resistance to imatinib in that context.<sup>21-23</sup>



**Figure 3. Genomic Structure of the 4q12 Chromosomal Region.**

Panel A shows the region of chromosome 4q12 between the Fip1-like 1 (*FIP1L1*) and *KDR* genes, indicating the deleted region between *FIP1L1* and platelet-derived growth factor receptor  $\alpha$  (*PDGFRA*) (approximately 800 kb in size). Panels B and C show the break points (indicated by arrows) in *FIP1L1* and *PDGFRA*, based on the results of reverse transcriptase–polymerase chain reaction and genomic polymerase chain reaction. The exact position of the exon 12 *PDGFRA* break points is indicated in the lower part of Panel C for patients for whom the genomic break points were cloned and sequenced. Exon numbering in *FIP1L1* is based on a complementary DNA (cDNA) clone (GenBank accession number NM\_030917); alternative exons 1a, 7a, 7b, 8a, and 10a, which are present in other cDNA clones or were identified in this study, are also shown. TM denotes transmembrane, and JM juxtamembrane region. The splice acceptor sites are underlined.

**EFFECT OF FIP1L1-PDGFR $\alpha$** 

The expression of FIP1L1-PDGFR $\alpha$  transformed the murine hematopoietic cell line Ba/F3 to interleukin-3-independent growth (Fig. 5B) and was constitutively tyrosine-phosphorylated in these cells (Fig. 5D). Deletion of the FIP1L1 moiety (amino acids 4 to 233) abrogated this type of growth, indicating that this part of the fusion protein was essential for the activation of the chimeric kinase. Further mutational analysis showed that the first 29 amino acids of FIP1L1 (encoded by exon 1) were necessary and sufficient to activate the PDGFR $\alpha$  kinase domain (Fig. 5B). Analysis of the phosphorylation status of ERK1 or ERK2 and STAT5 indicated that STAT5, but not ERK, was a downstream target of FIP1L1-PDGFR $\alpha$  (Fig. 5E, and data not shown).

**INHIBITION OF FIP1L1-PDGFR $\alpha$  KINASE ACTIVITY AND RESISTANCE TO IMATINIB**

To confirm that FIP1L1-PDGFR $\alpha$  was a target of imatinib, we tested the effect of imatinib on the growth of Ba/F3 cells expressing FIP1L1-PDGFR $\alpha$  or FIP1L1-PDGFR $\alpha$  harboring the T674I mutation (Fig. 5C). Ba/F3 cells expressing FIP1L1-PDGFR $\alpha$  were efficiently inhibited by much lower concentrations of imatinib than Ba/F3 cells expressing BCR-ABL. The concentration of imatinib required to inhibit cells transformed by FIP1L1-PDGFR $\alpha$  by 50 percent (IC<sub>50</sub>) was 3.2 nM, whereas the IC<sub>50</sub> for BCR-ABL was 582 nM.<sup>23</sup> These data indicate that FIP1L1-PDGFR $\alpha$  is more sensitive to inhibition by imatinib than is BCR-ABL and correlate with the findings that the effective dose of imatinib is lower in patients with the hypereosinophilic syndrome (100 mg per day) than in patients with BCR-ABL-positive CML (300 to 400 mg per day). As compared with Ba/F3 cells expressing wild-type FIP1L1-PDGFR $\alpha$ , Ba/F3 cells expressing the FIP1L1-PDGFR $\alpha$  T674I mutant were more than 1000 times as resistant to imatinib, with a cellular IC<sub>50</sub> of 7498 nM (Fig. 5C).

Consistent with these findings, imatinib inhibited tyrosine phosphorylation of FIP1L1-PDGFR $\alpha$  and its downstream target STAT5 with an IC<sub>50</sub> of approximately 5 nM (Fig. 5D and 5E, respectively), whereas inhibition of the T674I mutant required concentrations of imatinib that were at least 1000 times as high (10,000 and 5000 nM, respectively) (Fig. 5F and 5G). STAT5 phosphorylation was restored by the addition of interleukin-3 (Fig. 5E and 5G), demonstrating the specificity of imatinib in

this context. Taken together, these data demonstrate that the PDGFR $\alpha$  kinase domain is a direct target of imatinib in patients with the hypereosinophilic syndrome.

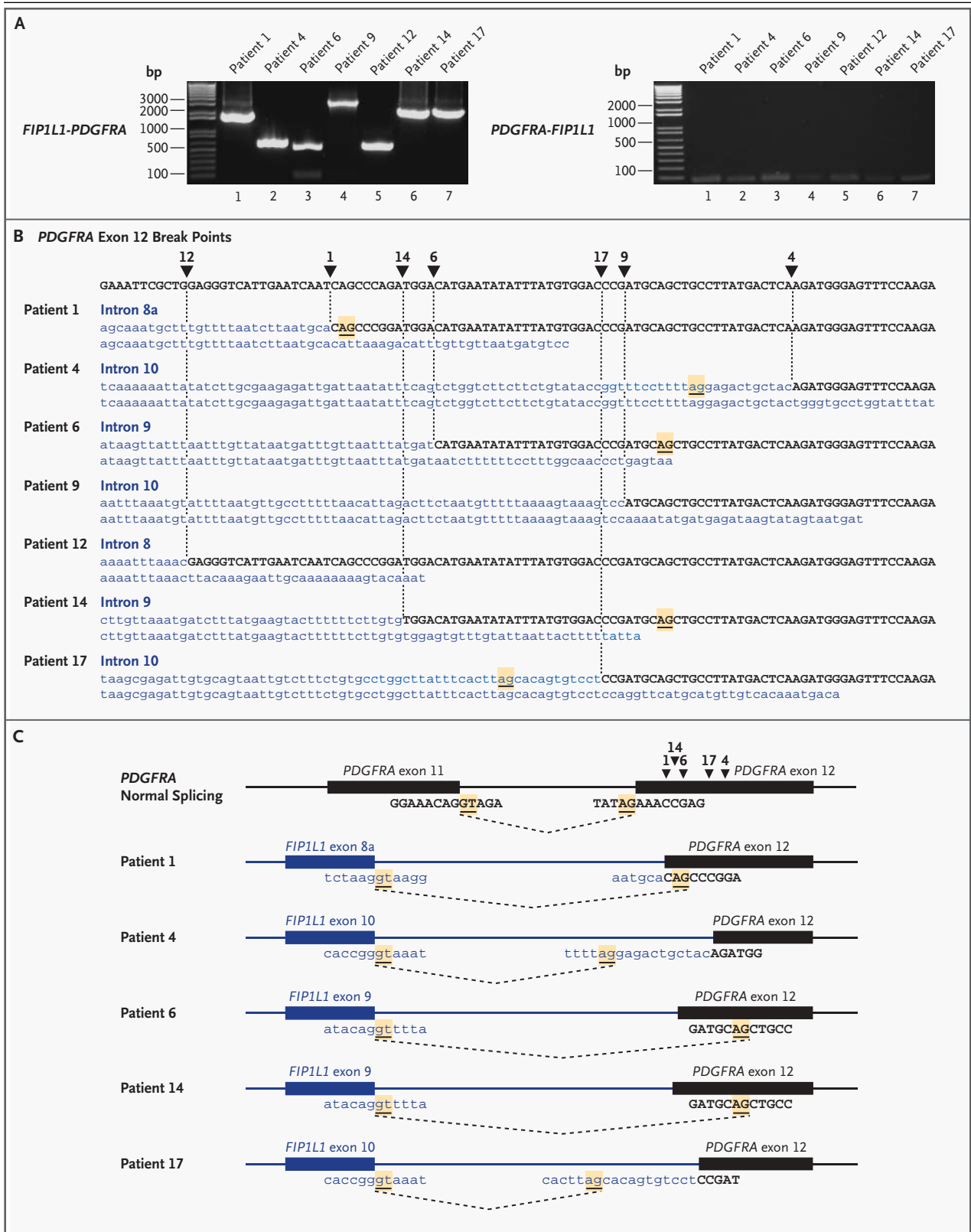
## DISCUSSION

Constitutive activation of tyrosine kinases is a key element in the pathogenesis of myeloproliferative diseases. In most cases the mutant kinases have been identified by cloning recurrent chromosomal translocation break points. Examples include the BCR-ABL,<sup>24</sup> ETV6-PDGFR $\beta$ ,<sup>25</sup> HIP1-PDGFR $\beta$ ,<sup>26</sup> ETV6-JAK2,<sup>27</sup> and H4-PDGFR $\beta$ <sup>28</sup> fusion proteins. The hypereosinophilic syndrome is a myeloproliferative syndrome, but most patients present with an apparently normal karyotype. We found a gene rearrangement, which is not evident on standard karyotyping, that results in a novel FIP1L1-PDGFR $\alpha$  fusion protein.

The FIP1L1-PDGFR $\alpha$  gene rearrangement is a clonal abnormality that raises several questions about the classification of eosinophilic syndromes. World Health Organization (WHO) criteria indicate that patients with clonally derived eosinophils should be classified as having chronic eosinophilic leukemia rather than the hypereosinophilic syndrome. On the basis of these criteria, at least seven of our patients with the hypereosinophilic syndrome should be reclassified as having chronic eosinophilic leukemia. Furthermore, it is plausible that most patients with the hypereosinophilic syndrome, or at least those with imatinib-sensitive disease, will have clonally derived eosinophils. It may

**Figure 4 (facing page). Genomic Break Points (Panels A and B) and Splicing (Panel C) of the Gene for Fip1-like 1 (FIP1L1) to the Gene for Platelet-Derived Growth Factor Receptor  $\alpha$  (PDGFRA).**

Panel A shows amplification of the genomic break points by long-range polymerase chain reaction in Patients 1, 4, 6, 9, 12, 14, and 17. The reciprocal fusion gene — PDGFRA-FIP1L1 — could not be amplified, suggesting that it does not exist and that fusion of FIP1L1 to PDGFRA is caused by a deletion. Panel B shows the sequence of the FIP1L1-PDGFRA fusion gene for the five patients. In Panel C, comparison of the genomic sequences of FIP1L1 and PDGFRA with the sequence from the corresponding complementary DNAs reveals that splicing occurs by means of cryptic splice sites present in introns of FIP1L1 or exon 12 of PDGFRA. Splice donor acceptor sites are underlined in Panels B and C.



therefore be appropriate to reevaluate the WHO classification in the light of our observations.

Several lines of evidence argue that the FIP1L1-PDGFR $\alpha$  fusion protein is a cause of the hypereosinophilic syndrome. First, it was found in a majority of our patients and has the biologic properties of other tyrosine kinase fusion proteins that are implicated in the pathogenesis of myeloproliferative disease. Second, most patients with the hypereosinophilic syndrome had a response to imatinib, a potent inhibitor of PDGFR $\alpha$ , PDGFR $\beta$ , KIT, and ABL.<sup>10</sup> Most such patients have a response to lower doses of imatinib than those required for the induction of hematologic and cytogenetic responses in patients with BCR-ABL-positive CML (e.g., 100 mg daily vs. 400 mg daily).<sup>8</sup> This difference correlates with the lower IC<sub>50</sub> of FIP1L1-PDGFR $\alpha$  than BCR-ABL. Third, clinical relapse and resistance to imatinib in a patient with the hypereosinophilic syndrome were associated with the acquisition of a point mutation in the ATP-binding domain of the FIP1L1-PDGFR $\alpha$  fusion protein that confers resistance to imatinib. The T674I substitution is analogous to the T315I mutation in the ABL kinase that occurs in some patients with BCR-ABL-positive CML in whom resistance to imatinib develops<sup>21</sup> and thus demonstrates that FIP1L1-PDGFR $\alpha$  is the therapeutic target of imatinib in the hypereosinophilic syndrome.

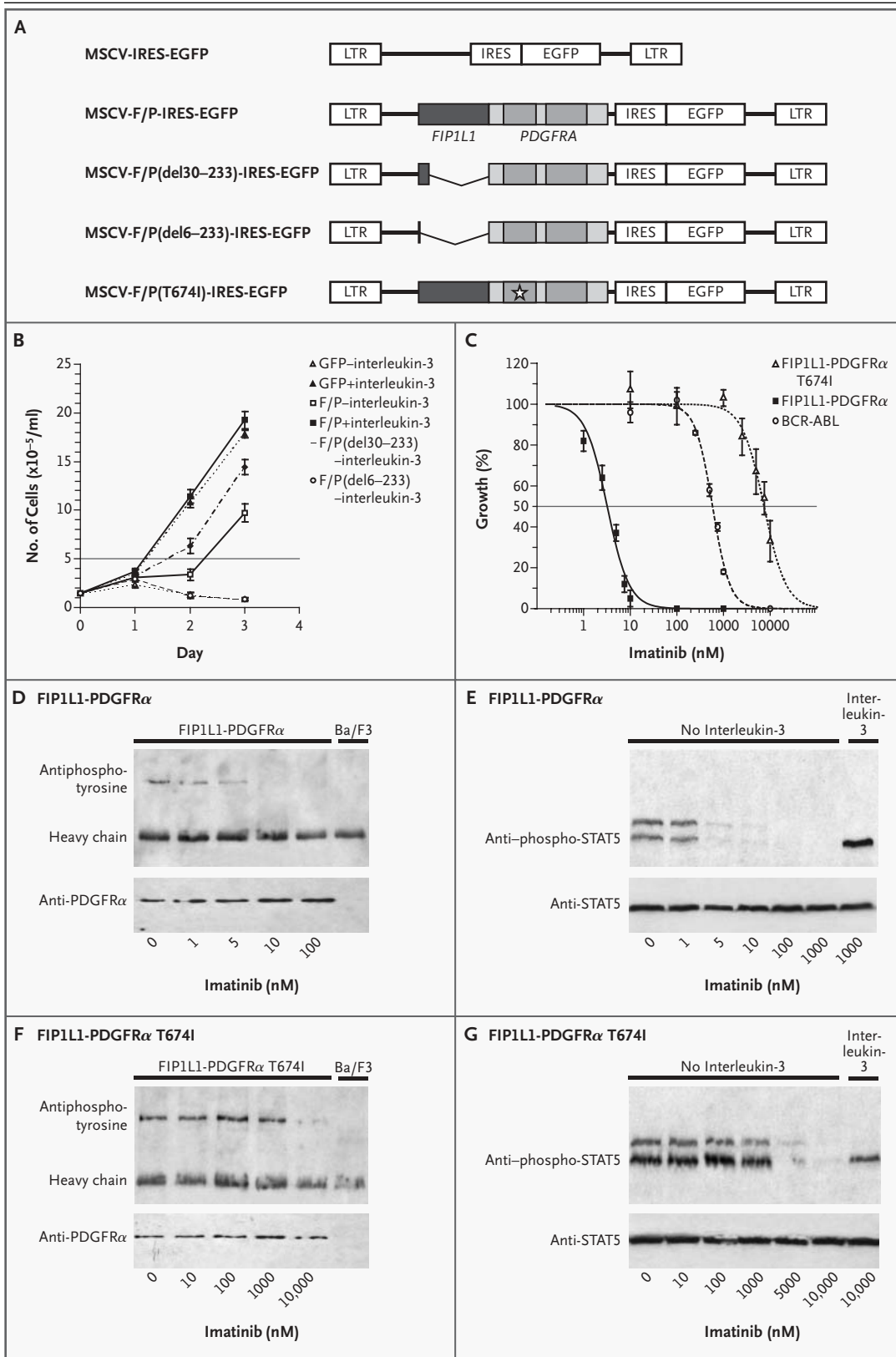
Nine of 11 patients with the hypereosinophilic syndrome had responses to imatinib that lasted more than three months, but only 5 of these 9 had a detectable FIP1L1-PDGFR $\alpha$  fusion. The basis for a response to imatinib in the other four patients is not known, but there are several possibilities. First, break points for the deletion may be widely distributed within the FIP1L1 gene. Our biochemical data indicate that a deletion that incorporated only the first exon of FIP1L1 would be sufficient to activate the PDGFR $\alpha$  kinase domain. However, reverse-transcriptase-PCR screening for such FIP1L1-PDGFR $\alpha$  variants was negative in these patients. Second, another gene near FIP1L1 could form a fusion protein with PDGFR $\alpha$ . Detailed fluorescence in situ hybridization with probes that span the deleted region will resolve this question, but we have not yet identified alternative fusion partners for PDGFR $\alpha$ . Third, in some patients with the hypereosinophilic syndrome, the KIT gene, located approx-

**Figure 5 (facing page). Transformation, Inhibition, and Signal Transduction Properties of the Fusion Tyrosine Kinase Formed by Fip1-like 1 (FIP1L1) and Platelet-Derived Growth Factor Receptor  $\alpha$  (PDGFR $\alpha$ ).**

Panel A shows the retroviral constructs used in the study. MSCV denotes murine stem-cell virus, IRES internal ribosomal entry site, EGFP enhanced green fluorescent protein, LTR long terminal repeat, and F/P FIP1L1-PDGFR $\alpha$ . The star indicates the position of the mutation. In Panel B, Ba/F3 cells retrovirally transduced with these constructs were grown in the absence or presence of interleukin-3, and their mean ( $\pm$ SD) growth was recorded over a period of three days. Panel C shows the dose-response curves and cellular 50 percent inhibitory concentration (IC<sub>50</sub>) of imatinib for Ba/F3 cells expressing FIP1L1-PDGFR $\alpha$ , FIP1L1-PDGFR $\alpha$  with the T674I mutation, or BCR-ABL. The IC<sub>50</sub> was 3.2 nM for FIP1L1-PDGFR $\alpha$ , 582 nM for BCR-ABL, and 7498 nM for FIP1L1-PDGFR $\alpha$  T674I. Panels D, E, F, and G show the phosphorylation status of PDGFR $\alpha$  and STAT5 in Ba/F3 cells expressing either the wild type or the mutant (T674I) FIP1L1-PDGFR $\alpha$ . Panels D and F show phosphorylation of PDGFR $\alpha$  detected with an antiphosphotyrosine antibody or anti-PDGFR $\alpha$  antibody (Control) after immunoprecipitation. Panels E and G show phosphorylation of STAT5 detected with an antiphospho-STAT5-specific antibody or anti-STAT5 antibody (Control) with the use of whole-cell lysates.

imately 400 kb downstream of PDGFRA, may be fused to FIP1L1. KIT is also sensitive to imatinib<sup>10</sup>; thus, a FIP1L1-KIT fusion protein in patients with the hypereosinophilic syndrome should also result in a clinical response. However, we have been unable to identify such a FIP1L1-KIT fusion gene in the four patients who had a response to imatinib but who did not have the FIP1L1-PDGFR $\alpha$  fusion gene. Fourth, a similar, cytogenetically silent gene rearrangement may occur, involving either ABL or PDGFR $\beta$  in some patients. Finally, an as yet unidentified kinase that is inhibited by imatinib may be constitutively activated by a mutation. Further analysis will be required to evaluate these possibilities.

Cloning of the FIP1L1-PDGFR $\alpha$  gene rearrangement identified chromosomal interstitial deletion as a novel molecular mechanism for a gain-of-function fusion gene. Most investigations of the loss of heterozygosity in human tumors have focused on the loss of function of one or both alleles of a putative tumor-suppressor gene. Our data suggest that a comprehensive analysis of human tumors for small deletions that may result in gain-of-function fusion genes should be undertaken.



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