Novel imatinib-sensitive PDGFRA-activating point mutations in hypereosinophilic syndrome induce growth factor independence and leukemia-like disease

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The FIP1L1-PDGFRα fusion is seen in a fraction of cases with a presumptive diagnosis of hypereosinophilic syndrome (HES). However, because most HES patients lack FIP1L1-PDGFRα, we studied whether they harbor activating mutations of the PDGFRα gene. Sequencing of 87 FIP1L1-PDGFRα-negative HES patients revealed several novel PDGFRα point mutations (R4681G, L507P, I562M, H570R, H650Q, N659S, L705P, R748G, and Y849S). When cloned into 32D cells, N659S and Y849S and—on selection for high expressers—also H650Q and R748G mutants induced growth factor-independent proliferation, clonogenic growth, and constitutive phosphorylation of PDGFRα and Stat5. Imatinib antagonized Stat5 phosphorylation. Mutations involving positions 659 and 849 had been shown previously to possess transforming potential in gastrointestinal stromal tumors. Because H650Q and R748G mutants possessed only weak transforming activity, we injected 32D cells harboring these mutants or FIP1L1-PDGFRα into mice and found that they induced a leukemia-like disease. Oral imatinib treatment significantly decreased leukemic growth in vivo and prolonged survival. In conclusion, our data provide evidence that imatinib-sensitive PDGFRα point mutations play an important role in the pathogenesis of HES and we propose that more research should be performed to further define the frequency and treatment response of PDGFRα mutations in FIP1L1-PDGFRα-negative HES patients.

Introduction

The WHO classification of myeloproliferative neoplasms (MPNs) comprises a variety of diseases, including BCR-ABL1-positive chronic myelogenous leukemia, chronic neutrophilic leukemia, polycythemia vera, primary myelofibrosis, essential thrombocytopenia, and systemic mastocytosis (SM). Patients with nonreactive eosinophilia and recurrent genetic abnormalities involving the PDGFRα, PDGFRβ, or FGFR1 genes have been classified in a separate subgroup termed “Myeloid and lymphoid neoplasms ends-polymerease chain reaction (RACE-PCR) of the PDGFRα gene in a subset of patients failed to identify any cytogenetically silent gene fusions.

Here, we report several novel activating point mutations within the coding region of PDGFRα in patients with HES. We show that 4 of these mutations induce constitutive PDGFR phosphorylation and growth factor independence. Our in vitro and in vivo experiments show that PDGFRα mutant cells remain sensitive to imatinib, suggesting that patients harboring these mutations should be treated with imatinib similarly to patients with PDGFR fusion genes.


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Methods

Primary patient samples and sequencing of PDGFRA

Samples of idiopathic HES cases (n = 87 [67% men], all confirmed to be negative for FIP1L1-PDGFRα) were randomly selected from diagnostic peripheral blood (PB) samples which were referred to our hospital between 2005 and 2009, as well as healthy donors (n = 35) and patients with chronic lymphocytic leukemia (CLL, n = 40). Informed consent was obtained from all patients according to the Declaration of Helsinki.

Cloning of PDGFRA mutants

Human PDGFRA cDNA (a kind gift of Carl-Hendrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) was cloned into pEmt:1A (Invitrogen), and the different mutations were introduced by site-directed mutagenesis using the following primer sets:

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R481G: 5'-GATCCACTCCCGAGGACGGAATACGTACGAGGGCGC-3', 5'-GCCCTCCACGGTAGTCCTCGGCTAGTGGAC-3'; L507P: 5'-CTGAGAATCTCCCTGGAGCTGAGAACC-3', 5'-GGCTCCCTGAGAGCTGAAACC-3'.
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Cells and mice

32D cells were obtained from the ATCC and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 IU/mL penicillin, 50 μg/mL streptomycin), and 10% WEHI-3B (Walter and Eliza Hall Institute) cell supernatant as a source of interleukin-3 (IL-3). Four- to 6-week-old C57BL/6 mice were purchased from Janvier. Approval for the animal research was obtained from the local authorities of North-Rhine Westphalia, Germany.

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\]
Table 1. Results from PDGFRA gene sequencing

<table>
<thead>
<tr>
<th>Patients, n (%)</th>
<th>Point mutations</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>46 (52.9)</td>
</tr>
</tbody>
</table>

In a screen 87 patients with idiopathic HES were sequenced for PDGFRA mutations.

Results

PDGFRA expression and gene mutations in HES patients

Sequencing of the PDGFRA gene in 87 HES patients, in whom the absence of FIP1L1-PDGFRα was confirmed, revealed the presence of known single nucleotide polymorphisms (SNPs; Table 1) as well as 9 novel PDGFRA gene point mutations in 7 patients. Of the latter patients, 4 patients had only 1 mutation (R481G, N659S, L705P, Y849S), while 3 patients had 2 mutations (I562M and H570R, H650Q and R748G, as well as Y849S and L507P; Table 2). None of these novel mutations has been described as a SNP. No point mutation was found in a separate set of 35 healthy controls. In addition, because 1 of the HES patients had a history of CLL, we sequenced PDGFRA in 40 patients with CLL without eosinophilia but found no PDGFRA mutations in CLL patients (data not shown).

PDGFRA mutations transform myeloid cells

To screen which of the PDGFRA point mutants possess transforming activity, we retrovirally expressed each mutant in factor-dependent murine myeloid 32D cells and analyzed the resulting PDGFRA overexpression (among all patients) 36 (42.5)

Table 2. Patients with PDGFRA point mutations

<table>
<thead>
<tr>
<th>ID</th>
<th>Clinical diagnosis</th>
<th>Mutation</th>
<th>Polymorphism</th>
<th>PDGFRA overexpression</th>
<th>Sex</th>
<th>Age, y</th>
<th>Eosinophil count, /μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HES</td>
<td>R481G</td>
<td>P567P</td>
<td>Yes</td>
<td>Male</td>
<td>76</td>
<td>276*</td>
</tr>
<tr>
<td>2</td>
<td>HES</td>
<td>I562M, H570R</td>
<td>S478P, A603A</td>
<td>Yes</td>
<td>Male</td>
<td>76</td>
<td>810*</td>
</tr>
<tr>
<td>3</td>
<td>HES and CLL</td>
<td>H650Q, R748G</td>
<td>P567P</td>
<td>Yes</td>
<td>Female</td>
<td>65</td>
<td>4320</td>
</tr>
<tr>
<td>4</td>
<td>HES</td>
<td>L705P</td>
<td>P567P</td>
<td>Yes</td>
<td>Male</td>
<td>70</td>
<td>5334</td>
</tr>
<tr>
<td>5</td>
<td>HES</td>
<td>Y849S</td>
<td>P567P, V824V</td>
<td>No</td>
<td>Male</td>
<td>67</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>HES</td>
<td>Y849S, L507P</td>
<td>None</td>
<td>No</td>
<td>Male</td>
<td>25</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>HES</td>
<td>N659S</td>
<td>P567P</td>
<td>No</td>
<td>Female</td>
<td>45</td>
<td>204*</td>
</tr>
</tbody>
</table>

HES indicates idiopathic hypereosinophilic syndrome; CLL, chronic lymphocytic leukemia; and NA, not available.

*During treatment with prednisone and cyclophosphamide.
mutants retained sensitivity to imatinib, as detected by an MTS assay (Figure 2B). A dose-dependent curve fitting algorithm from GraphPad Prism 5.0 was used to determine IC50 values of all 32D mutants (2.0nM H650Q; 159nM N659S; 0.9nM R748G; 2.6nM Y849S; 1.8nM FIP1L1-PDGFRA; 689nM BCR-ABL1), showing that H650Q, R748G, and Y849S mutants were as sensitive to imatinib as FIP1L1-PDGFRA, whereas mutant N659S was less sensitive but still more sensitive than BCR-ABL1 (Figure 2B). As expected, JAK2V617F mutants were insensitive to imatinib. Stat5 phosphorylation was inhibited by imatinib in all sensitive cell lines (Figure 2C). Imatinib exposure induced cell death as shown by 7-AAD positivity and loss of EGFP expression (Figure 2D).

**PDGFRA mutants H650Q and R748G induce leukemia-like disease in vivo similar to FIP1L1-PDGFRA**

Mutants involving the 659 and 849 amino acids had previously been shown to possess transforming potential in GIST, and our own results in 32D cells harboring the N659S and Y849S mutants showed that these 2 mutants immediately induced IL-3–independent growth.4,9,10 However, IL-3–independent clonogenic growth of H650Q and R748G cell lines was only seen after sorting for highly expressing clones. We sought to investigate whether these mutant cell lines were able to grow in vivo and induce a leukemia-like disease. Intravenous injection of 32D cell lines harboring the H650Q or R748G point mutants, or FIP1L1-PDGFRA, into syngeneic C3H/HeJ mice readily induced fatal leukemia-like disease within 20 to 40 days after injection (Figure 3A), while animals receiving 32D cells overexpressing wild-type PDGFRA showed no signs of disease for at least 8 months (data not shown) and showed no EGFP-positive cells on autopsy (Figure 3E). Diseased mice unequivocally showed splenomegaly (Figure 3B,D) and lymphadenopathy (Figure 3C). Intriguingly, the degree of lymphadenopathy was significantly higher in the recipients of the H650Q and R748G mutants than those receiving FIP1L1-PDGFRA–expressing cells (Figure 3C). FACS analysis demonstrated EGFP-positive donor cells in all affected organs analyzed (PB, BM, spleen, and lymph nodes; Figure 3E). In addition, histologic analysis showed infiltrates in the perivascular regions of the liver and a disturbed follicular structure of the spleen as well as displacement of normal granulocytic cells by blastlike cells in the BM (Figure 3F).

**In vivo imatinib treatment prolongs survival of mice injected with PDGFRA mutant cell lines**

To investigate whether imatinib treatment can rescue mice injected with PDGFRA-mutant cells, we treated mice injected with wt PDGFRA, H650Q, R748G, or FIP1L1-PDGFRA–overexpressing cells twice daily with imatinib by oral gavage starting on day 5 postinjection. Imatinib treatment of mice injected with wt PDGFRA cells had no negative impact on survival but significantly prolonged survival in all other groups (Figure 4A). In addition, there was a significant decrease of lymphadenopathy in all treated mice (Figure 4B). Splenomegaly was significantly decreased at the time of the final analysis in the case of the H650Q and R748G single mutants, while differences in spleen weight were not significantly reduced by imatinib in mice receiving FIP1L1-PDGFRA cells (Figure 4C).

**Discussion**

Considerable progress has been achieved in our understanding of the pathogenesis of CEL/HES through the identification of tyrosine kinase fusion genes and abnormal clonal T cells that overproduce eosinophilopoietic cytokines. However, these abnormalities are only found in a maximum of 20%-35% of patients and, therefore,
the molecular pathogenesis still remains unknown for the majority of patients. Here, we report on the identification of several novel point mutations within functionally relevant domains of the receptor tyrosine kinase PDGFRA in HES patients.

Several of these mutations were functionally not related to growth factor independence (R481G, L507P, I562M, H570R, L705P). In contrast, 4 mutations possessed transforming potential (H650Q, N659S, R748G, and Y849S). The N659S and Y849S mutations were identified in 1 and 2 patients with HES, respectively, and similar mutations had previously been identified in patients with GIST.4,10,11 The H650Q and R748G mutations were found in a 64-year-old female who had a 3-year history of B-CLL and was in remission after rituximab-based immunochemotherapy when eosinophilia first occurred. Eosinophil counts fluctuated between 1000/μL and 4560/μL until B-CLL relapsed approximately 2 years later, and the patient died approximately 10 months later despite chemotherapy. Transforming activity of H650Q and R748G in 32D cells was only detected on sorting for high expressor cells. Interestingly, the patient harboring these mutants showed overexpression of PDGFRA, suggesting that these 2 mutants may be weaker transforming mutants compared with the strong oncogenic mutations N659S and Y849S. A similar phenomenon was described for BCR-ABLtransduced CD34+ cells from human cord blood,12 where BCR-ABLhigh but not BCR-ABLLow expressors showed factor-independent proliferation. Nevertheless, cells overexpressing H650Q and R748G were able to induce fatal leukemia-like disease in mice, suggesting that they were indeed involved in the pathogenesis of HES in this patient.

While the Y849S mutation was identified in 2 patients, none of the other mutations occurred more than once in our cohort of patients (Table 2). However, previously reported novel mutations such as CBL mutations in acute myeloid leukemia (AML) were initially only found in 1 of 150 patients,13 but subsequently confirmed in up to 9% of secondary AML14 and myeloproliferative and myelodysplastic syndromes.15 We found that CD3+ T lymphocytes from the patient with the N659S mutation did not harbor this mutation (data not shown), showing that the mutation is acquired. In addition, we clearly demonstrate biologic relevance of this and
Figure 3. PDGFRA point mutations show increased lymphadenopathy compared with the FIP1L1-PDGFR fusion in a syngeneic transplantation model. (A) H650Q and R748G are transforming in vivo. Kaplan-Meier plot shows survival of C3H/HeJ mice injected with \(1.2 \times 10^6\) cells of 32D cell lines retrovirally expressing PDGFRA wt (n = 29), H650Q (n = 4), R748G (n = 15), and FIP1L1-PDGFR (n = 5). (B) Diseased mice show significant enlargement of the spleen. Mice were killed on day 22 after injection for analysis. The numbers below the photographs depict spleen weight. (C-D) Lymph node and spleen weight of injected mice. Moribund or dead mice were analyzed for spleen and lymph node weight, controls were analyzed at various time points (range days 22-57; PDGFRA wt n = 17; H650Q n = 14; R748G n = 14; FIP1L1-PDGFR n = 10). Included are untreated mice and mice treated with water by oral gavage. No statistical difference was observed between the 2 control groups (untreated vs water-treated) with the exception of R748G mice, because of 3 outliers which harbored greatly enlarged lymph nodes but marginally significantly smaller spleens. Statistical significance was tested with the nonparametric Mann-Whitney U test. (E) Hematopoietic and lymphatic organs show invasion of GFP-positive 32D cells. Bone marrow (BM), spleen (Spl), lymph nodes (LN), and peripheral blood cells (PB) were analyzed by flow cytometry for the presence of GFP-positive 32D cells. Depicted gates and percent values represent GFP-positive cells. Shown is 1 mouse of each group, analyzed 22 days after injection. (F) Histologic analysis of spleen, liver, and BM on day 22 after injection was performed after HE (hematoxylin/eosin) or NACE (Naphthyl acetate (chloro)-esterase) staining and showed infiltrates in the perivascular regions of the liver (arrows), a disturbed follicular structure of the spleen and remarkable reduction of NACE-positive cells in BM. Spleen and liver slides are depicted at \(\times 10\) magnification, BM at \(\times 100\).
was tested with the Mann-Whitney test. Animals surviving for 57 days were killed and included. Statistical significance was tested with the log-rank test.

Survival of mice. Kaplan-Meier plots show survival of injected mice (survival curves for PDGFRA wt overlap). Statistical significance was tested with the log-rank test.

Mice were injected with 1.2 \times 10^6 cells of indicated cell lines (32D PDGFRA wt [−imatinib/−imatinib \(n = 5/5\)], H650Q [\(n = 5/5\)], R748G [\(n = 5/5\)], and FIP1L1-PDGFRA [\(n = 4/5\)]) and treated with 62.5 mg of imatinib per kilogram body weight twice daily by oral gavage from day 5 after cell injection. One mouse was excluded because of late death on day 50 supposedly because of infection without comparable lymphadenopathy. Kaplan-Meier plots show survival of injected mice (survival curves for PDGFRA wt overlap). Statistical significance was tested with the log-rank test.

Treatment with imatinib consistently decreases lymphadenopathy and spleen weight of 32D PDGFRA H650Q and R748G-injected mice as shown by lymph node and spleen weight. Moribund or dead mice were analyzed for spleen and lymph node weight; animals surviving for 57 days were killed and included. Statistical significance was tested with the Mann-Whitney U test. Autopsy of the 4 surviving imatinib-treated FIP1L1-PDGFRA mice showed splenomegaly in 3 of the mice, while 1 of the mice showed a normal spleen weight. Therefore, although splenomegaly did develop in some of these mice, the difference was not statistically significant.

Our data show that the H650Q and R748G mutations were not tested for their transforming potential as well as tyrosine kinase domain.10 Up to 97% of PDGFRA mutations are located in exon 18 which codes for the tyrosine kinase domain (D842V/Y/I, D846Y, Y849C as well as deletions and longer mutations spanning at least 1 of the amino acid residues D842 or D846)11,19,10,20,29 and approximately 3% in exon 12 which codes for the juxtamembrane domain (V561D and deletions/insertions of 2 or more amino acids including the V561 residues or residue S566).2,9,21,22,27,28,30 To date, only 3 patients have been described with mutations in exon 14 which codes for the tyrosine kinase domain 1 (N659K).10 In inflammatory fibroid polyps (IFPs), rare cases of PDGFRA mutations in exon 12 and 18 have been reported (V561D, D842V/I mutations, different deletions spanning position 556 and 571 as well as small deletions around position 824 to 848).31 In addition, 2 AML cases were described with PDGFRA mutations in exon 17 and 19 (F808L, N870S)32 and 1 acute lymphoblastic leukemia (ALL) case with a mutation in exon 10 which encodes the extracellular fifth Ig-like domain (A509D).33 Lastly, 4 cases with PDGFRA mutations (C235Y in exon 5, W349C in exon 7 [\(n = 2\]), V536E in exon 11)34 and 2 cases with deletions34,35 have been reported in patients suffering from glioblastoma multiforme.

The finding of imatinib-sensitive PDGFRA point mutations in patients with HES and CEL suggests that all patients without underlying tyrosine kinase fusion genes should be screened for the presence of activating point mutations and treated with imatinib. However, in our series, PDGFRA-overexpressing cases were overrepresented and lymphocytic variants of HES were not formally excluded. These data need extension as well as confirmation, and we suggest that more research should be performed to identify the frequency of PDGFRA mutations in larger cohorts of patients. Importantly, novel point mutations should be rigorously tested for their transforming potential as well as tyrosine kinase inhibitor (TKI) sensitivity to exclude passenger mutations such as those seen in our own screen as well as those described for FLT3-ITD in AML.16 In addition, we need to learn more about the imatinib sensitivity of all of the mutant cells in vivo in patients before we can confirm or exclude that these mutations are involved in the pathogenesis of the disease. Finally, the interesting finding of PDGFRA point mutations and deletions in IFPs31 which are characterized by an inflammatory infiltrate rich in eosinophils...
raises the issue of a potential common pathogenesis of IFPs and hypereosinophilic neoplasms with PDGFRA mutations. It will be interesting in the future to dissect spindle cells and eosinophils from IFPs and define the exact cell population harboring the PDGFRA mutation.

Surprisingly, animals injected with 32D PDGFRA H650Q and R748G mutant cells showed a significantly stronger degree of lymphadenopathy compared with mice injected with 32D FIP1L1-PDGFRα cells (P < .001, P = .007, P < .001, respectively, Figure 3C) while the degree of splenomegaly was not different (Figure 3D). One major difference between the PDGFRA point mutants (H650Q and R748G) and the FIP1L1-PDGFRα fusion protein is that the PDGFRA point mutants but not FIP1L1-PDGFRα retain surface expression through transmembrane localization. Along this line, our results demonstrate that the ligand (PDGF-AA) was able to induce phosphorylation of downstream signaling molecules such as Akt only in the point mutant-transduced cells but not in the FIP1L1-PDGFRα-transduced cells (Figure 2A). This fact renders the PDGFRA point mutant-expressing cells responsive to exogenous PDGF which has been described to be expressed in lymph node stromal cell lines,36 suggesting that this may play a role in the preferential lymphadenopathy seen with these mutants.

In conclusion, we describe novel PDGFRA point mutations that transform growth factor–dependent hematopoietic cell lines in vitro and induce leukemia-like disease in vivo. Moreover, our in vitro and in vivo findings suggest that imatinib may be effective in patients with activating PDGFRA point mutations. Although only found in a minority of patients, the screening of HES patients for those mutations may therefore allow selection of patients with a response to imatinib or alternative tyrosine kinase inhibitors. In addition, our data extend the spectrum of diseases harboring PDGFRA point mutations and suggest common oncogenic pathways in idiopathic HES and GIST. If our results are confirmed by other groups, the current WHO classification of MPN may have to be extended to include PDGFRA point mutations.

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**Authorship**


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**References**


