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

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Running Title: Novel PDGFRA point mutations induce MPN

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Abstract
The FIP1L1-PDGFRA fusion is seen in a fraction of cases with a presumptive diagnosis of hypereosinophilic syndrome (HES). However, since most HES patients lack FIP1L1-PDGFRA, we studied whether they harbor activating mutations of the PDGFRA gene. Sequencing of 87 FIP1L1-PDGFRA negative HES patients revealed several novel PDGFRA point mutations (R481G, L507P, I562M, H570R, H650Q, N659S, L705P, R748G, and Y849S). When cloned into 32D cells, N659S and Y849S and, upon selection for high expressors, also H650Q and R748G mutants induced growth factor-independent proliferation, clonogenic growth, and constitutive phosphorylation of PDGFRA and STAT5. Imatinib antagonized STAT5 phosphorylation. Mutations involving positions 659 and 849 had been shown previously to possess transforming potential in gastrointestinal stromal tumors. Since H650Q and R748G mutants possessed only weak transforming activity, we injected 32D cells harboring these mutants or FIP1L1-PDGFRA 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 PDGFRA 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 PDGFRA mutations in FIP1L1-PDGFRA negative HES patients.
Introduction

The WHO classification of myeloproliferative neoplasms (MPN) 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).\(^1\) Patients with non-reactive eosinophilia are included in this group and should be termed “chronic eosinophilic leukemia (CEL)” if a clonal aberration is present, or “idiopathic hypereosinophilic syndrome (HES)” if no clonal aberration is detected. In addition, patients with eosinophilia and recurrent genetic abnormalities involving the PDGFRA, PDGFRB, or FGFR1 genes have been classified in a separate subgroup termed “Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1”.\(^1\)

However, such genetic lesions are detected only in a minority of patients with non-reactive eosinophilia, with the FIP1L1-PDGFRA fusion gene accounting for 5-15% of cases and other translocation products involving PDGFRA, PDGFRB, or JAK2 being even less common.\(^2\) Another 15-20% have been shown to belong to the so-called lymphocytic variant group, which is characterized by atypical clonal T lymphocytes secreting eosinophilopoietic cytokines.\(^3\) Nonetheless, 65-80% of these patients remain without known underlying genetic aberration\(^2\).

Given that gastrointestinal stromal tumors (GIST) are also negative for FIP1L1-PDGFRA but display activating point mutations of the PDGFRA gene, we reasoned that FIP1L1-PDGFRA negative HES patients may harbor similar PDGFRA point mutations. Therefore, fusion gene-negative cases were analyzed by sequencing of exons 9-19 which harbor the vast majority of activating PDGFRA point mutations in GIST.\(^4\) The karyotype of these cases, where known, did not reveal any rearrangement of chromosome 4q11-12, and rapid amplification of cDNA ends (RACE)-PCR of the PDGFRA gene in a subset of patients failed to identify any cytogenetically silent gene fusions\(^2\).

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

Primary patient samples and sequencing of PDGFRA
Samples of idiopathic HES cases (n=87 [67% males], all confirmed to be negative for FIP1L1-PDGFRA) 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. Hemi-nested RT-PCR for amplification of PDGFRA was performed using the following forward primers F1 5’-TTCAGCCAGTTGGAAGCTGTCA; F2 5’-CAGACGGTGAGGTGCACAG and reverse primer R1 5’ TGTCAAGATGCTCTCAGGAGC, spanning exons 9-19 of the PDGFRA gene. PCR products were bidirectionally sequenced using the amplification primers. In addition, PDGFRA expression levels were assessed in all patients by quantitative RT-PCR as described2.

Cells and mice
32D cells were obtained from the ATCC and cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640, Gibco/Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (FBS), antibiotics (100IU/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). 4 to 6 week old C3H/HeJ mice were purchased from Janvier (Janvier, St. Berthevin, France). Approval for the animal research was obtained from the local authorities of North-Rhine Westphalia, Germany.

Cloning of PDGFRA mutants
Human PDGFRA cDNA (kind gift of Carl-Hendrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) was cloned into pEntr.1A (Invitrogen, Germany, Karlsruhe), and the different mutations were introduced by site-directed mutagenesis using the following primer sets.
R481G:
5’-GATCCACTCCCGAGACGGAAGTACCGTGGAGGGC-3’
5’-GCCCTCCACGGTGAGGTGCACAG

L507P:
5′-GCTAAGAATCTCCCTGGAGCTGAGAACC-3′
5′-GGTTTCAGCTCCAGGGAGATTCTTAGC-3′

I562M:
5′-CGCTGGAGGGTGCTGGAATCAATCAGCCC-3′
5′-GGGCTGTATGGATTCCATGACCCCTCCAGCG-3′

H570R:
5′-CAATCAGCCAGATGGACGTGAATATATTTATGG-3′
5′-CCACATAATATATTCACGTCCATCTGGGCTTATG-3′

H650Q:
5′-CTGAAGATAATGACTCAACTGGGGCCACATTTGAAC-3′
5′-GTTCAAATGTGGCCCCAGTTGAGTCATTATCTTCAG-3′

N659S:
5′-CATTTGACATTGTAAGCTTGGAGGACCTG-3′
5′-CAGGCTCCAGCAAGCTTCAAATGTTCAAATG-3′

L705P:
5′-GAGAAGCCGAAAGAAGAGCCCGATATCTTTGGATTGAACC-3′
5′-GGTTCACATCCAGATATCCTGGCTTTCTTTGGGCTTC-3′

R748G:
5′-CAGTATGTCCCATGCTAAGGGCAAGAGGTTTCTAAATATTC-3′
5′-GAATTTAGAAACCTTTGGCTTTCTACATGGGACATCTG-3′

Y849S:
5′-GCATGATTCAACTCTGTGCGAAGGCAG-3′
5′-CTGCTTTTCGACACAGATCGAGTTCAATG-3′

Constructs were sequenced and shuttled into the pMY-IG vector (a kind gift of Dr. Toshio Kitamura, University of Tokyo, Tokyo, Japan)⁵. The pMY-IG is a retroviral vector harboring a “gateway” cassette for insertion of the respective gene which is followed by an internal ribosomal entry site and an EGFP sequence for flow cytometric selection of transduced cells.

**Retroviral transduction of 32D cells**

For generation of retroviral supernatants, Plat-E packaging cells were transfected with pMY constructs (empty vector, wildtype [wt] PDGFRA, and different PDGFRA mutations) and supernatants collected every 12 hours. Stable cell lines (32D empty
vector, 32D PDGFRA wt, and other analyzed cell lines) were generated by three rounds of retroviral transduction of 32D cells, followed by selection of EGFP positive or highly EGFP positive cells by fluorescence-activated cell sorting (FACS) (top 10% quantile; except for FIP1L1-PDGFRA and N659S and Y849S mutants, where this was not necessary).

**Growth curves and proliferation assays**

For growth analysis, 1x10^6 cells per well were plated without IL-3 into a 6-well dish containing 5 ml medium and counted daily. Wells were adjusted to 5 ml and split daily to maintain cells in a logarithmic growth phase. Total cell numbers were calculated by cell counting after trypan blue staining, and cell growth was calculated by taking into account the dilution factors of the previous days. Cells were cultured in triplicates. For proliferation assays, 2x10^4 cells per well were seeded in triplicates without IL-3 in a 96-well dish. Cells were incubated with different concentrations of imatinib for 72 hours. At the end of the culture period an MTS assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was performed. 20 µl MTS/phenazine methosulfate (PMS) solution (Promega) was added and optical density at OD_{490} was measured after two hours of incubation.

**CFU assay**

Colony-Forming Unit (CFU) assays were performed in Methocult M3231 methylcellulose (Stem cell Technologies, Vancouver, BC, Canada), containing no supplemented cytokines to assay for growth-factor independent clones. Cells were IL-3-deprived and seeded in triplicates into 35 mm-diameter dishes containing 200 cells in 1 ml of methylcellulose each. Numbers of CFU were determined 6 days after plating.

**Western blotting**

Stable 32D PDGFRA cell lines were starved from IL-3 and serum for 12 hours in 0.5% FCS. Subsequently, cells were washed once with ice-cold PBS and lysed with buffer containing 150 mM sodium chloride, 1% NP-40, 50mM Tris pH 8.0, 0.5% sodium deoxycholate, 0.1% SDS and protease/phosphatase inhibitors (Na3VO4,
Sigma-Aldrich, St.Louis, MO, USA; complete EDTA-free, Roche, Basel, Switzerland). Cell lysates were spun at 20 000g for 15 minutes. Lysates were resuspended in SDS sample buffer, boiled, and separated by SDS-PAGE. Proteins were blotted onto Immobilon P membranes (Millipore, Bedford, MA, USA) and stained with the indicated antibody (pSTAT5, 05-495, Upstate/Millipore, Billerica, USA; STAT5, sc835, Santa Cruz, San Jose, USA; pAKT, 4058S; AKT, 9272; pAKT, 3164; PDGFRA, 3164, pPDGFRA, 2992, Cell Signaling, Beverly, USA; β-actin, Sigma ). Detection was done with a secondary antibody, labeled with horseradish peroxidase (HRP)-coupled secondary antibodies and chemiluminescence reagent (ECL-Plus, GE Healthcare, Munich, Germany) were used for detection of the bands.

**Flow cytometry analysis**

32D cells and cells from the PB, bone marrow (BM), or spleen from the transplanted mice were isolated as described previously. Lysis of enucleated red blood cells was achieved by applying AKC buffer. After washing, cells were analyzed using a FACSCalibur machine (BD Biosciences, Heidelberg, Germany) for EGFP.

**32D cell transplantation and imatinib treatment**

Retrovirally transduced 32D cells were resuspended in PBS (1.2x10^6/ 200µl) and 1.2 x 10^6 cells injected into the tail veins of 6- to 8-week-old syngeneic C3H/HeJ mice. Mice were analyzed at certain indicated time points or when moribund. PB was collected from the retro-orbital plexus. Imatinib treatment of injected mice started 5 days after injection. Administration was performed two times daily by oral gavage of 100 µl imatinib solution (62.5 mg imatinib per kilogram body weight solved in water) (LC Labs, Woburn, MA, USA).

**Statistical analysis**

Statistical analyses were performed using non-parametric Mann-Whitney U test and presented as mean ± SD. p<0.05 was considered statistically significant. Statistical analysis of survival was performed using the Log Rank test.
Results

1. 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 nine novel PDGFRA gene point mutations in seven patients. Of the latter patients, four patients had only one mutation (R481G, N659S, L705P, Y849S), while three patients had two 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 thirty-five healthy controls. Also, since one of the HES patients had a history of CLL, we sequenced PDGFRA in forty patients with CLL without eosinophilia but found no PDGFRA mutations in CLL patients (data not shown). Since we had previously shown that a fraction of patients with HES show PDGFRA overexpression, we analyzed all patients for PDGFRA expression by quantitative RT-PCR. As depicted in Tables 1 and 2, 36 of 87 (42.5%) of patients showed PDGFRA overexpression, including the patients harboring the R481G, I562M and H570R, H650Q and R748G, or L705P mutations.

2. PDGFRA mutations transform myeloid cells

In order 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 cell lines for factor-independent cell growth. While all of the mutant cell lines grew in the presence of WEHI as a source of IL-3, four mutant cell lines (H650Q, N659S, R748G, and Y849S) survived in the absence of IL-3. The N659S and Y849S mutants immediately showed exponential growth in liquid culture and clonogenic assays. In addition, the H650Q and R748G PDGFRA mutant cell lines also showed exponential growth and increased colony formation in the absence of IL-3 when they were sorted for high EGFP expression (top 10%) (Fig. 1A and 1B). These effects were similar to those seen with 32D cells transduced with FIP1L1-PDGFRα, Flt3-ITD, or BCR-ABL1 (Fig. 1A). No relevant cell or colony growth was detected for R481G, L507P, I562M, H570R, L705P mutants or 32D cells transduced
with wild-type (wt) PDGFRA, wt JAK2, or the empty vector (Fig.1B), regardless of whether they were sorted for high EGFP expression or not. PDGFRA transcripts were readily detected in all of the transduced cells (not shown), and all of the cells expressed EGFP and PDGFRA, as assessed by Western blotting (Fig.1C) and flow cytometry (data not shown). Since H650Q and R748G were present in the same patient (Table 2), we investigated whether both mutations occurred on the same allele. To do this, cloning of the PCR products from this patient was performed and revealed that the resulting forty E. coli clones which were analyzed contained either the H650Q or the R748G mutation. These results suggest that the H650Q and R748G mutations occurred on separate alleles. The SNP P567P was present in all PCR clones and was thus not informative (homozygous SNP).

3. PDGFRA mutants are constitutively active and are sensitive to imatinib

We first sought to investigate whether the mutants were able to induce STAT5 activation. Indeed, after IL-3 deprivation, we found that the four transforming mutants, H650Q, N659S, R748G, Y849S as well as FIP1L1-PDGFRA but none of the other mutants or wt PDGFRA-transduced cells showed constitutive phosphorylation of STAT5 (Fig. 1C).

To investigate whether the mutant receptors retained responsiveness to ligand, we stimulated IL-3 starved 32D PDGFRA mutant cells with PDGF-AA. We found that none of the cells showed an additional increase of STAT5 phosphorylation upon PDGF-AA stimulation (Fig. 2A). However, AKT phosphorylation was strongly increased in both PDGFRA wt cells and the H650Q, N659S, R748G, and Y849S mutant cells but not FIP1L1-PDGFRA mutants (Fig. 2A), demonstrating that the PDGFRA point mutations do not alter sensitivity to PDGF ligand.

Imatinib is a strong PDGFRA and -B inhibitor, but imatinib resistance has been described both for BCR-ABL1 and FIP1L1-PDGFRA mutants.4,7,8 We assessed whether the transforming mutants were sensitive to imatinib treatment in vitro. Indeed, all mutants retained sensitivity to imatinib, as detected by an MTS assay (Fig. 2B). A dose-dependent Curve fitting algorithm from GraphPad Prism 5.0 was used to
determine IC50 values of all 32D mutants (H650Q 2.0 nM; N659S 159 nM; R748G 0.9 nM; Y849S 2.6 nM; FIP1L1-PDGFRA 1.8 nM; BCR-ABL1 689 nM), 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 (Fig. 2B). As expected, JAK2V617F mutants were insensitive to imatinib. STAT5 phosphorylation was inhibited by imatinib in all sensitive cell lines (Fig. 2C). Imatinib exposure induced cell death as shown by 7-AAD positivity and loss of EGFP expression (Fig. 2D).

4. 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 two mutants immediately induced IL-3 independent growth. 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 (Fig. 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 upon autopsy (Fig. 3E). Diseased mice unequivocally showed splenomegaly (Fig. 3B, D) and lymphadenopathy (Fig. 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 (Fig. 3C). FACS analysis demonstrated EGFP positive donor cells in all affected organs analyzed (PB, BM, spleen, and lymph nodes) (Fig. 3E). In addition, histological 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 blast-like cells in the bone marrow (Fig. 3F).
5. *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-PDGFRα overexpressing cells twice daily with imatinib by oral gavage starting on day 5 post injection. Imatinib treatment of mice injected with PDGFRA wt cells had no negative impact on survival but significantly prolonged survival in all other groups (Fig. 4A). Also, there was a significant decrease of lymphadenopathy in all treated mice (Fig. 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-PDGFRα cells (Fig. 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, four mutations possessed transforming potential (H650Q, N659S, R748G, and Y849S). The N659S and Y849S mutations were identified in one and two 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 immuno-chemotherapy when eosinophilia first occurred. Eosinophil counts fluctuated between 1000/µl and 4560/µl until B-CLL relapsed approximately two years later, and the patient died approximately 10 months later despite chemotherapy. Transforming activity of H650Q and R748G in 32D cells was only detected upon sorting for high expressor cells. Interestingly, the patient harboring these mutants showed overexpression of PDGFRA, suggesting that these two mutants may be weaker transforming mutants as compared to the strong oncogenic mutations N659S and Y849S. A similar phenomenon was described for BCR-ABL-transduced CD34+ cell 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 two 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 AML were initially only found in
one of 150 patients\textsuperscript{13} but subsequently confirmed in up to 9\% of secondary AML\textsuperscript{14} and myeloproliferative and myelodysplastic syndromes.\textsuperscript{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 biological relevance of this and the other three activating mutants. This suggests that these mutations are driver mutations for malignant growth. In contrast, the remaining mutations are likely to be mere passenger mutations as described for the Flt3 locus in AML,\textsuperscript{16} although we cannot completely rule out that some of these mutants do possess weak transforming potential, possibly in conjunction with other yet undefined aberrations.

Our data show that the H650Q and R748G mutations were not located on the same allele since none of the sequencing products showed both mutations, as analyzed by individual bacterial clones. Since the P567P SNP was present in all bacterial clones and was therefore homozygous, we cannot distinguish between the possibility that both mutations in this patient occurred on separate alleles in the same cell or in different cell clones. As both mutations harbor transforming potential on their own, it is likely that two different cell clones were present.

The H650Q and N659S mutations are located in the first tyrosine kinase domain of PDGFRA (exon 14) in close proximity to the ATP binding site (amino acid 627) and close to the kinase insert domain that separates the two kinase domains. The replacement of a basic charged histidine by the neutral non-polar glutamine residue is not located in the proximity of any known protein binding site, and therefore, the mechanism of receptor activation resulting from this amino acid exchange remains elusive at this point. In contrast, the R748G mutation is located in the 104 amino acid-spanning kinase insert domain (amino acids 691 to 795) (exon 16). This region is rich of tyrosine residues known to be autophosphorylated upon activation of the receptor, thus enabling the receptor to directly recruit different SH2 domain-containing proteins such as Src and SHP2 or indirectly as proposed for GRB2.\textsuperscript{17}

Finally, the Y849S mutation is located in the activation loop of PDGFRA (exon 18) and affects the main tyrosine residue that leads to increased catalytic efficiency of the PDGFRA upon autophosphorylation.\textsuperscript{18} Interestingly, the H650Q, R748G, and Y849S mutants were highly sensitive to imatinib-mediated kinase inhibition, while the N659S mutant showed a lower sensitivity.
Activating mutations in PDGFRA have been described in diverse malignancies. Most frequently, they are found in approximately 8% of all patients with GIST. 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 one of the amino acid residues D842 or D846) and about 3% in exon 12 which codes for the juxtamembrane domain (V561D and deletions/insertions of two or more amino acids including the V561 residue or residue S566). To date, only three patients have been described with mutations in exon 14 which codes for the tyrosine kinase domain 1 (N659K). 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). In addition, two AML cases were described with PDGFRA mutations in exon 17 and 19 (F808L, N870S) and one acute lymphoblastic leukemia (ALL) case with a mutation in exon 10 which encodes the extracellular fifth Ig-like domain (A509D). Lastly, four cases with PDGFRA mutations (C235Y in exon 5, W349C in exon 7 (n=2), V536E in exon 11) and two cases with deletions 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, since 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 mutants in larger cohorts of patients. Importantly, novel point mutants should be rigorously tested for their transforming potential as well as TKI sensitivity to exclude passenger mutations such as those seen in our own screen as well as those described for FLT3-ITD in AML. 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 IFPs 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 to mice injected with 32D FIP1L1-PDGFRA cells (p<0.001, p=0.007, p<0.001 respectively, Fig 3C) while the degree of splenomegaly was not different (Fig. 3D). One major difference between the PDGFRA point mutants (H650Q and R748G) and the FIP1L1-PDGFRA fusion protein is that the PDGFRA point mutants but not FIP1L1-PDGFRA 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-PDGFRA-transduced cells (Fig. 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 lines36, suggesting that this may play a role in the preferential lymph adenopathy 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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CE, PE, CW, HS, AR, and SK designed research. PE, CW, and AR sequenced patient samples. CE, MF, MS, MSt, and SK performed cell line and mouse research. CE, PE, CW, HS, NCPC, WEB, CMT, AR, AH, WKH, and SK analyzed the data. CE, PE, AR, and SK wrote the paper. Conflict of Interest Disclosure: Novartis Advisory Board: AH, AR, SK. Novartis Honoraria: AH, AR. Novartis Research Funding (although not for the present project): SK.
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Tables

Table 1: Results from PDGFRA gene sequencing

<table>
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<th>Point mutations</th>
<th>patients (n)</th>
<th>patients (%)</th>
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<td>PDGFRA overexpression</td>
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In a screen 87 patients with idiopathic HES were sequenced for PDGFRA mutations.

Table 2: Patients with PDGFRA point mutations

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<th>ID</th>
<th>Clinical diagnosis</th>
<th>Mutation</th>
<th>Polymorphism</th>
<th>PDGFRA overexpression</th>
<th>Gender</th>
<th>Age (ys)</th>
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Figure Legends

Figure 1: Expression of specific PDGFRA mutations in 32D cells overcomes IL-3 dependence.

32D cells were retrovirally transduced with PDGFRA vectors containing mutations as specified in the results section or empty vector, FIP1L1-PDGFRA, JAK2wt, JAK2V617F, BCR-ABL1 or FLT3-ITD. (A) Growth capacity of all mutant PDGFRA cell lines without IL-3. PDGFRA H650Q, N659S, R748G and Y849S induce IL-3-independent growth in 32D cells. Cells expressing the indicated mutations were seeded without IL-3 and counted daily for 5 days. The graph depicts mean viable cell numbers taken from a representative experiment performed in triplicates. The results were confirmed in three independent experiments. (B) Clonogenic growth in semisolid medium. The 32D cells expressing the indicated mutations were plated in triplicates in methyl cellulose media without IL-3 at a concentration of 200 cells per dish. Colonies were counted on day 6. These results were confirmed in three independent experiments. (C) Western blot experiments of 32D cell lines. Total cell lysates of IL-3- and 0.5% FCS-deprived (12h starvation) 32D cells were conducted. Blots were stained with the indicated phosphorylation-specific or total antibodies. PDGFRA and pPDGFRA antibodies recognize two bands, the mature form of the receptor with a size of approximately 180 kDa and an incompletely glycosylated precursor at about 160 kDa. STAT5 and STAT5 phosphorylation were detected on a second membrane using same lysates and same amounts of protein.

Figure 2: 32D cells expressing mutated PDGFRA retain ligand dependent phosphorylation of AKT and imatinib sensitivity in vitro.

(A) Activation of different signaling pathways by PDGF-AA. Cell mutants were starved overnight in medium containing 0.5% FCS and stimulated with 10 ng/ml PDGF-AA for 10 minutes. Total cell lysates were prepared, and the western blot membranes stained with the indicated phosphorylation-specific antibodies. (B) MTS Assay in presence of Imatinib and calculated IC50 values. The same 32D cells were used in a Cell Titer 96 Aqueous Solution-Assay to measure the influence of imatinib on proliferation after 72 hours of Imatinib exposure. Each point represents the mean percentage of growth compared to the untreated cells from three independent
experiments. Data were used to calculate IC50 values with GraphPad Prism 5 (C). Inhibition of intracellular signaling by imatinib treatment. 32D cell mutants were starved overnight in medium containing 0.5% FCS and treated with the indicated concentrations of imatinib for two hours. Total cell lysates were separated by SDS-PAGE. After blotting, the blots were stained with a phosphorylation specific STAT5, total STAT5, or ß-actin antibody. (D) FACS analysis of imatinib treated mutant 32D cell lines. Cells were incubated with 0.5 or 5 µM Imatinib or vehicle for 72h and 7-AAD stained. Depicted percentages and gates represent remaining GFP positive and 7-AAD negative cells. The Figure shows one representative experiment of two independent experiments.

Figure 3:
PDGFRA point mutations show increased lymphadenopathy compared to 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*10E6 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 sacrificed 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 two control groups (untreated vs. water-treated) with the exception of R748G mice, due to 3 outliers which harbored greatly enlarged lymph nodes but marginally significantly smaller spleens. Statistical significance was tested with the non-parametric 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 analysed by flow cytometry for the presence of GFP positive 32D cells. Depicted gates and percent values represent GFP positive cells. Shown is one mouse of each group, analyzed 22 days after injection. (F) Histological analysis of spleen, liver and bone marrow 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 bone marrow. Spleen and Liver slides are depicted at 10x magnification, bone marrow at 100x.

**Figure 4:**
*Imatinib treatment of mutant 32D PDGFRA cell-injected mice prolongs survival and decreases lymphadenopathy*

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 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 due to infection without comparable lymphadenopathy (PDGFRA wt). (A) Injected cells are susceptible to imatinib treatment, prolonging survival of mice. Kaplan-Meier plots show survival of injected mice (survival curves for PDGFRA wt overlap). Statistical significance was tested with Log Rank test. (B,C) 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 sacrificed and included. Statistical significance was tested with the Mann-Whitney U test. Autopsy of the four surviving imatinib-treated FIP1L1-PDGFRA mice showed splenomegaly in three of the mice, while one of the mice showed a normal spleen weight. Therefore, although splenomegaly did develop in some of these mice, the difference was not statistically significant.
Fig. 1C

<table>
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<th></th>
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<th>PDGFRA wt</th>
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<th>H570R</th>
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- pPDGFRA
- PDGFRA
- pStat5
- Stat5
- beta-actin
**Fig. 2A**

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<tr>
<th>Protein</th>
<th>PDGFRA wt</th>
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<th>R748G</th>
<th>Y849S</th>
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- 83 kDa
- 83 kDa
- 62 kDa
- 62 kDa

**Fig. 2B**

MTS value after 72 hr (mean % of untreated +/- SEM)

- H650Q
- N659S
- R748G
- Y849S
- FIP1L1-PDGFRα
- JAK2 V617F
- BCR-ABL

Imatinib (log nM)
Fig. 2C
Fig. 3A

Cumulative survival

Survival time
(days after cell injection)

PDGFRA wt
H650Q
R748G
FIP1L1-PDGFRα

Fig. 3B

PDGFRA wt   H650Q   R748G   FIP1L1-PDGFRα

96.6 mg  589.3 mg  651.7 mg  556 mg
Fig. 3E

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Fig. 3F

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

Christian Elling, Philipp Erben, Christoph Walz, Marie Frickenhaus, Mirle Schemionek, Martin Stehling, Hubert Serve, Nicholas C.P. Cross, Andreas Hochhaus, Wolf-Karsten Hofmann, Wolfgang E. Berdel, Carsten Müller-Tidow, Andreas Reiter and Steffen Koschmieder