

Cell clonality in hypereosinophilic syndrome: what pathogenetic role?

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Abstract

Objective

Idiopathic hypereosinophilic syndrome (HES) is a heterogeneous disorder, including either a myeloproliferative or a lymphoproliferative variant (l-HES).

In l-HES, T-lymphocytes could be involved in the pathogenesis through several cytokines, including IL5.

Methods

We assayed both TCR β - and δ -rearrangements by fluorescent PCR, characterizing 14 patients affected by HES. Lyn activation (a src-kinase involved in the IL5 pathway) was also tested in 6 cases.

Results

FIP1L1-PDGFR α was detected in 4 cases (28.6%); a clonal TCR was found in 10 cases (71.4%), including cases FIP1L1-PDGFR α -positive; four cases did not show any molecular marker. In this series, levels of IL5, IL4, IL2 and γ IFN were measured, without any significant difference among different subgroups.

All pathological samples tested did not show Lyn activation.

Immunophenotype was also characterized: only one case showed an atypical CD3-/CD4+ population in the bone marrow.

Conclusion

This study would suggest that a real distinction between m- and l-HES is not wholly convincing and that clonal T-cell expansion could not be the "primum movens" but an epiphenomenon in HES.

Key words

HES, TCR, FIP1L1/PDGFR α .

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Introduction

Idiopathic hypereosinophilic syndrome (HES) is defined as blood eosinophilia of unknown origin exceeding 1500 cells/ml, persisting for over 6 months, and leading to organ damage or dysfunction due to infiltration by eosinophils (1).

According to WHO criteria, diagnosis of HES is made after excluding all causes of reactive eosinophilia, presence of an abnormal T-cell population and other concomitant clonal myeloid disorders. All cases with demonstrated clonal cytogenetic abnormality or presenting blasts (> 2% in the peripheral blood or 5% -19% in the bone marrow) are defined as affected by chronic eosinophilic leukemia (CEL) (2).

Among possible differential diagnosis, Churg-Strauss syndrome has to be carefully excluded on the basis of clinical and laboratory tests: this is relevant on the light of a possible T cell involvement in this vasculitis (3).

More recently, other Authors have described two HES variants: 1) the "myeloproliferative" form (m-HES), characterized by increased serum vitamin B12, high tryptase levels and splenomegaly; 2) the "lymphocytic variant" (l-HES), when hypereosinophilia is secondary to a primitive T-lymphoid disorder demonstrated by the presence of a circulating T-cell clone (4, 5).

The renewed interest in HES derives principally from the efficacy of Imatinib in many patients: cytogenetic evidence of an interstitial deletion on 4q12 in some patients responsive to Imatinib allowed cloning the FIP1L1-PDGFR α fusion gene that characterizes 17-36% of all patients (6, 7).

In these cases, juxtaposition of FIP1L1 to PDGFR α leads to a deregulated PDGFR α kinase activity and abrogates normal growth factor-dependent stimulation of the receptor.

PDGFR α is a member of the family of type III-receptor tyrosine kinases and represents one of the targets of Imatinib. Consequently, the diagnosis of FIP1L1-PDGFR α -positive HES is now recognized as a fundamental step in diagnosis and management of these patients.

Even the lymphocytic variant seems

to be particularly interesting due to its clinical and biological features. Several studies showed that T-cell clones derived from peripheral blood of patients with HES displayed eosinophilopoietic activity in the presence of stem cells from healthy subjects, thus suggesting that T-lymphocytes could be involved in HES pathogenesis, probably through release of several cytokines (IL5, IL4, and IL13) (8, 9, 10).

IL5 and mechanisms of its signal transduction are quite well-characterized today.

Lyn, Jak2 and Raf-1 kinases are necessary for the anti-apoptotic effect of IL5, whereas Raf-1 is also essential for eosinophil activation and degranulation (11).

Lyn is a src-kinase constitutively associated with the β c receptor for IL5 and represents one of the earliest activated kinases after IL5 stimulation.

Through activation of various adapter proteins and GTPases, the final result is the activation of the Ras-MAPK pathway, with consequent activation of transcription factors such as c-fos, c-jun and NF- κ B (12).

In some cases of l-HES, an abnormal T-cell population (CD3-/CD4+, CD3-/CD8+/CD56+) or an inverted ratio CD4/CD8 in the absence of an abnormal phenotype, were reported (13).

In contrast to m-HES, l-HES appears to affect females more frequently, with cutaneous manifestations and lung and digestive system involvement; notwithstanding a better short-term prognosis, long-term outcome may be less favorable, due to the occurrence of T-cell malignancies (14).

Considering the interesting clinical and biological features of l-HES, we planned to analyze both TCR β - and δ -chain gene rearrangements in 14 patients with hypereosinophilia observed in our Center between 1991 and 2004.

The aim of study was to investigate the possible pathogenetic role of T-cell clonality in HES cases by a sensitive molecular method (fluorescent PCR) (15, 16).

Patients and methods

Patients

This retrospective study included 14

adult patients (3 female and 11 male) affected by HES, observed in our center over the period 1991 - 2004. All patients gave their informed consent for storage and use of biological samples. The clinical diagnosis of HES was based on the diagnostic criteria of Chusid *et al.* (1). Causes of secondary eosinophilia, including Churg-Strauss syndrome, have been carefully excluded. In all cases, persistent hypereosinophilia and organ infiltration were documented.

Patient's characteristics are listed in Table I. Median age of the patients was 60 years (range 38-76 years). Median number of WBC was $11.3 \times 10^9/l$ (range 5-35), with a median eosinophil count of $4.8 \times 10^9/l$ (range 1.5-20.3).

Regarding treatment, prior to 2003 (when T-cell clonality was first demonstrated) patients received α -Interferon (IFN) 3×10^6 UI/day, three times a week (nine cases); four cases received pegylated Interferon, 50 mcg/week.

In absence of any demonstrated T-cell clonality, treatment included hydroxyurea (1 g/day) or steroids.

After 2003, all FIP1L1-PDGFR α -posi-

tive cases received Imatinib, 100 mg/day. A clinical and hematological complete remission (CR) included, at minimum, normalization of the peripheral eosinophil count and disappearance of all symptoms and signs of HES/CEL (17).

APR was defined as reduced eosinophil count $> 50\%$, without any progression of organ infiltration or damage.

Cytogenetic analysis

Conventional cytogenetic analysis was performed on bone marrow aspirates, following the recommendations of the International System for Human Cytogenetic Nomenclature (18). At least 20 metaphases were analyzed for each sample.

Molecular assays

All RT-PCR analyses were performed on the same bone marrow samples employed for the cytogenetic analysis. Total RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. Suitable aliquots were utilized for PCR tests

after spectrophotometrical quantitative evaluation.

cDNA synthesis reaction was performed with 1 μ g of total RNA in a total volume of 20 μ l containing 200 units of M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 1X First-Strand Buffer (50 mM Tris-HCl (pH 8.3) 75 mM KCl, 3 mM MgCl₂), 1 mM dNTPs, 32 units of RNaseOUT (Invitrogen, Carlsbad, CA, USA), 10mM DTT, 5 μ M random primers. The synthesis program included an initial incubation at 37°C for 10 min, followed by incubation at 42°C for 45 min. Reaction was inactivated by heating at 99°C for 3 min and the efficacy of transcription was assayed by amplification of beta2-microglobulin (adopted as the housekeeping gene).

Qualitative PCR reactions were performed by using specific primers for *ABL* exon 3 and *BCR* exon 13, according to recommendations by European Concerted Action Biomed 1 (19).

The FIP1L1-PDGFR α fusion gene was amplified according to Cools *et al.* (6). High molecular weight DNA was extracted by Genomic DNA Isolation Re-

Table I. Clinical characteristics of patients and responses to treatments.

PZ	Sex	Age	WBC $\times 10^9/l$	BM EO %	Organ Involvement	TCR Beta	TCR Delta	Fipili PDGFR α	Therapy	Clinical response	Molecular monitoring
1	M	38	35.0	30	spleen	+	-	+	IFN	CR	TCR - FIP1L1PDGFR +
2	M	59	24.8	35	skin	+	-	+	HU PDN	PR PR	TCR + FIP1L1PDGFR +
3	M	61	10.0	30	Spleen, lungs	+	-	+	STI	CR	ND
4	M	40	10.9	22	skin	-	+	-	IFN	PR	TCR +
5	M	47	15.0	50	Spleen, liver	+	-	+	IFN STI	PR CR	TCR - FIP1L1PDGFR -
6	M	64	11.4	15	heart	-	-	-	HU IFN STI	NR NR NR	TCR +
7	M	75	8.2	20	GI	-	+	-	IFN	CR	TCR -
8	M	74	11.2	20	Liver, GI	-	-	-	PDN IFN	PR PR	TCR +
9	M	75	13.8	18	Liver, skin	-	-	-	IFN	NR	ND
10	M	40	17.5	23	Spleen	-	+	-	IFN	CR	ND
11	F	76	17.5	30	heart	-	-	-	IFN	PR	ND
12	F	46	8.6	70	Spleen, liver, skin	-	+	-	STI	PR	ND
13	F	76	10.5	15	lungs	-	+	-	HU	PR	TCR +
14	M	46	5.0	25	Spleen	-	+	-	NO Tx	STABLE	ND

IFN: interferon; HU: hydroxyurea; PDN: prednisone; STI: imatinib; No Tx: no treatment; ND: not evaluated.

agent DNAZOL™ (Gibco BRL, Milan, Italy); after precipitation with ethanol 100%, the DNA was suspended in TE buffer (pH = 8). After quantitative spectrophotometric evaluation suitable aliquots were utilized for PCR tests.

Delta TCR rearrangements were amplified according a protocol already adopted in our laboratory for evaluation of T-cell clonality in multiple myeloma patients (16); PCR-amplified products were resolved by capillary electrophoresis on ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA).

Genescan 2.1 software was then used to analyze the PCR products, with accurate sizing and quantification of the peak areas.

TCR β chain rearrangement was evaluated by using a new purchasable kit, according to manufacturer's instructions (*In vivo* Scribe Technologies, San Diego, CA, USA).

TCR β and δ profiles were also analyzed on bone marrow samples from 15 healthy adults. In these cases, PCR products were typically distributed in a Gaussian fashion, with an average of 7-9 different peaks.

Cytokine levels

Commercial two-site sandwich ELISA kits were used to determine concentrations of IL5, IL4, IL2 and γ IFN.

Flow cytometry

Immunophenotype was identified by flow cytometry analysis performed by two- and three-color immunofluorescence (fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin-chlorophyll-a-protein (PerCP)-conjugated monoclonal antibodies against CD3, CD4, CD8, CD2, CD45, CD25, TCR α/β and γ/δ) (Becton Dickinson, Milan, Italy).

Lyn activation

Paraffin-embedded samples (bone marrow, stomach, small bowel) from six patients were assayed for Lyn activation, using Phospho-Src (Tyr416) antibody (Cell Signaling Technology, MA, USA), according to manufacturer's instructions.

Slides were evaluated blindly by two

independent operators on a Leica microscope at 1000 x enlargements.

Positivity was detected as dark brown staining of cytoplasm.

In each experiment appropriate negative and positive controls were performed.

Negative controls were performed by substituting the specific antibody for irrelevant rabbit isotype immunoglobulin.

Gastric enterochromaffin cells chromogranin A-positive and bone marrow megakaryocytes were adopted as positive controls.

Results

With a median follow-up of 30 months (range 1-169 months), all patients but one survived.

Four patients progressed, with a median PFS for the overall series of 37 months. Conventional karyotype was normal in all cases.

On the basis of molecular data, three subgroups have been distinguished: 1) FIP1L1-PDGFR α -positive cases; 2) cases showing TCR clonality (Figs. 1 and 2); 3) cases without any molecular marker (see Table I).

First subgroup

RT-PCR assays detected the FIP1L1-PDGFR α fusion gene in 4 of the 14 evaluated cases (28.6%); all patients carrying this fusion gene were male.

Three of these four patients presented splenomegaly, and one case also had concomitant hepatomegaly; one patient had skin and another one lung involvement.

Because the study started in 1994 and detection of FIP1L1-PDGFR α -fusion gene was often performed retrospectively, serum tryptase levels were measured in an insignificant percentage of cases.

In the FIP1L1-PDGFR α -positive subgroup, median leukocyte count was $19.9 \times 10^9/l$; median values of cytokines were: 3 pg/ml for IL5 (normal values 0-25.8 pg/ml), 6.8 pg/ml for IL4 (normal values 0-5.8), 0.1 U/ml γ IFN (normal values 0-1.2 U/ml).

Cytofluorimetric analysis did not reveal any T-abnormal clonal population.

Clinical features of these patients at

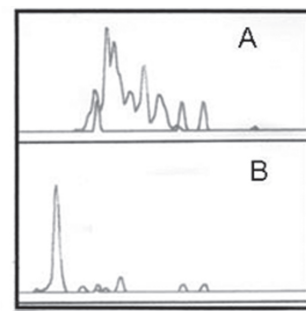


Fig. 1. TCR δ rearrangement detection: in A) a polyclonal; in B) a clonal pattern

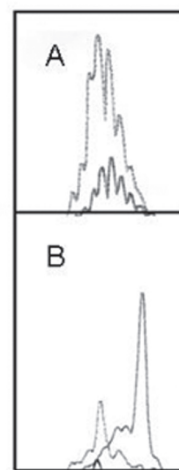


Fig. 2. TCR β rearrangement detection: in A) a polyclonal; in B) a clonal pattern.

diagnosis and progression rate did not differ from FIP1L1-PDGFR α -negative cases.

Second subgroup

Clonal TCR rearrangement was detected in 10 of the 14 evaluated cases (71.4%); interestingly, all four FIP1L1-PDGFR α -positive cases showed a clonal T-cell population.

In this subgroup, median leukocyte count was $13.1 \times 10^9/l$ and cytokine median values were in the normal ranges. IL5 levels were moderately increased (median 32 pg/ml, normal ranges 0-25.8 pg/ml) in four cases.

About immunophenotype, only one case showed an atypical CD3-/CD4+ population in the bone marrow.

Third subgroup

Four cases did not present any molecular marker. No peculiar clinical characteristics at diagnosis were noted, but none of these patients achieved a complete hematological response after

both to IFN and Imatinib. Even in this subgroup cytokine levels did not differ from normal values and atypical immunophenotype profiles were not detected.

Lyn detection

Lyn activation was assayed in samples from six cases (two presenting isolated TCR clonality, two FIP1L1-PDGFR α -positive, the two cases without any molecular marker).

In spite of the evident eosinophilic infiltration in assayed tissues, phosphorylated (activated) Lyn was not detected in any case, independently on molecular profile and measured serum cytokine levels (Fig. 3).

Clinical results

In the subgroup showing T-cell clonality, α IFN offered three complete (CR) and two partial (PR) responses, including two FIP1L1-PDGFR α -positive cases that achieved a CR and a PR, respectively.

Imatinib was adopted in four cases; both FIP1L1-PDGFR α -positive patients achieved a CR. Of the remaining two cases, one showing a TCR clonality and another one without any molecular marker, the first achieved a PR and the second did not respond to treatment.

Interestingly, after 5 years of therapy with Interferon, one FIP1L1-PDGFR α -/TCR-positive case showed persistent hepato-splenomegaly in spite of the normalization of the peripheral blood count. Three months after the beginning of Imatinib treatment, the patient showed significant improvement of hepato-splenomegaly, with complete resolution of ascites. Molecular assays performed after 12 months showed disappearance of the FIP1L1-PDGFR α -fusion gene. After a further 6 months, TCR clonality also disappeared.

On the other hand, another FIP1L1-PDGFR α -positive patient who refused Imatinib and continued therapy with α IFN, did not achieve molecular remission after 9 years of treatment, in spite of a good clinical response.

Overall, 8 patients were molecularly evaluated after almost 6 months of treatment: at that time, 4 of them

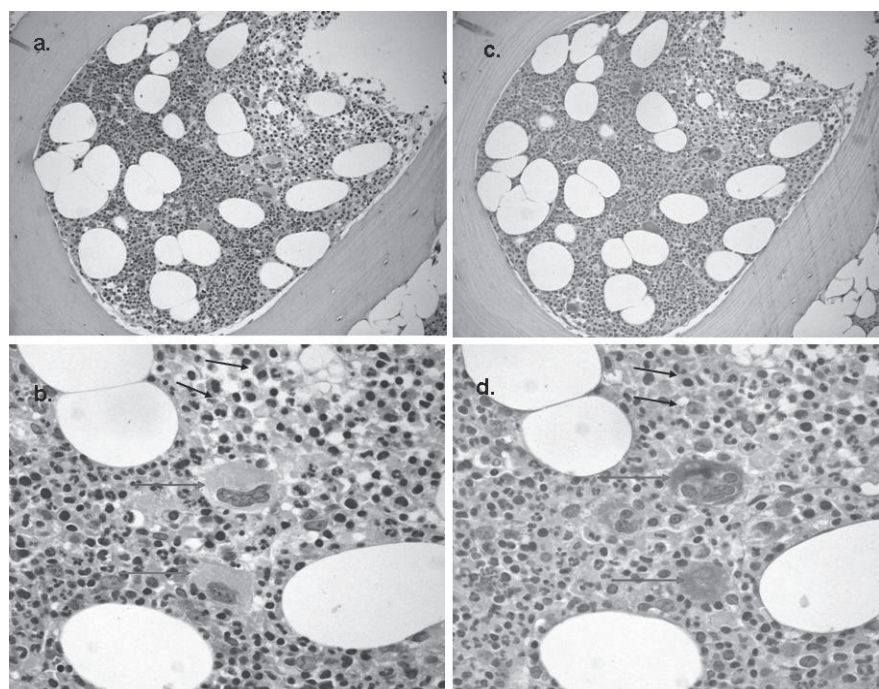


Fig. 3. Bone marrow immunoistochemistry.

In "a" and "b" Hematoxylin-eosin sections.

"b": original magnification 1000x; black arrows indicate eosinophils that present red/purple granules in the cytoplasm; red arrows indicate megakaryocytes that appear blue pale.

In "c" and "d" correspondent sections after treatment with anti-PLyn antibody.

"d": original magnification 1000x; black arrows indicate eosinophils that don't react with antibody; red arrows indicate megakaryocytes that show brown cytoplasmic granules.

achieved clinical partial remission, 3 a complete clinical response whereas one did not respond.

In cases in clinical partial remission or resistant PCR-negativity was not achieved; in two of three cases in clinical complete remission TCR clonality disappeared.

Discussion

The high rate of TCR clonality reported in our series (71%) would suggest that the "*primum movens*" of the large majority of HES cases could be a clonal T-cell proliferation.

Recently, Roche-Lestienne *et al.* performed molecular characterization of HES in 35 patients with normal cytogenetics. TCR γ clonality was detected in 11 (31%) patients; it should be noted that this clonality was not found in the six FIP1L1-PDGFR α -positive cases (7).

Two observations could explain the discrepancy noted between our data and those from these authors: 1) all cases were evaluated for both β and δ chain rearrangements; this analysis has

not been reported before; 2) the high sensitivity of the molecular technique employed. Regarding this topic, as recently reported by the BIOMED network (19) and also by our group (15), fluorescent PCR offers higher sensitivity and specificity than other molecular methods.

Nevertheless, several items argue against this T-cell-dependent pathogenetic hypothesis:

1) If T-cells are the first pathogenetic step in HES, increased levels of cytokines would probably be expected. In our series, median cytokine levels were not significantly increased. Nevertheless, this observation is in agreement with previously reported data showing that serum levels of IL5 do not aid in distinguishing HES patients with an underlying clonal T-cell disorder (20, 21).

2) All pathological samples tested did not show Lyn activation. As reported above, phosphorylation of Lyn is a fundamental step in the IL5-driven eosinophil proliferation, thus indirectly confirming the IL5-independence of

eosinophils growth in our patients.

3) Observation of the patient where TCR clonality disappeared few months after disappearance of the FIP1L1-PDGFR α fusion gene appears to suggest that the T-cell clone proliferation would be dependent upon the eosinophil clone controlled by the Imatinib therapy.

This last observation could be supported by data from the literature that reported a possible cross-talk between eosinophils and T-lymphocytes sustained by the B7 molecule, typically expressed on APC but highly expressed on eosinophils of HES patients as well.

It should be noted that APC would induce proliferation of clonal T populations through engagement of CD2 and CD28 surface molecules and initiation of an IL2/IL2R loop (22).

Consequently, it can be hypothesized that cross-talk between eosinophils and clonal T-cells contributes to activation of the latter, eosinophils being the real "primum movens" of the autocrine IL2-dependent T-cell growth (23).

The efficacy of Interferon in a large majority of cases does not counteract this hypothesis and could be justified by the presence of specific receptors on eosinophils (24) in addition to a direct inhibitor effect, as already reported in other chronic myeloproliferative disorders (25).

In conclusion, on the basis of above-reported data, we suggest that a real distinction between m- and l-HES is not wholly convincing and that clonal T-cell expansion is probably not the "primum movens" in HES.

It appears possible that the appearance of a T-cell clone would be an expression of reactivity to the eosinophil proliferation (both leukemic or not) as it frequently happens – especially for B cells – in autoimmune diseases.

On this basis, the suggested classification (4) of non-leukemic hypereosinophilic syndromes based on the presence of T-cell clonality could not be fully correct.

Further studies on a larger number of

patients and including non HES hypereosinophilia are necessary to confirm our hypothesis.

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