T-cell abnormalities are present at high frequencies in patients with hypereosinophilic syndrome

Grzegorz Helbig,[1] Agata Wieczorkiewicz,[1] Joanna Dziaczkowska-Suszek,[1] Miroslaw Majewski,[1] and Slawomira Kyrcz-Krzemien[1]

[1]Departament of Haematology and Bone Marrow Transplantation, Silesian Medical University, Katowice, and [2]Institute of Haematology and Transfusion Medicine, Medical University, Warsaw, Poland

ABSTRACT

Background

A T-cell clone, thought to be the source of eosinophilopoietic cytokines, identified by clonal rearrangement of the T-cell receptor and by the presence of aberrant T-cell immunophenotype in peripheral blood defines lymphocytic variant of hypereosinophilic syndrome (L-HES).

Design and Methods

Peripheral blood samples from 42 patients who satisfied the diagnostic criteria for HES were studied for T-cell receptor clonal rearrangement by polymerase chain reaction according to BIOMED-2. The T-cell immunophenotype population was assessed in peripheral blood by flow cytometry. The *FIP1L1-PDGFRA* fusion gene was detected by nested polymerase chain reaction.

Results

Forty-two HES patients (18 males and 24 females) with a median age at diagnosis of 56 years (range 17-84) were examined in this study. Their median white blood cell count was 12.9×10^9/L (range 5.3-121), with an absolute eosinophil count of 4.5×10^9/L (range 1.5-99) and a median eosinophilic bone marrow infiltration of 30% (range 11-64). Among the 42 patients, clonal T-cell receptor rearrangements were detected in 18 patients (42.8%). Patients with T-cell receptor clonality included: T-cell receptor β in 15 patients (35%), T-cell receptor γ in 9 (21%) and T-cell receptor δ in 9 (21%) patients, respectively. Clonality was detected in all three T-cell receptor loci in 4 cases, in two loci in 7 patients and in one T-cell receptor locus in the remaining 7 patients. The *FIP1L1-PDGFRA* fusion transcript was absent in all but 2 patients with T-cell receptor clonality. Three patients out of 42 revealed an aberrant T-cell immunophenotype. In some patients, an abnormal CD4:CD8 ratio was demonstrated.

Conclusions

T-cell abnormalities are present at high frequencies in patients with HES.

Key words: T cells, T-cell receptor, hypereosinophilic syndrome, *FIP1L1-PDGFRA*.

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Correspondence:
Grzegorz Helbig MD, PhD, Department of Haematology and Bone Marrow Transplantation, Silesian Medical University, Dabrowski Street 25, 40-032 Katowice, Poland.
E-mail: ghelbig@o2.pl

Introduction

Hypereosinophilic syndrome (HES) is a rare heterogenous group of diseases characterized by chronic, unexplained hypereosinophilia with organ involvement.[1] A small subset of HES patients has an interstitial deletion in chromosome 4q12, that leads to the expression of the imatinib-sensitive *FIP1L1-PDGFRA* fusion gene.[2] The lymphocytic variant of HES (L-HES) represents a distinct clinical entity. Hypereosinophilia in these patients results from the overproduction of eosinophilopoietic cytokines, mainly interleukin 5 (IL-5), by clonal T cells. The detection of the aberrant T-cell phenotype in peripheral blood by flow cytometry and the presence of T-cell receptor (TCR) clonal rearrangement are required for diagnosis.[3]

In this paper, we screened 42 HES patients to estimate the proportion of cases with evidence for T-cell clonality and to compare the clinical features between patients with clonal and non-clonal TCR gene rearrangements.

The assessment included: (i) analysis of the peripheral blood T-cell immunophenotype, (ii) analysis of T-cell receptor rearrangement, (iii) detection of the *FIP1L1-PDGFRA* fusion transcript and (iv) determination of IL-4, IL-5 and tryptase serum levels.

Design and Methods

Patients and methods

This prospective study included 42 treatment-naive HES patients. Informed consent was obtained from all patients according to the Declaration of Helsinki and the study was approved by the institutional ethics committee. All patients included in this study satisfied three main Chusid's criteria for HES: (i) chronic hypereosinophilia i.e., two consecutive eosinophil readings of $>1.5\times10^9$/L; (ii) exclusion of reactive causes of eosinophilia and (iii) eosinophil organ involvement.[1]

Patients were recruited between 2002 and 2008 from 4 centers in Poland. Before entering the study, the following tests were performed: a complete history and physical examination, a complete blood count with differential, routine chemistries, electrocardiogram, echocardiogram, pulmonary function tests, chest X-ray, ultrasound/computed tomography, bone marrow aspirate and biopsy. Additional organ-specific evaluations were performed based on clinical findings. On entering the study, all patients had to exhibit symptoms, signs, and imaging or histological evidence of eosinophilic organ involvement. More frequent causes of organ damage were carefully excluded before the diagnosis of eosinophilic-related organ dysfunction was established.

An intensive work-up was implemented to exclude secondary hypereosinophilia. Infectious causes were excluded by repeated clinical evaluation, blood and stool cultures, serological and imaging studies. Serological studies for HIV, CMV, EBV, HBV and HCV were also performed. Additional laboratory evaluation included a wide panel of autoantibodies in order to exclude connective tissue disease. Hematologic malignancies and solid tumors associated with eosinophilia were excluded by standard laboratory and imaging studies. In case of lymphadenopathy, a lymph node excision was made to exclude lymphoma. Bone marrow mast cell infiltration was excluded by tryptase stained marrow biopsy samples and/or flow cytometry analysis with monoclonal antibodies against CD2, CD25 and CD117. Molecular analysis for the detection of 816KIT mutation was performed when necessary.

Tryptase, Interleukin-4 (IL-4) and Interleukin-5 (IL-5) quantitation

Serum tryptase levels were measured using the UniCAP Tryptase fluoroenzymeimmunoassay (Pharmacia, Uppsala, Sweden). Serum IL-4 and IL-5 levels were measured using the Bender MedSystems tests (Vienna, Austria).

Immunofluorescence analysis

Peripheral blood T lymphocytes were analyzed by standard flow cytometric techniques. Initial screening for T cells with an aberrant immunophenotype was performed using a panel of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanin (PC 5.1) against the lineage-asscociated T-cell markers CD2, CD3, CD4, CD5, CD6, CD7 and CD8 using a three-color immunofluorescence analysis. For the identification of individual markers, monoclonal antibodies against CD16, CD56 and CD57 were used (monoclonal antibodies provided by Becton Dickinson, San Jose, USA). Relevant isotype-matched controls were used to determine background fluorescence. Data were collected on at least 10,000 viable cells using EPICS-XL-MCL flow cytometer (Beckman Coulter, Miami, USA) and System II Software.

TCR gene rearrangement analysis

Genomic DNA was extracted using the QiAmp DNA Blood minikit (Qiagen, Germany) according to the manufacturer's instructions. All amplification reactions were performed using an automated thermocycler according to the BIOMED-2 multiplex polymerase chain reaction (PCR) protocol.[4] PCR products for heteroduplex analysis were denatured at 95°C for 5 min and subsequently renatured at 4°C for 60 min to induce duplex formation. Afterwards, the duplexes were loaded into 6% polyacrylamide gels containing 0.5x Tris-boric acid-EDTA buffer for 50 min, and visualized by ethidium bromide staining. Monoclonal PCR products give rise to homoduplexes, whereas polyclonal PCR products form heteroduplexes, resulting in a smear of slow-migrating fragments. Sensitivity limit of this method is about 5%. Clonal rearrangement was defined as a distinct band of expected size. TCR rearrangement analysis was also performed in 10 healthy adults matched for age.

FIP1L1-PDGFRA detection

RNA was extracted from 106 PBMCs using RNAEasy RNA isolation kit (Qiagen, Valencia, CA, USA). cDNA synthesis and RT-PCR were performed from 2 μg of total RNA using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with gene-spe-

cific primers. The *FIP1L1-PDGFRA* fusion was detected by nested PCR as described by Cools *et al.*[2]

Statistical analysis

Differences in distribution were determined using the Kolgomorow-Smirnow test and significant differences were compared using the Mann-Whitney and Wilcoxon tests for independent and dependent variables. Data are expressed as median and range and values < 0.05 were considered statistically significant.

Results

Forty-two HES patients with a median age at diagnosis of 56 years (range 17-84) were screened for the presence of the HES-associated T-cell clonality. Eosinophilic organ damage/dysfunction identified: splenomegaly (n=13), cardiac involvement (n=12), skin manifestations (n=10), pulmonary infiltration (n=8), hepatomegaly (n=8), mild lymph node enlargement (n=5), gastrointestinal manifestation (n=3) and neuropathy (n=1). Clinical manifestations at presentation are detailed in Table 1.

Cardiac manifestation included cardiomyopathy, congestive heart failure and mitral regurgitation. Cutaneous manifestation presented as diffuse maculopapular, pruritic rash and mucosal ulceration. Bilateral non-specific diffuse infiltrates were the most common pulmonary manifestations whereas eosinophilic enterocolitis dominated the digestive system involvement. There was peripheral neuropathy in one case.

In the majority of HES patients an organ involvement was documented by imaging studies e.g. CT, abdominal ultrasound, chest X-ray. An eosinophilic tissue infiltration was also proven by histology in 15 cases: lymph node (n=5), myocardium (n=3), digestive system (n=3), skin (n=2) and bronchoalveolar lavage (n=2). Cytogenetic analysis revealed normal karyotypes in 24 cases, one case had t(6;11)(q21;q23) and metaphases were not present in the remaining 17 patients. IL-4, IL-5, vitamin B12, serum IgE and tryptase levels were measured in all patients. Serum IL-4 level was below detection level in 11 cases whereas unmeasurable serum IL-5 level was revealed in 5 patients.

Among the 42 HES patients, clonal TCR rearrangement by PCR was detected in 18 patients (42.8%). TCR clonality included: TCRβ in 15 patients (35%), TCR gamma in 9 (21%) and TCRδ in 9 (21%), respectively. Clonality was detected in all three TCR loci in 4 cases, in two loci in 7 patients and in one TCR locus in the remaining 7 patients. TCRβ rearrangements were complete in 11 and incomplete in 8 patients. Thorough analysis of obtained results revealed no evidence for oligoclonality of TCR rearrangements. We also did not observe any sub(clonal) TCR gene rearrangements in any of the analyzed 10 healthy subjects. Details of TCR analysis are presented in Table 2. The *FIP1L1-PDGFRA* fusion transcript was detected in 2 patients with TCR clonality and this fusion was also demonstrated in 4 patients in the non-clonal TCR group. In all 6 cases the treatment with low-dose imatinib appeared to be highly effective at 100 mg/day.

Three out of 42 studied patients revealed an abnormal T-cell phenotype by flow cytometry. One male patient with skin manifestations and severe AEC presented with $CD2^+CD3^-CD4^+CD5^+CD7^-CD8^-$ T cells. Among lymphocytes there were 77.4% of $CD3^-CD4^+$ cells, 7.23% of $CD3^+CD8^-$ cells, 79.5% of $CD2^+CD7^-$ cells and 73.3% of $CD3^-CD5^+$. Molecular studies identified TCR clonal rearrangement: TCRβ, TCRδ and TCRγ and his

Table 1. Clinical manifestation at presentation.

Parameter	TCR negative n=24 (%)	TCR positive n=18 (%)
Constitutional symptoms[1]	33	17
One organ involvement[2]	54	44
2 organs	29	33
> 2 organs	17	22
Cardiac involvement	25	33
Lung involvement	17	22
Splenomegaly	29	33
Hepatomegaly	21	17
Cutaneous manifestation	17	33
Other manifestation (lymph nodes, gastrointestinal, neurological)	8	11

[1]Constitutional symptoms included: weakness, fatigue, fever, weight loss, night sweats, cough, dyspnea; [2]no organ involvement in one patient with detectable FIP1L1-PDGFRA fusion transcript.

Table 2. Results of BIOMED-2 TCR multiplex polymerase chain reaction in 18 HES patients with detected TCR clonality.

Patient	TCRβ			TCRγ		TCRδ
	Tube A (Vβ-Jβ)	Tube B (Vβ-Jβ)	Tube C (Dβ-Jβ)	Tube A (Vχ-Jχ)	Tube B (Vχ-Jχ)	Tube (Vδ-Jδ)
1	NC	C	C	NC	NC	NC
2	NC	C	NC	NC	NC	C
3	C	C	NC	NC	C	NC
4	C	C	NC	NC	NC	C
5	NC	NC	C	NC	NC	C
6	C	C	NC	C	NC	NC
7	C	NC	NC	NC	NC	NC
8	NC	C	C	NC	NC	NC
9	C	C	NC	NC	C	C
10	NC	C	C	C	C	C
11	NC	NC	C	NC	NC	NC
12	NC	C	NC	C	C	NC
13	NC	NC	C	C	NC	C
14	NC	NC	NC	NC	C	NC
15	NC	NC	NC	NC	NC	C
16	NC	NC	NC	NC	NC	C
17	NC	NC	C	C	C	NC
18	NC	C	C	C	C	C

C: clonal; NC: non-clonal.

serum IgE and IL-4 level were elevated: (840 IU/mL and 13 pg/mL, respectively). In contrast, serum IL-5 levels were undetectable. He achieved hematologic remission while on steroids, but the aberrant T-cell clone was still present. A second female patient, presented with increased WBC counts (21×10^9/L), hypereosinophilia (3.5×10^9/L) and lymphocytosis (13×10^9/L). Serum IgE levels were 13,966 IU/mL. In addition, her blood immunophenotyping results were abnormal revealing a predominant $CD3^+CD4^+$ T-cell population i.e., 98% of total lymphocytes were $CD3^+$ $CD4^+$ (an absolute $CD4^+$ count was $12.\times10^9$) while B and NK cells comprised only 1.5% and 0.5%, respectively. These $CD3^+$ cells also expressed other pan T-cell markers including $CD2^+$, $CD5^+$ and $CD7^+$. TCR sequence analysis revealed clonal rearrangement i.e., TCRβ and TCRδ. Her serum IL-4 levels were elevated whereas serum IL-5 levels were undetectable. Cytogenetic studies identified t(6;11). This condition progressed to peripheral T-cell lymphoma after three years of persistent eosinophilia.[5] A third male patient presented with an abnormal peripheral blood T-cell population defined by $CD2^+CD3^+CD4^-$ $CD5^+CD7^+CD8^+CD16^-CD56^+$ and $CD57^+$ cells. These cells occupied 32% of total lymphocytes. Molecular studies revealed clonal TCR gene rearrangements, i.e., TCRβ and TCRγ. His blood eosinophilia was moderate (1.6×10^9), serum IgE was normal and serum IL-4 and IL-5 were undetectable. Serological and molecular studies for cytomegalovirus infection were negative. In summary, among 18 patients with evidence for T-cell clonality only 3 had phenotypically abnormal T-cell subsets whereas among the 24 patients without T-cell clones, no phenotypic abnormalities were observed.

In some patients, an abnormal CD4:CD8 ratio was identified. The other abnormalities included increases or decreases in $CD3^+CD4^+$ or $CD3^+CD7^+$ cells.

We compared clinical and biological features of patients with clonal and non-clonal TCR gene rearrangements. No significant differences were found between these two analyzed groups except that the CD4:CD8 ratio was increased in the non-clonal TCR population (p=0.03). Study group characteristics are shown in Table 3.

Discussion

The detection of an abnormal T-cell population (TCP) in peripheral blood and TCR clonal rearrangement (TCC) are required for the diagnosis of L-HES. It was demonstrated that hypereosinophilia may precede the development of overt T-cell lymphoma, but it remains unclear how often the abnormal T-cell population is associated with prolonged eosinophilia.[3] The first HES patient with an aberrant TCP and overproduction of IL-5 was described in 1994.[6] Three additional patients with abnormal $CD3^-CD4^+$ cells in peripheral blood by flow cytometry were identified. These cells produced excessive amounts of IL-4 and IL-5 compared to $CD3^+CD4^+$ cells from healthy volunteers. Involvement of the skin was the most common presentation in this population.[7] Among 60 patients with idiopathic eosinophilia studied by Simon *et al.*, 16 had T cells with an aberrant immunophenotype. It was demonstrated that purified clonal T cells from 2 patients secreted large amounts of IL-5.[8] Serum IL-5 level was not measured in this study. However, it should be mentioned that serum cytokine levels are not indicative of T-cell mediated disease. The standard for L-HES diagnosis should be the determination of cytokine profile in supernatants of *ex vivo* T cells, but it is inapplicable in daily routine practice due to high cost and complexity. In 4/16 patients with the abnormal TCP, T-cell lymphoma was ultimately diagnosed.[8] In a larger study involving 99 patients with idiopathic eosinophilia $>0.6\times10^9$/L, clonal T cells were detected in 14 patients (14%). In 6 patients, an overt T-cell malignancy was already present at the time of evaluation; 7 out of the remaining 8 patients with the aberrant T-cell clone presented with cutaneous manifestation and an increased serum IgE level was demonstrated in 5 (serum IL-4 and IL-5 levels were not measured in this study).[9] In contrast, the majority of studied HES patients had detectable TCR clonal rearrangement in one recently published report.[10] TCR clonal rearrangement by PCR was seen in 10 out of 14 patients (71.4%), but only one case presented with an aberrant T-cell immunophenotype by flow cytometry (i.e., $CD3\text{-}CD4^+$). It should be underlined that authors used very sensitive diagnostic methods for TCR clonal detection compared to those used by others.[8,9,11] In conclusion, the results demonstrated by Galimberti *et al.*,[10] suggested that T-cell prolif-

Table 3. Study group characteristics.

Parameter	Non-clonal TCR	Clonal TCR
Number of patients	24	18
Gender: male/female	33%/67%	56%/44%
Median age (range)	59 (17-84) yrs	54 (18-77) yrs
Median WBC[a] count ($\times10^9$/L)	14.1 (5.9-121.0)	9.6 (5.4-68.9)
Median hemoglobin (g/dL)	13.1 (8.2-16.1)	13.5 (11.8-16.0)
Median platelet count ($\times10^9$/L)	303 (104-891)	230 (90-481)
Median AEC[b] ($\times10^9$/L)	6.1 (1.5-99.0)	3.4 (1.5-31.6)
Median ALC[c] ($\times10^9$/L)	2.0 (1.0-5.6)	2.2 (1.0-13.0)
CD4/CD8 ratio	1.7 (0.4-10.0)	1.3 (0.5-6.2)
Median eosinophils in bone marrow (%)	30 (11-50)	35 (14-64)
Median serum IgE (IU/mL)[d]	78 (4.0-1795)	147 (6.7-13966)
Serum IgE >N[d]	50%	56%
Median serum B12 vitamin (pg/mL)[d]	250 (149-1918)	385 (178-781)
Serum B12>N[d]	12%	0%
Median serum IL-5 (pg/mL)[d]	9 (0-65)	13 (0-69)
Serum IL-5 >N[d]	21%	22%
Median serum IL-4 (pg/mL)[d]	2 (0-15)	2 (0-13)
Serum IL-4>N[d]	29%	17%
Median tryptase level (ng/mL)[d]	4(2-399)	9 (2-190)
Tryptase>N[d]	25%	28%

[a]WBC:-white blood cell; [b]AEC-absolute eosinophil count; [c]ALC-absolute lymphocyte count; [d]normal ranges (N): IgE <100 IU/mL; for vitamin B12 level: 157-1057 pg/mL; tryptase level: <11.5 ng/mL; IL-4 and IL-5: <5.8 pg/mL and <25.8 pg/mL, respectively, n.s. not significant, 0-undetectable levels of IL-4 and IL-5.

Table 4. Summary of T-cell HES studies.

Parameter	Roufosse *et al.*[7] N=9 (%)	Simon *et al.*[8] N=60	Roche-Lestienne *et al.*[11] N=35	Galimberti *et al.*[10] N=14	Vaklavas *et al.*[9] N=99	This report N=42
Clonal T cells	44	27	30	71.4	14	42.8
LPD	Yes	Yes	ND	ND	Yes	Yes
Population TCR^+/FIP^+	ND	ND	NO	Yes	NO	Yes
Serum IL-5	↑↑↑*	ND	↔	↑	ND	↔
Serum IgE	↑↑	↑↑	ND	ND	↑↑	↑
Organ involvement	Skin	Skin	ND	Skin, spleen	Skin	Skin

*LPD: lymphoproliferative disorder; ND: no data, ↑slightly elevated; ↑↑markedly elevated; ↑↑↑significantly high; ↔normal range; *in 1 out of 4 patients.*

eration is probably not the *primum movens* of sustained hypereosinophilia, but rather an epiphenomenon in the majority of cases. A high proportion of the patients who tested positive for TCR clonal rearrangement in our study did so for two main reasons: (i) high sensitivity of the molecular technique used in our study, (ii) the patient population who fulfilled the stringent criteria for hypereosinophilic syndrome. Results of T-cell studies in patients with HES are summarized in Table 4.

In our current study, the F/P fusion transcript was detected in 2 patients with clonal TCR rearrangement. Both patients responded promptly to low doses of imatinib and remained in complete hematologic and molecular remission. TCR analysis was not repeated after imatinib was administered. The *FIP1L1-PDGFRA* fusion transcript was undetectable in 6 patients with T-cell clone in one recently published study.[9] On the other hand, Galimberti *et al*. revealed the presence of the *FIP1L1-PDGFRA* fusion transcript in 4 out of 10 patients with TCR clonality. It is worth noting that clonal T cells were no longer detected after the disappearance of *FIP1L1-PDGFRA* following imatinib.[10] It was demonstrated that F/P mutation arises in a pluripotent stem cell and it was detected in different cells including T lymphocytes.[12] Moreover, some data showed the association of the F/P fusion gene with T-cell lymphoma.[13,14]

Detection of the abnormal T-cell immunophenotype and/or TCR clonality in patients with long-term hypereosinophilia may precede the development of T-cell lymphoma.[8] This was also seen in one patient in our study. However, there was a single report of a patient with hypereosinophilia and clonal $CD3^-CD4^+$ lymphocytosis for more than 13 years who did not develop lymphoma[15] suggesting that the disease had an indolent course in at least some cases. It is noteworthy that steroids remained a treatment of choice in these patients.

The therapy may have lowered the number of peripheral eosinophils and the absolute numbers of phenotypically abnormal T cells. On the other hand, there were also patients who responded to corticosteroid treatment with a decrease in eosinophil level, but an aberrant T-cell population remained unaffected.[7] It seems likely that progression of L-HES to overt lymphoma resulted from partial deletion of chromosome 6q in $CD3\text{-}CD4^+$ cells; however, this association was demonstrated only in one patient.[16] One patient from our study group presented with an abnormal T-cell population, TCR clonal rearrangement and t(6;11)(q21;q23). This patient progressed to peripheral T-cell lymphoma after three years of eosinophilic prodrome.[5] This case indicated the need for close cytogenetic monitoring in patients with L-HES.

We found no significant differences in clinical or serological features when we compared patients with clonal and non-clonal TCR gene rearrangements, except for the decreased CD4:CD8 ratios in patients with clonal TCRs. Patients with detectable TCR clonal rearrangement had increased serum IL-5, IgE and lymphocytosis, but these values did not reach statistical significance. We proved that T-cell abnormalities in HES did not necessarily correlate with blood lymphocytosis.

In addition, the occurrence of the aberrant T-cell population may not always indicate the presence of clonal TCR and viceversa. In addition to the typical case with T-cell clonality and aberrant T-cell population in peripheral blood, for which diagnosis of L-HES appears certain, the presence of TCR clonal rearrangement without abnormal TCP raises the problem of interpretation. According to a recently published review the demonstration of isolated clonal TCR rearrangement seems to be insufficient to classify patients as having L-HES.[17]

It should, however, be noted that clonal T cells are found in peripheral blood of the elderly,[18] and in patients with severe viral infection.[19] This implies that results of molecular clonality studies should always be interpreted together with clinical and morphological features. We would like to underline that more than 60% of TCR positive cases (11/18) in our study revealed TCR clonality in two or more TCR loci and this finding is in favor of true clonality (*A. Langerak, personal communication, February 16, 2009*).

We did not reveal TCR clonality in healthy subjects matched for age. To our knowledge, we present up-to-date the largest prospective analysis so far of T-cell abnormalities in patients fulfilling the HES criteria using very sensitive and modern diagnostic tools. Although in mature T-cell malignancies changes of antigen expression are frequently observed, flow cytometry cannot be applied for T-cell clonality assessment.Thanks to international co-operative BIOMED-2 efforts, PCR analysis of *TCR* gene rearrangements became a gold standard of

T-cell clonality detection.[20] The drawback of this PCR based approach for TCR clonality detection is that it is not quantitative. In summary, T-cell clone was identified in a significant proportion of patients with HES. These cases may progress into overt T-cell lymphomas, therefore, close monitoring of these patients is indicated. The timing of treatment is controversial and therapeutic approaches should be designed on a case-by-case basis.

Authorship and Disclosures

GH planned the research, collected and analyzed data, wrote the manuscript; AW collected data; JDS performed immunophenotyping analysis; MM performed molecular analysis; SKK critically reviewed the manuscript.

The authors reported no potential conflicts of interest.

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