A combination of cytometry, cytogenetic analysis, fluorescence in situ hybridization and reverse transcriptase polymerase chain reaction for establishing clonality in cases of persisting hypereosinophilia

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To evaluate the frequency of clonal abnormalities in patients with unexplained persisting eosinophilia we analyzed 40 patients (27 males, 13 females) using cytometry, cytogenetic analysis, interphase fluorescence in situ hybridization (FISH), and reverse transcriptase polymerase chain reaction (RT-PCR). Cytogenetic analysis revealed clonal abnormalities in five patients (four of whom were males) including t(8;9)(p21;p24), ins(9;4)(q34;q12q31), del(6)(q24), and trisomy 8 (n=2). RT-PCR confirmed a PCM1-JAK2 fusion underlying the t(8;9). FISH analysis suggested a rearrangement involving PDGFRA in the ins(9;4). A FIP1L1-PDGFR fusion gene was identified in four male patients by interphase FISH and RT-PCR. These methods in combination demonstrated clonality in 8/40 patients (20%) with a male predominance (6/8; 75%).

Key words: hypereosinophilia, CMPD, PDGFRA, cytogenetics, PCR.

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Hypereosinophilia most frequently represents a reactive condition associated with autoimmune disorders, asthma, allergies, infectious diseases, and a number of other rare non-hematologic and hematologic malignancies such as Hodgkin's lymphoma or T-cell non-Hodgkin's lymphoma. Persistent eosinophilia is only rarely of clonal origin and usually occurs in cytomorphologically overlapping disorders: idiopathic hypereosinophilic syndrome (HES), chronic eosinophilic leukemia (CEL), systemic mast cell disease, chronic myeloid leukemia, chronic myeloproliferative disorders (CMPD) other than chronic myeloid leukemia and chronic myelomonocytic leukemia. HES is a heterogenous disorder characterized by hypereosinophilia exceeding 1500 eosinophils/µL peripheral blood for more than 6 months and tissue infiltration by eosinophils ultimately leading to end organ damage or dysfunction. For yet unknown reasons HES is more common in males and occurs predominantly between the age of 20 and 50 years. According to the WHO classification CEL is defined by an increase of >2% blasts in the peripheral blood or of >5%-19% in bone marrow or evidence of clonality. Chromosomal abnormalities in CEL and eosinophilia-associated CMPD or myelodysplastic syndromes include a variety of balanced translocations, most commonly involving the chromosome bands 5q31-35 and 8p117, deletions of 20q, trisomy 8, and monosomy 7. Occasional reciprocal balanced translocations, e.g. t(1;4)(q44;q12)1, t(5;11)(p15;q13), t(8;9) (p22;p23), or t(5;9)(q32;q35), have also been identified. Analysis of individuals who present with eosinophilia-associated CMPD and acquired reciprocal chromosomal translocations has revealed diverse tyrosine kinase fusion genes, most commonly involving the tyrosine kinase receptors PDGFRA, PDGFRB, FGFR1 and JAK2. The cytogenetically invisible FIP1L1-PDGFR fusion gene results from an interstitial deletion on chromosome 4q12 and is the most frequent molecular rearrangement in CEL. It is usually detected by interphase fluorescence in situ hybridization (FISH) with differentially labeled probes for CHIC2, FIP1L1, and PDGFRA or reverse transcriptase polymerase chain reaction (RT-PCR). Eosinophilia is a frequent condition in the routine clinical setting. Following the identification of cytogenetic and molecular markers of clonality some patients with previously unexplained hypereosinophilia must be classified as having CEL. We here report a retrospective study on 40 patients with persisting unexplained eosinophilia combining cytometry, cytogenetic analysis, interphase FISH, and RT-PCR to determine the value of these methods for demonstrating clonality in such cases.

Design and Methods

We performed a retrospective study on 40 patients (27 males, 13 females) with unexplained peripheral hypereosinophilia >1500/µL persisting for more than 6 months. The median age of these patients was 60 years (range, 19-89 years). Their median white blood count was 14.7×10³/µL (range: 6-91×10³/µL) and the median percentage of...
Eosinophils in the peripheral blood was 50% (range: 20%-70%). Cytomorphologic evaluation was performed in all cases on Pappenheim stains of peripheral blood and bone marrow. Mast cells were identified by toluidine blue staining. In 37/40 cases cytogenetic analysis was performed according to standard procedures. The International System for Human Cytogenetic Nomenclature was used for the designation of the chromosomes.

Interphase FISH for the \textit{CHIC2} deletion and for the \textit{FIP1L1-PDGFRA} fusion as well as for other \textit{PDGFRA} rearrangements was performed in all cases. Three bacterial artificial chromosome (BAC) probes (RPCI11-120K16, RPCI-3H20, and RPCI11-24O10) were used, as published by Gotlieb et al. and Vandenberghe et al. These probes were kindly provided by Reiner Siebert, University of Kiel. BAC 3H20 (mapping between \textit{PDGFRA} and \textit{FIP1L1}), BAC 120K16 (mapping centromeric to \textit{FIP1L1}), and BAC 24O10 (mapping telomeric to \textit{PDGFRA}) were used in double-color experiments to study the cryptic deletion leading to a \textit{FIP1L1-PDGFRA} fusion in all cases. At least 100 cells were evaluated in every sample. Seventeen cases were screened for the \textit{FIP1L1-PDGFRA} fusion by RT-PCR. RT-PCR for the \textit{PCM1-JAK2} fusion gene was performed as previously described. In addition, all cases were analyzed with a FISH probe (Vysis) for \textit{BCR-ABL} to exclude a diagnosis of chronic myeloid leukemia. Depending on the cytogenetic results, in some cases FISH analyses with additional probes were performed.

### Results and Discussion

Cytomorphologic information on bone marrow was available in eight of nine patients with evidence of clonal aberrations. Bone marrow cellularity was increased in seven of eight patients. The percentage of bone marrow blasts was ≤5% in five patients and between 5-10% in three patients. The percentage of eosinophils ranged between 10% and 30%. Mast cells were increased in five of seven patients (UPN 1, 2, 3, 5, and 6) for whom bone marrow cytomorphology was available. A normal karyotype was seen in 31 of 37 patients (85%). Clonal aberrations were observed in six of 37 (15%) patients (male, n=5, 83%). These aberrations were a t(8;9)(p21;p24), an ins(9:4)(q34;q12q31), a del(6)(q24), trisomy 8 (n=2), and loss of chromosome Y in a 90-year old patient. As loss of chromosome Y is not

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Clinical data</th>
<th>Karyotype</th>
<th>Interphase FISH</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>s-AML following CEL</td>
<td>male</td>
<td>64</td>
<td>diagnosis of CEL 1998; s-AML following CEL</td>
<td>47,XY,+8 [20]</td>
<td>CHIC2 deletion positive</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CEL</td>
<td>male</td>
<td>61</td>
<td>CEL following T-NHL in CR</td>
<td>46,XY [25]</td>
<td>CHIC2 deletion positive</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>35</td>
<td>diagnosis 5/2002; no therapy, stable disease</td>
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</tr>
<tr>
<td>4</td>
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<td>41</td>
<td>n.a.</td>
<td>46,XY [25]</td>
<td>CHIC2 deletion positive</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>s-AML following CEL</td>
<td>female</td>
<td>72</td>
<td>diagnosis 4/04; s-AML after imatinib for 5 weeks 6/04</td>
<td>46,XX,ins(9;4)(q34;q12q31) [5]</td>
<td>PDGFRA splitting n.a.</td>
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<tr>
<td>6</td>
<td>MPS/MDS</td>
<td>female</td>
<td>84</td>
<td>diagnostis 6/04; palliative chemotherapy with HU 6/04-7/04</td>
<td>46,XX,+8 [17]</td>
<td>CHIC2 negative negative</td>
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</tr>
<tr>
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<td>diagnosis 11/02; allo-PBSCT 8/03</td>
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<td>77</td>
<td>diagnosis 1/04</td>
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<td>CHIC2 negative negative</td>
<td></td>
</tr>
</tbody>
</table>

s-AML: secondary acute myeloid leukemia; MDS: myelodysplastic syndrome; MPS: myeloproliferative syndrome; CR: complete remission; T-NHL: T-cell non-Hodgkin’s lymphoma; PBSCT: peripheral blood stem cell transplantation; HU: hydroxyurea; n.a.: not available.

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Table 1. Clinical data, cytogenetic, and molecular findings in eight patients with persisting unexplained hypereosinophilia and clonal aberrations.

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necessarily associated with hematologic malignancies in elderly men, this case was not included in the cohort with proven hematologic disorders.

A CHIC2 deletion was demonstrated by interphase FISH in four of the 40 patients (UPN 1-4). The FIP1L1-PDGFRA fusion transcript was confirmed in all four cases by RT-PCR. Three patients had a normal karyotype, whereas one had trisomy 8 (UPN 1). All four patients were males and were 33, 33, 60, and 63 years old. In the patient with ins(9;4)(q34;q12q31), a 72-year-old female, interphase FISH analysis demonstrated separation of one FIP1L1 and one PDGFRA signal. One PDGFRA signal was localized on the derivative chromosome 1, whereas the FIP1L1 signal remained on chromosome 4. This suggested a yet unknown rearrangement involving PDGFRA.

RT-PCR analysis of the case with t(8;9)(p21;p24), a 65-year-old male patient, demonstrated the PCM1-JAK2 fusion gene. Details concerning this patient have been published elsewhere.16

So far, cellularity in HES or CEL could only be proven by conventional cytogenetic analysis showing an increase of blasts4 or by X-linked DNA analysis in female patients.17 The latter is, however, only of limited value, because more than 90% of HES patients are male. The recent identification of recurrent gene fusions clarifies the diagnosis in some cases of HES and suspected CEL.1,4,7,15 The most commonly involved genes are the receptor tyrosine kinases PDGFRα, PDGFRβ, and FGFR1 and the non-receptor tyrosine kinase JAK2. The FIP1L1-PDGFRA fusion gene currently represents the third most frequently detected genetic aberration in CMPD beside BCR-ABL and the recently identified JAK2 V617F mutation.1

It was shown that a subset of HES/CEL patients who present with rearrangements of PDGFRα and PDGFRβ have rapid and complete clinical and hematologic responses to treatment with imatinib.1,2,3,15 Responses lasting more than 3 months were reported by Cools et al. in nine of 11 FIP1L1-PDGFRA positive patients,1 and by Apperley et al. in all four patients with CEL and rearrangements of PDGFRβ who were treated with imatinib.16 Klion et al. recorded molecular remission in five of six patients and reversal of myelofibrosis in all seven patients with the myeloproliferative variant of HES with imatinib treatment.20 In the analysis by Vandenbreghe et al. imatinib induced a complete molecular remission in two of three of evaluable cases with FIP1L1-PDGFRA-positive CEL.21

Because of the clinical heterogeneity of HES and the low number of cytogenetically and molecularly analyzed cases, the real frequency of the fusions genes in this syndrome is not yet established.16 However, the excellent therapeutic option provided by imatinib makes a prompt, correct diagnosis of CEL mandatory.1,4,7,15 The 10% frequency of FIP1L1-PDGFRA-positive CEL found in this study is similar to that found by Pardanani et al., who identified the CHIC2 deletion in 12% of 89 patients with eosinophilia.4 In our cohort the combination of cytology, cytogenetics, interphase FISH and RT-PCR led to the detection of clonal aberrations in 20% of cases with persisting unexplained hypereosinophilia. However, this complex battery of diagnostic investigations should only be applied after exclusion of underlying conditions causing reactive eosinophilia according to the classical diagnostic algorithm.

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