Bone marrow morphology is a strong discriminator between chronic eosinophilic leukemia, not otherwise specified from reactive idiopathic hypereosinophilic syndrome

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This is a Bone Marrow Pathology Group Study

Running title: BM morphology of CEL,NOS/idiopathic HES

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Abstract

Chronic eosinophilic leukemia, not otherwise specified can be difficult to distinguish from idiopathic hypereosinophilic syndrome according to the current World Health Organization guideline. To examine if BM morphological features might aid in the differential diagnosis of these two entities, we studied a total of 139 patients with a diagnosis of chronic eosinophilic leukemia, not otherwise specified (n=17) or idiopathic hypereosinophilic syndrome (n=122). As a group, abnormal BM morphological features, resembling myelodysplastic syndromes, myeloproliferative neoplasm or myelodysplastic/myeloproliferative neoplasm were identified in 40/139 (27%) patients, 16 (94%) chronic eosinophilic leukemia and 24 (20%) hypereosinophilic syndrome. Abnormal bone marrow correlated with an older age (p<0.001), constitutional symptoms (p<0.001), anemia (p=0.041), abnormal platelet counts (p=0.002), organomegaly (p=0.008), elevated LDH (p=0.005), an abnormal karyotype (p<0.001), as well as the presence of myeloid-neoplasm related mutations (p<0.001). Patients with abnormal bone marrow had a shorter survival (48.1 months vs. not reached, p<0.001), which was independent of other confounding factors (p<0.001). Similar survival significance of abnormal bone marrow was also observed in hypereosinophilic syndrome patients alone. In summary, most chronic eosinophilic leukemia, not otherwise specified and a proportion of cases of idiopathic hypereosinophilic syndrome show abnormal bone marrow features similar to those encountered in patients with myelodysplastic syndromes, myelodysplastic/myeloproliferative neoplasm or BCR-ABL1-negative myeloproliferative neoplasm. Of patients who are currently considered to have idiopathic hypereosinophilic syndrome, abnormal bone marrow is a strong indicator of clonal hematopoiesis. Like other myeloid neoplasms, bone marrow morphology should be one of the major criteria in the assessment patients with chronic eosinophilic leukemia, not otherwise specified or clonal hypereosinophilic syndrome versus those with truly reactive idiopathic hypereosinophilic syndrome.

Key words: Chronic eosinophilic leukemia, not otherwise specified, idiopathic hypereosinophilic syndrome, bone marrow, morphology, mutation, karyotype, survival
Introduction

Hypereosinophilia (HE) is defined by the presence of $\geq 1.5 \times 10^9/L$ peripheral blood (PB) eosinophils and may be reactive, neoplastic or idiopathic (1-3). Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) (4) is a myeloproliferative neoplasm (MPN), characterized by an expansion of eosinophils but lacking well-defined molecular genetic alterations such as \textit{BCR-ABL1} and rearrangements of \textit{PDGFRA, PDGFRB, FGFR1 and PCM1-JAK2}. In idiopathic hypereosinophilic syndrome (HES), there is tissue/organ damage related with eosinophilic infiltrate/activation, but the cause of HE is unknown.

Due to their substantial overlapping features, idiopathic HES is currently described alongside CEL, NOS in the current World Health Organization (WHO) classification monograph (4); this categorization remains largely unmodified in the 2016 WHO revision (5). According to the current guidelines, CEL, NOS, can only be reliably separated from idiopathic HES by the presence of increased blasts in bone marrow (BM) and or peripheral blood (PB), or proof of clonality. Clonality had been mainly determined by chromosomal analysis or testing mutations well known to occur in MPN, such as \textit{JAK2, MPL, CALR} or \textit{KIT}. However, the latter mutations are uncommon in these eosinophilic diseases (6, 7). More recently, next-generation sequencing (NGS) approaches have been applied to these eosinophilic disorders. Anderson and colleagues conducted whole-exome sequencing of 9 idiopathic HES patients (8) and identified somatic missense mutations in 3 patients. The mutations they found involved spliceosome gene \textit{PUF60} and the cadherin gene \textit{CDH17}. More recently, by using NGS with a gene panel targeted to somatic mutations commonly associated with myeloid neoplasms, we detected the presence of mutations at a relatively high variant allele frequency ($\geq 10\%$) in 25-30\% of idiopathic HES (7). These mutations mostly occurred in genes involving in DNA methylation and chromatin modification, such as \textit{AXSL1, TET2, EZH2, and DNMT3A}. While such mutations would imply that some idiopathic HES are clonal stem cell neoplasms, they have also been reported in some aging individuals lacking evidence of a myeloid neoplasm (9) (10), mandating caution in the use of mutations as definitive evidence of a neoplastic myeloid process. On the other hand, the detection of mutations by NGS study relies on the testing panel used, which may vary in the number of genes sequenced as well as the depth of sequencing.

Although some studies have suggested that abnormal eosinophil morphology is associated with clonal eosinophilia, it is generally felt that such cytologic abnormalities lack sufficient specificity to differentiate a neoplastic process from a reactive eosinophilia (4) (11-14). As a result, BM morphology is not an
integrate part in the diagnosis and classification of hypersinophilia. This is in apparent contrast with other myeloid neoplasms, where abnormal BM features play a major role in establishing the diagnosis. In particular, BM morphology represents a “gold standard” in the diagnosis of myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative (MDS/MPN) neoplasms. Of MPNs, morphology has become one of the major criteria in the WHO classification (2016) (5) of essential thrombocytopenia, polycythemia vera, and primary myelofibrosis. In contrast, in the case of CEL, NOS or of HES with clonal eosinophilia, there is limited published information in relation to BM morphology.

In our previous study(7), with the knowledge of molecular genetic information, we observed some BM features that appeared to be preferentially present in cases with molecular genetic alterations. In this study, we conducted a thorough review of BM morphology of a large series of CEL, NOS and idiopathic HES cases collected from 7 large medical centers in the United States using a defined set of morphologic criteria, blinded to the original diagnosis and molecular genetic data. We used the morphologic features to define an “abnormal” BM morphology, and correlated the morphological results with clinical and laboratory features, cytogenetics, mutation data, and patient outcomes. We sought to determine if morphology can be utilized in the distinction of CEL, NOS and clonal HES from truly reactive idiopathic HES.

METHODS

Patients

Cases were collected from MD Anderson Cancer Center, Stanford University Medical Center, Cleveland Clinic, Massachusetts General Hospital, Weill-Cornell Medical College, the Hospital of the University of Pennsylvania and the University of New Mexico between year 2005 and 2014. All included cases had persistent hypereosinophilia (≥1.5x10⁹/L) that were not acute leukemias, chronic myeloid leukemia (CML), MDS, chronic myelomonocytic leukemia (CMML), systemic mastocytosis, or cases with rearrangements of PDGFRα, PDGFRβ, FGFR1 or PCM1-JAK2. For idiopathic HES, every patient had “end-organ damage” according to the definition by the working group on eosinophil disorders (15). Lymphocytic/T-cell variant HES (1), was excluded based on the identification of aberrant T cells by flow cytometry with or without TCR gene rearrangement PCR studies. Clinical information was retrieved from the electronic medical records. This study was approved by the Institutional Review Boards of all participating institutions.

Bone Marrow Morphologic and Histologic Assessment
BM morphology was assessed for the following parameters (Table 1): cellularity, megakaryocyte numbers, morphology and distribution, fibrosis, dysgranulopoiesis, dyserythropoiesis, myeloid:erythroid (M:E) ratio, and eosinophil morphology. PB smears were also reviewed for eosinophil morphology and evidence of dysgranulopoiesis. Hypercellularity was defined by a cellularity at least 20% higher than the age-appropriate cellularity, and overall ≥70% in patients age 50-60 years; ≥60% in patients >60 years; and ≥90% in patients <30 years of age. Megakaryocyte morphology was recorded as predominantly MDS-like (small with hypolobated/non-lobated nuclei or separated nuclear lobes), MPN-like (medium to large megakaryocytes with hyperlobulated, hyperchromatic, or bulbous nuclei, often with clustering and increase in numbers), mixed MDS and MPN-like, or within normal limits (WNL). In order to strictly define dysgranulopoiesis and dyserythropoiesis, the features had to be seen in ≥20% of cells of the assessed lineage. Myelofibrosis grade was assessed according to the European Bone Marrow Fibrosis Consensus criteria(16). For eosinophil morphology, abnormal features were markedly abnormal granulation (hypogranulation or uneven granulation), cytoplasmic vacuoles, abnormal nuclear lobation (nonlobated or multilobated), unusually large size or markedly increased immature forms. These features had to be observed in at least 20% of the eosinophils on the BM smears, since mild nuclear hypersegmentation and mild abnormal granulation in the PB can be seen with eosinophil activation(17) or treatment with hydroxyurea(18). All cases were assessed by the members from the respective institution using the same set of criteria, which were developed by the BMP members after reviewing representative cases as a group. The features were reassessed by one observer (SAW); cases with borderline morphologic abnormalities or discrepancy were again centrally reviewed by the group and scored by consensus. Approximately 10% of cases (n=13) had some disagreements on some of the parameters, but all members agreed on “abnormal” or “not abnormal” morphology for all cases. The disagreements mainly related to eosinophilic morphology, since the criteria were not previously defined; disagreements on scoring megakaryocyte morphology were present in a smaller subset of cases and centered on if the features were MDS-like or mixed MDS/MPN like. All morphology review was blinded to clinical features, molecular genetic data, original diagnoses and patient outcomes.

**Cytogenetics, Fluorescence in situ hybridization and Molecular Testing**

Conventional cytogenetic analysis was performed on G-banded metaphase cells prepared from unstimulated BM aspirate cultures using standard techniques. Twenty metaphases were analyzed and the results were reported using the International System for Human Cytogenetic Nomenclature. Fluorescence in situ hybridization and/or molecular genetic methods for detecting *BCR-ABL1*,
PDGFRA, PDGFRB, or FGFR1, were performed at respective institutions as part of the routine clinical work-up if indicated.

**Targeted next-generation sequencing**

Targeted next-generation sequencing (NGS) had been performed on 57 patients previously(7) and was performed on an additional 19 patients on DNA samples extracted from frozen unfractionated BM cells collected at the time of diagnosis, using the same method we described previously(7). The coding sequences of 44 genes (sequencing >90% gene coding regions), including ABL1, ASXL1, BCOR, BRAF, CALR, CBL, CEBPA, DNMT3A, ETV6, EZH2, FAM5C, FLT3 (ITD and TKD), GATA1, GATA2, HNRNPK, IDH1, IDH2, IKZF1, JAK1, JAK2, KDM6A, KIT, KRAS, MPL, NFE2, NOTCH1, NPM1, NRAS, PHF6, PTPN11, RAD21, RUNX1, SF3B1, SH2B3, SMC1A, SMC3, STAG2, SUZ12, TET2, TP53, U2AF1, WT1, and ZRSR2, were performed specifically for this study. Variant calling was performed with Illumina MiSeq Reporter Software 1.3.17. using human genome build 19 (hg 19) as a reference.

**Statistical Analyses**

For continuous variables, data are reported as median and range. For nominal variables, data are reported as the number of patients unless otherwise specified. Survival was calculated from the date of diagnosis to the date of last follow-up or death not attributable to causes that were clearly not associated with disease (car accident, suicide et al). Patients who received hematopoietic stem cell transplant (HSCT) were censored at the time of the procedure. Distribution of survivals was estimated by Kaplan-Meier curves. Multivariable analysis was performed using Cox regression model. Fisher’s exact and Chi-square tests were used for categorical comparisons. All p values are two-tailed and were considered significant when <0.05. No adjustments for multiplicity were made.

**Results**

**Patients, clinical data and molecular genetic data**

A total of 139 patients were included in the study, either meeting the criteria of CEL, NOS (17 patients) or idiopathic HES (122 patients) after applying the exclusion and inclusion criteria and had sufficient material for morphological assessment. An abnormal karyotype was seen in 16/17 patients of the CEL, NOS patients; the detailed karyotype information on these cases was published previously (7). In brief, 5 patients had a complex karyotype, 1 had 2 cytogenetic abnormalities, 9 had a single abnormality, and
1 was identified by FISH to have a del(9p) abnormality. Three patients had ≥5% BM blasts, including 1 patient with a normal BM karyotype who was also classified as CEL, NOS according to the WHO Classification criteria. The clinical and laboratory features of these patients as a group are shown in Table 2.

NGS was performed in 76 patients. In total, mutations were found in 21/76 patients (27.6%). The mutation data and frequency are shown in Figure 1. In brief, the mutations in decreasing frequency were ASXL1 (7/76, 9.2%); TET2 (5/76, 6.6%); EZH2 (5/76, 6.6%); DNMT3A (5/76, 6.6%), NOTCH1 (4/76, 5.3%), SETBP1 (3/76, 4.0%); CBL (2/76, 2.6%); U2AF1 (2/76, 2.6%), and one each (1.3%) for TP53, JAK2 exon13, NRAS, BCOR, GATA2, CSF3R and ETV6. Two or more mutations were found in 8/76 (11%) patients. Overall, 18/70 (25.7%) tested idiopathic HES and 3/6 tested CEL, NOS (50%) had mutations identified.

**Bone Marrow Morphology**

BM morphology was evaluated in conjunction with PB smears, blinded to all clinical, laboratory, molecular genetic data and patient outcome. Increased BM eosinophils were seen in the majority of the cases, comprising a median of 21% (range 4-91%) of BM cells; only 3 patients had <10% eosinophils in the BM. In two-thirds of the cases (71%), BMs were unremarkable except for increased eosinophils (Figure 2). In contrast, in one-third of cases (29%), besides the increased eosinophils, a number of other BM changes were observed; these are shown in Table 1. The most common abnormalities (Figure 3) were BM hypercellularity, abnormal eosinophils, abnormal megakaryocytes, a markedly elevated M:E ratio ≥10; moderate to marked fibrosis, dysgranulopoiesis, and dyserythropoiesis.

Cases were considered to be morphologically abnormal if they showed overtly abnormal megakaryocytes (resembling MDS or MPN), significant dysgranulopoiesis or dyserythropoiesis, or increased (≥5%) BM blasts. These included 25 cases with abnormal megakaryocytes, most of which showed MDS-like morphology (Supplementary Table 1). Of these 25 cases, 15 cases also showed abnormal eosinophils; 3 had ≥5% BM blasts, 6 had MF2 or MF3 myelofibrosis; 19 had hypercellularity, 6 had dysgranulopoiesis, and 8 showed dyserythropoiesis. Of patients whose BM did not show abnormal megakaryocytes or had insufficient megakaryocytes for assessment, 4 patient BMs were concluded to be abnormal, including 3 with marked dysgranulopoiesis and 1 with marked dyserythropoiesis (2 of these also showed abnormal eosinophils; 2 with a hypercellularity and 1 with MF2 fibrosis). An additional 11 cases were scored as “abnormal” because of the presence of at least two of the following changes, including BM hypercellularity (n=10), MF2 or MF3 fibrosis (n=6), abnormal eosinophils (n=4); M:E ratio >10 (n=1), markedly decreased/near absence of megakaryocytes (n=2),
and 1 with a subset MDS-like megakaryocytes (see supplemental Table 1). There were also increased macrophages/histiocytes, stromal cells, vessels, and a disarray distribution of the BM cellular elements in some of these cases. These 11 cases were centrally reviewed, and one example is shown in Figure 3.

Thus, 40/139 cases (29%) were considered to have abnormal BM morphology and 99 had either normal morphology or only 1 morphologic abnormality that did not include significant dysplasia, abnormal megakaryocytes, or excess blasts. In total, 16 of 17 (94%) CEL, NOS and 24 of 122 (22%) of the HES cases were morphologically abnormal. If the current WHO definitions of CEL, NOS and HES were used to anchor the reviewed cases as “true positives” for each diagnosis, abnormal morphology would have a sensitivity 94.1% (95% CI 71.3-99.8%) and 84.7% (95% CI 77.8-90.2%) specificity for CEL, NOS.

**Correlation of BM Morphology with Clinical Features**

The 40 patients with an abnormal BM had clinical presentations that differed from the 99 patients who lacked significantly abnormal BM findings (Table 2). The patients with abnormal BM were older and presented with a higher WBC and a higher absolute eosinophil count. These patients also presented with lower hemoglobin levels and more commonly abnormal platelet counts (either thrombocytopenia or thrombocytosis)(p=0.002). Clinically, more patients with abnormal BM morphology presented with constitutional symptoms (19/40 vs 17/97, p<0.001), but fewer allergy/hypersensitivity (2/40 vs 30/97, p<0.001); cough, bronchitis, or pneumonitis (1/40 vs 24/97, p<0.001); gastrointestinal symptoms (5/40 vs 28/97, p=0.049); and cardiac insufficiency, myocardial infarction or pericardial effusion (2/40 vs 20/97, p=0.012). Skin rashes or various forms of dermatitis were common in both groups of patients. An abnormal BM also correlated with more frequent organomegaly and elevated LDH (Table 2).

**Correlation of BM Morphology with Molecular Genetic Data**

Of 17 patients with abnormal cytogenetics and/or increased BM blasts (CEL, NOS by the current WHO criteria), 16 had an abnormal BM. The one CEL-NOS patient lacking abnormal BM morphology was a 64 year old female who presented with chest pain, and was found to have elevated troponin, pericardial and pleural effusions. This patient had del(16)(q23q24) and BM showed 40% eosinophils, but otherwise had normal morphology. FISH for *CBFB and CHIC2* were both negative. The patient was alive after 24 months of follow-up.
Mutations by NGS study were more frequently seen in patients with an abnormal BM (12/20 vs 11/56, p=0.002). Mutations involving 2 or more genes were significantly more common in patients with an abnormal BM (7/20 vs 1/56, p<0.001). Moreover, TP53, EZH2, SETBP1, NRAS, JAK2 exon13 and CSF3R were only seen in patients with an abnormal BM. Of patients who lacked abnormal BM findings, mutations included single gene mutation in TET2 (n=2), DNMT3A (n=3, 2 with 5-10% variant allele frequency-VAF); ASXL1 (n=1), CBL (n=1), and NOTCH1 (n=1). The only patient who had 2 mutations (TET2, VAF 25% and DNMT3A, VAF 15%) without abnormal BM findings, was a 24 year-old man who presented with fever and chest pain, dizziness and sensory abnormalities in both hands. The patient was found to have abnormal MRI findings in brain and lung, likely due to eosinophilic infiltrates. He showed some response to corticosteroids, but did not tolerate imatinib. He was alive at 28 months of follow-up.

**Correlation of BM Morphology with Outcome data**

These patients were treated with various agents recommended for patients with idiopathic HES/CEL, NOS, including corticosteroids with or without interferon, hydroxyurea for cytoreduction, cyclosporine, methotrexate, and alemtuzumab. Tyrosine kinase inhibitors (TKI), mostly imatinib and some dasatinib, were used in 54/126 (43%) patients over the course of the disease. Hypomethylating agents (HMAs), single agent chemotherapy, and high-dose chemotherapy were also used in some patients when disease showed progression or was refractory to other treatment modalities. A total of 7 patients received hematopoietic stem cell transplant (HSCT).

The median follow-up for all 139 patients was 38.9 months (range 0-405.3 months). Three unrelated deaths, due to suicide, car accident and diffuse large B cell lymphoma, were censored at the time of death. Of 40 patients with an abnormal BM, there were 18/40 deaths, including 3 due to AML progression. The other causes of death included infection, bleeding and organ failure. Among the 5 patients in this group who received HSCT, 4 were alive and one died of disease recurrence. In contrast, of 99 patients without abnormal BM morphology, none experienced AML progression. There were 8 deaths in this group, including 4 due to myocardial infarctions or heart failure, 2 due to chronic obstructive lung disease, one due to the complication of bone fracture as a result of long-term steroid use, and another of unknown cause. Both patients who received HSCT were alive at the last follow-up. The median overall survival for patients with an abnormal BM was 48.1 months (range 1-120.1 months), significantly inferior to patients with a normal BM (not reached, range 0-277.2) (Kaplan Meier Log rank, p<0.001) (Figure 4A). Survival comparison was also performed in patients with a normal karyotype and <5% BM blasts, who would be considered as idiopathic HES by the current WHO
criteria. Within this group of patients, abnormal BM morphology remained to be a predictor for an inferior survival (median 125.5 months vs not reached, Kaplan Meier Log rank, p<0.001) (Figure 4B).

The prognostic significance of an abnormal morphology was tested in multivariable analysis. The variables included age, gender, WBC, absolute eosinophil count (AEC), hemoglobin, LDH, organomegaly, constitutional symptoms, heart or brain involvement, cytogenetics, mutations, and presence of two or more mutations. In the final multivariable Cox regression model, only age, thrombocytopenia, heart and/or brain involvement and abnormal BM morphology emerged as significant prognostic factors (Table 3); abnormal karyotype, mutations, and other clinical and laboratory parameters were not independently significant. Multivariable analysis was also performed in the subset of 122 patients with a normal karyotype and <5% BM blasts, who would be classified as idiopathic HES by the current WHO criteria; an abnormal morphology remained to be an independent predictor for inferior survival (Table 3).

Discussion

In this study, we reviewed the BMs of 139 patients who presented with hypereosinophilia without recurrent molecular genetic alterations or a known reactive cause. Following the current WHO classification criteria, 17(12%) patients would be classified as CEL, NOS, either due to the presence of an abnormal karyotype and or increased BM blasts. However, abnormal bone marrow (BM) morphology, with features resembling MDS, MDS/MPN or MPN was observed in 40 of these patients, including 16 of 17 (94%) patients who were classified as CEL, NOS and 24 of 122 (20%) patients who would be classified as idiopathic HES.

For BM morphology, the assessment criteria were in part derived from what we had observed previously (7) by comparing cases with molecular genetic abnormalities versus no identifiable abnormalities. These included increased blasts, hypercellularity, abnormal megakaryocytes, dyserythropoiesis and dysgranulopoiesis, markedly elevated M:E ratio and fibrosis, and abnormal eosinophils. The definitions of “abnormal” BM findings were similar to those characteristically found in other myeloid neoplasms, including MDS, MPN or MDS/MPN, except for the inclusion of eosinophil morphology. In these patients, a BM eosinophilic infiltrate was invariably present, with only 3 patients having <10% eosinophils in the BM. However, in two-thirds of the patients, an increase in BM eosinophils either did not significantly alter or only led to a slight increase in BM cellularity. In patients who showed a significant BM hypercellularity, it was frequently due to increased neutrophils and their
precursors, megakaryocytes, and in some, erythroid precursors, or less commonly to an increased number of macrophages. Additional changes included increased stromal cells, histiocytes, vessels, and disarray of the cellular distribution, which are often referred by others as altered BM topography (19). Recognizing that cytologic eosinophil atypia may be seen in reactive eosinophilia (4) (11-14), we arbitrarily considered abnormal eosinophil morphology only if it involved at least 20% of the eosinophils based. Interestingly, we found that mild atypical changes in reactive eosinophils were more frequently observed in PB than in BM(Figure 2), suggesting that BM smears may be more reliable in the assessment of eosinophil morphology. Nevertheless, of the 25 patients who showed significant numbers of abnormal eosinophils, 22 patients also showed other BM abnormalities and only 3 patients had it as the sole alteration. Of the latter 3 patients, two had a long-standing history of allergy and gastrointestinal symptoms and one patient had deep venous thrombosis, endocardial fibrosis and Budd-Chiari syndrome. All three of these patients had a normal karyotype, and two tested by NGS were negative for mutations. Our findings suggest that alterations in eosinophil morphology can be used in conjunction with other BM findings in morphological assessment, but if it is the sole alteration, may be unreliable to differentiate a neoplastic process from reactive eosinophilia(4) (11-14).

Prior to this study, there has been very little description of megakaryocytes in the literature in patients with hypereosinophilia (20), even in well-defined entities such as PDGFRα and PDGFRβ rearranged myeloid/lymphoid neoplasms (21-23). In our series, abnormal megakaryocyte morphology was frequently observed, with cytologic features mostly resembling MDS-type megakaryocytes or mixed small and large megakaryocytes and with only a few cases showing megakaryocyte morphology similar to BCR/ABL1-negative MPN. Dysgranulopoiesis and/or dyserythropoiesis were seen in some patients. These findings in a patient with eosinophilia suggest a clonal neoplastic process. On the other hand, the presence of dysplastic changes in association with thrombocytopenia and anemia seen in some of these patients, may raise the question whether such cases should rather be considered more closely related to a MDS/MPN rather than to a true MPN, the current nosological attribution of CEL, NOS and HES in the current WHO classification scheme.

Clinically, patients with abnormal BM often showed features suggesting a myeloid neoplasm, with frequent constitutional symptoms, organomegaly, higher LDH, higher WBC and AEC, and more frequent abnormal platelet count and anemia. In contrast, they had fewer symptoms related to eosinophil activation, such as allergy, hypersensitivity, arthritis, muscle aches, gastrointestinal, pulmonary, and cardiac-related symptoms. There were 18 (45%) disease-associated deaths in patients with abnormal BM morphology, including 3 who progressed to AML; and many deaths were due to complications of BM failure. In contrast, there were only 8 (8%) deaths in patients with normal BM
morphology, and the causes of deaths were mainly due to cardiac or pulmonary complications. Patients with abnormal BM had a median survival that was significantly inferior to patients lacking significantly abnormal BM morphology. The median survival of 48.1 months appeared to be better than previously reported of 15-22 months (7, 20) in patients with CEL, NOS. However, the previous studies only included cases with an abnormal karyotype and/or increased blasts. Similar survival significance of abnormal BM morphology was also observed in HES patients with a normal karyotype and no increased BM or PB blasts, who otherwise would be diagnosed as idiopathic HES. These findings were underscored in the multivariable analysis, which showed that abnormal BM morphology, but not an abnormal BM karyotype, was an independent prognostic marker when other factors were co-analyzed.

In this study, we were also able to correlate mutational data with morphology and clinical data. There were 21/76 (27.6%) patients found to have mutations. Similar to what we have found previously (7), mutations frequently involved genes in DNA methylation and chromatin modification such as ASXL1, TET2, and DNMT3A. Although, most of these mutations are also frequently reported in normal aging individuals (9, 10), making it challenging to apply mutation data in the establishment of a clonal hematopoietic stem cell neoplasm, mutations involving at least one gene (60% versus 16%) as well as 2 or more genes were significantly more frequent in patients with an abnormal BM. The caveat was that the patients with abnormal BM morphology were significantly older (64.0 vs 47.7 years). Interestingly, it has been shown recently that in CMML, age-related somatic mutations through successive acquisition convert a myelomonocytic biased hematopoiesis into overt leukemia (24). Further we found in our patients that mutations more specific for a myeloid neoplasm (TP53, EZH2, SETBP1, NRAS, CSF3R, JAK2) were only found in patients with abnormal BM morphology. Similar findings have been reported in MDS patients that certain specific mutations, the number of mutations and VAF, are predicting MDS evolvement in cytopenic patients with clonal hematopoiesis of undetermined potential (9, 25). Nonetheless, based on our findings of the differences in mutation frequency and affected genes associated with BM morphology, we recommend that abnormal BM morphology should prompt NGS study using a myeloid mutation panel to try to establish evidence of clonality, and identify a neoplastic hematopoietic disease.

since abnormal morphology and myeloid mutations seem to correlate.

In summary, we found that most of the patients with CEL, NOS and about 20% of patients with idiopathic HES have abnormal BM morphology, while the remainders have unremarkable BM morphology with the exception of increased eosinophils. The abnormal BM findings seen in these
cases are similar to those seen in MDS, MDS/MPN and/or BCR-ABL1 negative MPN. Cytologic abnormality of eosinophils if alone, it is not entirely specific for a neoplastic process, but its presence should prompt a careful BM morphologic assessment and appropriate molecular genetic testing. We found that the abnormal BM morphology correlates with clinical presentations typically associated with a myeloid neoplasm, such as constitutional symptoms, splenomegaly, high LDH, anemia and abnormal platelet counts and less commonly with symptoms associated with an eosinophil activation syndrome, such as allergy, respiratory, or gastrointestinal symptoms, or cardiac involvement. Abnormal BM morphology significantly correlates with an abnormal karyotype and the presence of myeloid neoplasm-related mutations, and it is highly associated with an inferior patient outcome. The prognostic significance is independent of the effect of abnormal karyotypes, mutations or other risk factors. We conclude that abnormal BM morphology should be regarded as a critical parameter useful for identifying a neoplastic subset of patients considered to have idiopathic HES. The presence of abnormal BM findings should be added to the abnormal karyotype and excess PB and/or BM blasts as one of the defining criteria for diagnosing chronic eosinophilic leukemia, not otherwise specified.

Conflict of Interest

All authors have no conflict of interest related to this study for disclosure

Authors’ Contribution

SAW: design, review material, analyze data, and wrote the paper

WT: perform molecular tests

AO: design, perform and co-wrote the paper

All members: collect data, review material and edit the manuscripts

All authors read the manuscripts and edit the manuscript

References:

Table 1. Bone marrow morphological findings of patients with a diagnosis of CEL, NOS/idiopathic HES

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<thead>
<tr>
<th>Morphological features</th>
<th>Patients (n=139)</th>
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<tr>
<td>Eosinophil percentage, median (range)</td>
<td>21% (4-91%)</td>
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<td>• Patients with &gt;=10% BM eosinophils</td>
<td>136/139 (98%)</td>
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<tr>
<td>Cellularity, median (range)</td>
<td>60% (1-100%)</td>
</tr>
<tr>
<td>• Hypercellularity*</td>
<td>43/129 (31%)</td>
</tr>
<tr>
<td>MDS-like Megakaryocytes**</td>
<td>17/137 (12%)</td>
</tr>
<tr>
<td>MPN-like megakaryocytes**</td>
<td>2/137 (2%)</td>
</tr>
<tr>
<td>Mixed MDS and MPN-like megakaryocytes**</td>
<td>6/137 (4%)</td>
</tr>
<tr>
<td>Dyserythropoiesis **</td>
<td>9/135 (7%)</td>
</tr>
<tr>
<td>Dysgranulopoiesis **</td>
<td>9/135 (7%)</td>
</tr>
<tr>
<td>Abnormal eosinophils ***</td>
<td>25/134 (19%)</td>
</tr>
<tr>
<td>M:E ratio, median (range)</td>
<td>3.3(0.7-31.7)</td>
</tr>
<tr>
<td>• M:E ratio &gt;10</td>
<td>16/129 (12%)</td>
</tr>
<tr>
<td>MF2 or MF3 fibrosis</td>
<td>13/114 (11%)</td>
</tr>
<tr>
<td>Morphologically abnormal****</td>
<td>40/139 (29%)</td>
</tr>
</tbody>
</table>

* at least 20-30% higher than age-appropriate cellularity or ≥90%
** dysplastic/abnormal cells ≥20% of respective lineage
*** markedly abnormal granulation (hypogranulation or uneven granulation), cytoplasmic vacuoles, and/or abnormal nuclear lobation (monolobated or multinucleated); unusual large size or markedly increased immature forms, present in ≥20% cells
**** ≥20% abnormal megakaryocytes, erythroids, myeloids, or 2 of the following: hypercellularity, abnormal eosinophils, M:E ratio >10, or MF1-3 fibrosis
|                           | Total (n=139) | Morphologically abnormal (n=40) | Morphologically within normal limits (n=99) | *p  
|---------------------------|---------------|---------------------------------|---------------------------------|------
| **Age**                   | 53.2(13.5-90.0) | 64.0(28-89.5)                  | 47.7(13.5-90.0)                  | <0.001 |
| **Gender (male:female)**  | 79:60         | 28:12                           | 51:48                           | 0.059 |
| **WBC (x10^9/L)**         | 13.8(5.3-193.2) | 29.7(5.4-193.2)                 | 11.5(5.3-143.1)                 | <0.001 |
| **Eosinophils %**         | 37(10-92)     | 39(10-92)                       | 36(12-88)                       | 0.235 |
| **Absolute eosinophil count** | 4.8 (1.5-177.7) | 11.2(1.6-177.7)                 | 3.9(1.5-113)                    | <0.001 |
| **Hemoglobin (g/L)**      | 12.9 (6-18.0) | 12.2(6-15.3)                    | 13.1(6.6-18.0)                  | 0.041 |
| **Platelets (x10^9/L)**   | 273 (29-1744) | 236 (29-1744)                   | 279(72-734)                     | 0.476 |
| **• <140**                | 21/132 (16%)  | 13/39 (33%)                     | 9/93 (10%)                      | 0.002 |
| **• >450**                | 16/132 (12%)  | 6/39 (15%)                      | 10/93 (11%)                     |       |
| **• 140-450**             | 97/132 (73%)  | 20/39 (51%)                     | 74/93 (80%)                     |       |

**Clinical presentations**

- **Constitutional symptoms** 36/137 (26%) 19/40 (48%) 17/97 (18%) <0.001
- **Allergy/Hypersensitivity** 32/137 (23%) 2/40 (5%) 30/97 (31%) <0.001
- **Muscular/joints/fasciitis** 32/137 (23%) 5/40 (13%) 27/97 (28%) 0.075
- **Thrombotic events** 6/137 (4%) 4/40 (10%) 2/97 (2%) 0.060
- **Skin rashes/dermatitis** 46/137 (34%) 13/40 (33%) 33/97 (34%) 1.0
- **Endocrine (thyroid, pancreas)** 6/137 (4%) 0/40 (0%) 6/97 (6%) 0.180
- **GI symptoms** 33/137 (24%) 5/40 (13%) 28/97 (29%) 0.049
- **Pulmonary/upper respiratory** 25/137 (18%) 1/40 (3%) 24/97 (25%) 0.001
- **Heart/pericardium** 22/137 (16%) 2/40 (5%) 20/97 (21%) 0.012
- **CNS/peripheral neuropathy** 11/137 (8%) 6/40 (15%) 5/97 (5%) 0.080
- **Organomegaly** 17/123 (14%) 10/36 (28%) 7/87 (8%) 0.008
- **Elevated LDH** 30/83 (36%) 17/30 (57%) 13/53 (25%) 0.005

**Abnormal karyotype**

16/133 (12%) 15/39 (38%) 1/94 (1%) <0.001

**Mutations**

- Two or more mutations 8/76 (10%) 7/20 (35%) 1/56 (2%) <0.001
- **TP53, EZH1, SEBP1, NRAS, CSF3R, JAK2** 6/76 (8%) 6/20 (30%) 0/56 (0%) <0.001

**Patient outcomes**

- **Deaths** 26/139 (19%) 18/40 8/99 <0.001
- **Survival (months)** 48.1 (1-120.1) Not reached (0-277.2) <0.001

Note: * p values are comparison between morphologically normal vs abnormal bone marrows; * patient outcomes: censored for unrelated death if known and at the time of hematopoietic stem cell transplant.
Wang et al. BM morphology in CEL,NOS/HES

Table 3. Factors independently predicting an inferior survival of patients with CEL, NOS/idiopathic HES (n=139) as well as of patients with idiopathic HES (n=122) only in multivariable analysis*

<table>
<thead>
<tr>
<th>Variables</th>
<th>All patients (CEL,NOS/idiopathic HES)(n=139)</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per year increase)</td>
<td></td>
<td>1.050 (1.019-1.082)</td>
<td>0.001</td>
<td>1.061 (1.021-1.103)</td>
<td>0.003</td>
</tr>
<tr>
<td>Heart and/or brain involvement</td>
<td></td>
<td>7.875 (2.888-21.473)</td>
<td>&lt; 0.001</td>
<td>5.260 (1.636-16.912)</td>
<td>0.005</td>
</tr>
<tr>
<td>Platelets &lt;140 x10^9/L</td>
<td></td>
<td>4.411 (1.803-10.785)</td>
<td>0.001</td>
<td>7.575 (2.153-26.651)</td>
<td>0.002</td>
</tr>
<tr>
<td>Abnormal bone marrow morphology</td>
<td></td>
<td>7.818 (2.795-21.869)</td>
<td>&lt; 0.001</td>
<td>7.043 (2.191-22.639)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*bone marrow morphology were co-analyzed with age, gender, organomegaly, increased LDH, karyotype, mutation, hemoglobin levels, platelets, white blood cell count, absolute eosinophil count, brain/heart involvement.
Figure legends

Figure 1. Mutations detected in 21 patients with a diagnosis of CEL, NOS/idiopathic HES

Figure 2. Many cases with idiopathic HES show unremarkable bone marrow (BM) morphology. BM cellularity is either age appropriate (A. patient age 48 years) or only slightly increased (B. patient age 45 years), with increased BM eosinophils and normal-appearing megakaryocytes. C. Eosinophils in peripheral blood may show mild uneven granulation (D) but unremarkable on BM smear. No dysgranulopoiesis or dyserythropoiesis (BM biopsy, Hemtoxylin & Eosin, original magnification x400; PB and BM smears Wright-Giemsa, original magnification x1000)

Figure 3. Morphologically abnormal bone marrow (BM). BM hypercellularity (A and B) with increased eosinophils and neutrophilic granulocytic elements; frequent small hypolobated MDS-like megakaryocytes (A, arrows) or mixed MDS- and MPN-like megakaryocytes (B). C. Peripheral blood shows abnormal eosinophils with multiple lobes and marked hypogranulation or agranulation. D. The same changes are also observed in the BM from the same case. In addition, dysplastic erythroblasts and granulocytes (arrows) are also evident. E and F, a case with decreased megakaryocytes, a hypercellularity with disrupted BM topography (E) and a BM smear showing markedly increased immature eosinophils and dyserythropoiesis (F, arrows). (BM biopsy: Hemtoxylin & Eosin, original magnification x400; PB and BM smears: Wright-Giemsa, original magnification x1000)

Figure 4. Survival comparison of patients with chronic eosinophilic leukemia, not otherwise specified (CEL, NOS)/idiopathic hypereosinophilic syndrome (HES). A, with all patients included (n=139), patients with morphologically abnormal bone marrow (ABN) had a median survival of 48.1 months, significantly inferior to patients with a normal BM (WNL) (unreached, p<0.001). B. For patients who would be otherwise classified as idiopathic HES (a normal karyotype and or <5% blasts, n=122), an abnormal BM was also significantly associated with a shorter survival (p<0.001).
Supplementary Table 1: Detailed morphological features of bone marrow that were assessed to be abnormal (n=40)

| 25 cases with MDS-like, MPN-like or Mixed MDS and MPN-like megakaryocytes:                  |
|                                                                                          |
| • 15/23 have abnormal eosinophils,                                                        |
| • 3 with ≥5% blasts,                                                                     |
| • 6/24 MF2 or MF3 fibrosis,                                                              |
| • 19/25 with a hypercellularity,                                                          |
| • 6/24 with dysgranulopoiesis,                                                            |
| • 8/24 dyserythropoiesis                                                                  |
| 3 cases with dysgranulopoiesis and 1 case with dyserythropoiesis                          |
| • 1 also abnormal eosinophils,                                                            |
| • 2 with hypercellularity                                                                 |
| • 1 with MF2 fibrosis                                                                    |
| 11 cases showing at least two other abnormalities:                                       |
| • 10 with hypercellularity;                                                               |
| • 3 with MF3 fibrosis; 3 with MF2 fibrosis,                                               |
| • 4 with abnormal eosinophils:                                                            |
| • 1 with a M:E ratio >10;                                                                 |
| • 2 with markedly decreased megakaryocytes, 1 with abnormal megakaryocytes (subset)      |
Suppl. Figure: Comparison of patients with an abnormal karyotype, and or positive mutations, or increased blasts (n=36) versus all other patients (n=103). Of note, in the latter group, mutations were only tested in 52 (52%) patients. The survival was 80.3 months vs not reached.