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Clonality of neutrophilia associated with plasma cell neoplasms: report of a SETBP1 mutation and analysis of a single institution series

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Abstract

A rare but well-known association between plasma cell neoplasms and neutrophilia is known to exist. Whether the neutrophilia is secondary to the plasma cell neoplasm or this convergence represents two independent clonal disorders is unclear. We reviewed five consecutive cases from a single institution over a 3-year period, applying molecular, cytogenetic and cytokine-profiling approaches to determine whether neutrophilia associated with plasma cell neoplasms represents a reactive or clonal process. We report, for the first time, the occurrence of a SETBP1 mutation in two cases, as well as changes in G-CSF and IL-6 in SETBP1 wild type vs. mutated patients that are supportive of a hypothesis that neutrophilia associated with plasma cell neoplasms may sometimes be reactive and may sometimes represent a second clonal entity. Finally, using an *ex vivo* drug screening platform we report the potential efficacy of the multi-kinase inhibitor dasatinib in select patients.

Keywords

Chronic neutrophilic leukemia; neutrophilia; plasma cell neoplasm; SETBP1

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Introduction

Chronic neutrophilic leukemia (CNL) is a WHO-recognized myeloproliferative neoplasm defined by leukocytosis with a predominance of neutrophils and the absence of increased myeloblasts, a hypercellular bone marrow with increased neutrophilic granulocytes, hepatosplenomegaly, no secondary causes of neutrophilia or concomitant myeloproliferative neoplasm or myelodysplastic syndrome, and no evidence of the BCR-ABL1 fusion gene or PDGFRA, PDGFRB or FGFR1 gene rearrangements [1]. Up to 20% of neutrophilia cases diagnosed as CNL are associated with another malignancy; the most common association is with plasma cell neoplasms [1] (multiple myeloma, smoldering myeloma, monoclonal gammopathy of undetermined significance [MGUS]), which has been well-described in several dozen case reports [2]. Given the relative rarity of the conditions independently and the frequency of the association this relationship is regarded as unlikely to be coincidental. Indeed, observations that cytokines such as G-CSF and IL-6 are produced by plasma or stromal cells in the setting of neutrophilia associated with plasma cell neoplasms, and that treatment directed against plasma cell neoplasms resulted in decreases in these cytokines and the neutrophilia, suggest the neutrophilia is a secondary process [3-8]. Furthermore, X-linked polymorphism analysis in a single patient suggested the neutrophilia associated with a plasma cell neoplasm was polyclonal [3]. However, mutations in Janus kinase 2 (JAK2) and colony stimulating factor 3 receptor (CSF3R), markers of clonal myeloproliferative neoplasms [9,10], have been reported in neutrophilic patients with underlying plasma cell diseases [11-14], suggesting that patients may in fact have two independent clonal disorders. Recently, SET-Binding Protein 1 (SETBP1) has been shown to be recurrently mutated in *de novo* cases of CNL and related diseases [15-17], but it has not previously been reported to occur in neutrophilia associated with plasma cell neoplasms.

We examined five consecutive cases of neutrophilia with underlying plasma cell neoplasms from a single institution to further investigate the relationship between these associated diseases. We employed cytogenetic, molecular and cytokine profiling assays to explore the extent to which the observed neutrophilia is a reactive process, and performed an *ex vivo* drug screen to determine whether there would be potential for targeted therapeutic interventions for this rare entity.

Materials and methods

Patients

We retrospectively reviewed all cases of neutrophilia with concomitant plasma cell neoplasms from bone marrow biopsies at the University of Colorado from 2011 to 2014. Five untreated MGUS patients were selected to serve as controls. Informed consent for tissue acquisition was obtained from all patients.

Cytogenetic testing

Cytogenetic studies were performed using standard techniques. Unselected, unstimulated bone marrow cells were cultured overnight in Chang Mitogen Free (Irvine Scientific, Irvine, CA), Chang Marrow media (Irvine, CA); and Chang Mitogen Free with lipopolysaccharide

(0.5 µg/ml) (Sigma-Aldrich; St. Louis, MO). Following exposure to colcemid, fixed cells were prepared on VWR Superfrost White (VWR, Radnor, PA) microscope slides, stained for G-banding, and scanned on an automated LEICA scanning system, using an algorithm that maximizes capture of bone marrow metaphase spreads. Between 15 and 20 metaphase cells were analyzed for numerical or structural abnormalities.

Fluorescence *in situ* hybridization (FISH) studies for plasma cell dyscrasias were performed, using enumeration probes for 1p (CDKN2C), 1q21 (CKS1B), 11q23 (MLL/KMT2A), 13q14 (D13S319 with D12Z3 control), and 17p13.1 (p53, plus chromosome 17 centromere, alpha satellite control) as well as break apart probes for IgH (14q32) and translocation partners (Abbott Molecular, Des Plaines, IL). Imaging for both FISH and cytogenetics was performed on the Cytovision LEICA Biosystems (Buffalo Grove, IL).

Genetic sequencing

Peripheral blood or bone marrow aspirate samples were treated with Ammonium-Chloride-Potassium (ACK) solution to lyse red blood cells. Genomic DNA was extracted from 5×10^6 cells using a DNeasy Blood & Tissue kit (Qiagen). SETBP1 was amplified using 50–100 ng of genomic DNA and the AccuTaq DNA Polymerase (Invitrogen) and the following M13-tagged primers in exon 3 (SETBP1-2476F-exon3-M13F, gtaaaacgacggccagtACCTGGAAGCTGTCTCCACCCA and SETBP1-2753R-exon3-M13R caggaaacagctatgaccCGGTGGCCATGCCGGTTCTT). PCR products were purified using Amicon Ultra-0.5 mL Centrifugal Filters (EMD Millipore) and then Sanger sequenced using M13F (gtaaaacgacggccagt) and M13R primers (caggaaacagctatgacc) by Eurofins Genomics. CSF3R exons 14-17 were sequenced in a similar fashion, and Jak2, c-Kit and BCR-ABL were sequenced using standard methods in the clinical laboratory.

Cytokine profiling

Multiplex bead-based analysis was performed on peripheral blood or bone marrow plasma samples to analyze multiple cytokine parameters simultaneously. Peripheral blood or bone marrow samples were centrifuged and the plasma layer was removed and frozen at -80°C . Briefly, frozen samples were thawed and analyzed per the protocol for a combination of multiple human cytokines and chemokines using the HCYT0MAG-60K 38 Milliplex Assay (EMD Millipore, Billerica, MA). Plasma samples were incubated in the presence of magnetic beads and cytokine/chemokine levels were detected on the Luminex xMap platform (EMD Millipore, Billerica, MA) and analyzed in the Milliplex xPONENT Analyst software before final concentrations were graphed in Graphpad Prism.

Ex-vivo drug screen

Peripheral blood or bone marrow aspirate samples were treated with ACK solution to lyse red blood cells. Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Atlanta Biologicals, L-glutamine (Invitrogen), fungizone (Invitrogen), penicillin/streptomycin (Invitrogen), and 2-mercaptoethanol (10^{-4} M, Sigma). Cells were screened against a panel of kinase inhibitors as described previously [18]. IC50s for three replicates of dasatinib were graphed using Prism Software.

Results

Over a 3-year period, five cases of neutrophilia with a concomitant plasma cell neoplasm were seen at the University of Colorado. Baseline features of the five cases are detailed in Table I.

Patient summaries

Patient 1 was originally diagnosed at another facility with chronic myelogenous leukemia due to neutrophilia and a bone marrow biopsy that revealed hyperplastic granulopoiesis and hypercellularity. A monoclonal plasma cell population was noted at 20%, and BCR-ABL testing was negative, but he was started on imatinib. One month into treatment a lack of hematological response prompted an imatinib dose escalation to 800 mg, and when there was again no hematological response the imatinib was stopped and hydroxyurea was initiated. He was referred to our institution where a repeat bone marrow biopsy confirmed a 25% population of kappa restricted monotypic plasma cells, with a markedly hypercellular bone marrow with left-shifted granulopoiesis and no increase in blasts or evidence of dysplasia. He was diagnosed with neutrophilia associated with an underlying plasma cell neoplasm and *ex vivo* drug screening suggested sensitivity to dasatinib (Figure 1). He was started on dasatinib which was titrated from 10 mg PO daily ultimately to 100 mg PO daily over the course of 5 months with little change in the white blood cell count (WBC) or absolute neutrophil count (ANC) (Figure 2A). A bone marrow biopsy showed no decrease in plasma cells and persistent granulocytic hyperplasia after 12 months of therapy. Ultimately, 16 months after initiating dasatinib this medication was discontinued; he was started on bortezomib, dexamethasone and lenalidomide to treat his multiple myeloma. He had an immediate decrease in his WBC count (Figure 2A) and after 3 months of therapy a bone marrow biopsy showed a significant reduction in plasma cells (4%) and neutrophils that were only moderately increased. However, the peripheral leukocytosis persisted, and the decision was made to proceed to an autologous stem cell transplant with high dose melphalan conditioning. Nine months after transplant there is no increase in bone marrow plasma cells but WBC and ANC remain mildly elevated ($16.7 \times 10^9/L$ and $13.1 \times 10^9/L$, respectively).

Patient 2 was incidentally noted during a workup for back pain to have leukocytosis. A bone marrow biopsy was markedly hypercellular with left-shifted granulocytic hyperplasia; a monotypic population of kappa-restricted plasma cells accounted for 15% of the total bone marrow. After *ex vivo* drug screen results predicted responsiveness to dasatinib (Figure 1), the patient was started at 50 mg PO daily. After an immediate reduction in leukocytosis he became leukopenic; dasatinib was held, and then resumed when the leukopenia resolved. He was ultimately titrated to a dose of 20 mg/10 mg every other day, and remains on that schedule with a normal WBC and ANC, 18 months after initiating dasatinib. Bone marrow biopsies performed after 3 and 6 months of treatment showed no substantial changes in the plasma cell neoplasm burden, with continued 10–20% involvement, and ongoing significant hypercellularity. However, after 12 months of therapy, the bone marrow reverted to normocellularity with active trilineage hematopoiesis, and the plasma cell population had significantly decreased to 5% (Figure 2B-D).

Patient 3 was incidentally noted to have leukocytosis during a pre-operative workup for back surgery. A bone marrow biopsy revealed a myeloproliferative disorder not otherwise specified, and it was noted that a clonal plasma cell population co-existed. He was lost to follow up for over 12 months when he re-presented with pleural and pericardial effusions and a WBC in the $90,000 \times 10^9/L$ range. A bone marrow biopsy at this time revealed a markedly hypercellular bone marrow, with increased granulopoiesis, no increased blasts or dysplasia, and a population of monotypic kappa-restricted plasma cells that accounted for 7% of the bone marrow. He was maintained on hydroxyurea and anagrelide for 10 months, at which time he developed significant leukocytosis. This prompted a repeat biopsy which was concerning for disease progression, with new chromosome 7 abnormalities and the suggestion of a more immature myeloid phenotype; the clonal plasma cell population persisted. In addition, a SETBP1 D868N was identified (Figure 3). The patient was induced with 7 days of cytarabine and 3 days of idarubicin, complicated by neutropenic fevers due to influenza H1N1 and bacterial endocarditis. He experienced acute respiratory distress syndrome and renal failure and ultimately died 21 days after induction.

Patient 4 was noted to have neutrophilia during a routine annual physical. The bone marrow biopsy was markedly hypercellular with granulocytic hyperplasia; it was also notable for the presence of a lambda-restricted plasmacytosis of 20%. She was treated with bortezomib, lenalidomide and dexamethasone which resulted in a stringent complete response, and has remained on maintenance therapy for 30 months. Her neutrophilia entirely resolved rapidly with this treatment and has not returned.

Patient 5 had been noted to have persistent elevations in his WBC for many years. After a precipitous rise to $75,000 \times 10^9/L$, a bone marrow biopsy showed marked hypercellularity with 5% blasts and no evidence of dysplasia; in addition, a very small population of lambda-restricted plasma cells (<1%) was detected. A SETBP1 G870S mutation was identified (Figure 3), and the patient was managed with hydroxyurea. After 6 months there was an increase in leukocytosis with lymphadenopathy. The lymph node biopsy revealed involvement of the myeloproliferative disease process. The patient showed *ex vivo* sensitivity to dasatinib (Figure 1) but elected to remain on hydroxyurea. After 16 months of observation with an elevated but stable WBC and ANC, he developed a precipitous rise in his WBC and a bone marrow biopsy revealed evolution to AML. The patient opted for comfort care measures and expired.

Targeted gene sequencing identifies mutations in SETBP1

All patient samples were negative for mutations in JAK2 and CSF3R (Table I). Three were sequenced for c-KIT and were negative. SETBP1 was sequenced in all patients and found to be mutated in patients 3 and 5, with D868N and G870S mutations, respectively, both of which occur in the SKI-homologous region of the protein [15,19].

FISH analysis shows hyperdiploidy of 1q and trisomy 11q23 to be present only in the plasma cell population

Cytogenetic testing was performed on all patient samples (Table I). The BCR-ABL fusion gene was not identified in any samples. Hyperdiploidy of chromosome 1 (trisomy or

tetrasomy) was present in 3/5 patients and trisomy 11q23 (MLL) was present in 2/5 patients; these abnormalities were only noted in plasma cells and not in segmented or other cell populations (Figure 4).

Cytokine profiling

Due to prior reports hypothesizing that plasma cell dyscrasias can result in reactive neutrophilia by increasing G-CSF and IL-6 levels [6-8], G-CSF and IL-6 were quantified in all of the patient samples as well as in five matched control patients with untreated MGUS and no neutrophilia (Figure 5). IL-6 was 83.74 pg/ml in the SETBP1^{WT} patients, compared to 31.33 pg/ml in the SETBP1 mutated samples and 12.556 pg/ml in controls. G-CSF was 293.1 pg/ml in the SETBP1^{WT} patients, compared with 190.7 pg/ml in the SETBP1 mutated samples and 143.7 pg/ml in controls.

Discussion

Neutrophilia in the setting of an underlying plasma cell neoplasm is an uncommon but well-known phenomenon which has historically been ascribed to a reactive process emanating from the clonal plasma cell population. However, the availability of genomic sequencing has recently resulted in the observation that recurrent mutations typically associated with clonal myeloproliferative neoplasms occur in cases of neutrophilia with underlying plasma cell neoplasms. The availability of a single-institution series of five such patients represents the largest consecutive case series of this disease entity, and presents an opportunity to further analyze whether neutrophilia exists due to the co-occurrence of two independent clonal disorders or is the result of a secondary process.

We report here, for the first time to our knowledge, that SETBP1 is recurrently mutated in neutrophilia cases associated with plasma cell neoplasms, joining JAK2 and CSF3R as other genes previously reported to be mutated in this setting [12,13]. SETBP1 is a SET-binding 170 kDA nuclear protein located at 18q21.1; germline mutations occur in children with Shinzel-Giedion syndrome, a congenital disorder associated with skull abnormalities, hydronephrosis and motor and intellectual retardation [20]. In myeloid malignancies SETBP1 mutations are thought to result in inhibition of the serine/threonine phosphatase protein phosphatase 2A (PP2A) [21], and likely represent a poor risk disease feature [21,22]. SETBP1 mutations, like JAK2 and CSF3R, are highly specific to myeloid malignancies, and therefore their occurrence in the setting of neutrophilia associated with a plasma cell neoplasm suggests the presence of two independent clones.

This led to the hypothesis that concurrent neutrophilia with plasma cell neoplasms may sometimes be the result of a reactive process while at other times represents the convergence of two independent clones. We further hypothesized that the patients who were wild type for SETBP1 and all other relevant genetic mutations were examples of reactive neutrophilia, while those with SETBP1 mutations were examples of clonal neutrophilia, and that differences should be observed between these groups when analyzing expression of G-CSF and IL-6. Both of these cytokines had previously been implicated as causative of a plasma cell-driven reactive neutrophilia [6-8], and were predicted to be elevated in SETBP1^{WT} but not mutated patients. Using non-neutrophilic untreated MGUS patient samples as controls,

we did observe this trend, with expression of G-CSF and IL-6 in SETBP1^{wt} patients elevated compared to SETBP1 mutated patients, which approximated controls (Figure 5).

Further evidence to support the hypothesis that plasma cell-associated neutrophilia is sometimes clonal and sometimes non-clonal comes from the cytogenetic testing performed. Cytogenetic abnormalities were observed only in the SETBP1^{WT} samples; both of the SETBP1 mutated samples had a normal karyotype, suggesting that reactive neutrophilia may be related to recurrent cytogenetic abnormalities, and that non-reactive neutrophilia can be observed in the absence of cytogenetic abnormalities if there is a clonal driver. Intriguingly, the cytogenetic abnormalities in the SETBP1^{WT} patients are very similar. All three have trisomy/tetrasomy of 1q, typically present in less than 50% of smoldering myeloma patients [23] and 2/3 have a trisomy 11q23, suggesting these structural abnormalities may contribute to neutrophilia in an as-yet unknown manner. Finally, the FISH analysis, showing the cytogenetic abnormalities only existed in the myeloma population (Figure 4), also supports a reactive neutrophilia in those cases.

The use of an *ex vivo* drug screen was employed to determine whether a particular kinase inhibitor may have activity against the neutrophilia in all five patients. Patients 1 and 2 had half-maximal inhibitory concentration (IC50) levels for dasatinib, an orally available multi-targeted kinase inhibitor with particular activity against BCR-ABL and SRC family kinases [24] that were <10 nM, and therefore potentially clinically active. Patient 1 had no discernible response to increasing doses; however, patient 2 has had a durable response, and after a considerable period of time, has even had a decrease in the burden of underlying plasma cells. Dasatinib has not previously been shown to have activity against multiple myeloma [25], but the small cohort that reported this negative study did not contain any patients with trisomy 1, and it is possible that multiple myeloma characterized by the overexpression of cyclin-dependent kinase genes like CKS1B, located on chromosome 1q and downregulated by dasatinib [26], may be exceptions.

Normalization of the neutrophilia in response to treating the plasma cell neoplasm would be suggestive of a reactive process. This occurred in the case of patient 4, in whom there was no additional clonal driver identified. However, aggressive treatment of patient 1's plasma cell neoplasm did not result in full resolution of the neutrophilia. This could be due to the fact that this patient's response to chemotherapy was not as complete as that of patient 4, or suggestive that his neutrophilia was not a reactive process.

Based on our hypothesis, patients with identifiable mutations known to be associated with neutrophilia would be less likely to have resolution of neutrophilia through treatment of the myeloma. Unfortunately, our data set cannot provide insights into this question since the two patients with SETBP1 mutations did not receive treatment for their plasma cell neoplasms. In addition, one would predict dasatinib or other drug sensitivity only in those patients with identified mutations; however, patients 1 and 2, despite having predicted sensitivity, did not have identifiable mutations.

This report is limited by the small numbers associated with this disease entity. In addition, it is unknown whether the SETBP1 mutation was present in only the neutrophils or also in the

plasma cell population as well. Finally, there are almost certainly other as-yet unknown oncogenic driver mutations that may be relevant in the samples in which no mutations were found in the tested genes.

In conclusion, we report here for the first time that SETBP1 mutations are present in cases of neutrophilia associated with plasma cell neoplasms. In patients with these mutations, levels of G-CSF and IL-6 were lower than patients who did not have SETBP1 or any other identifiable oncogenic driver mutation, although the differences were not significant in this small sample set. One patient who was treated with chemotherapy directed against the plasma cell neoplasm had full resolution of the neutrophilia, and one patient found to have *ex vivo* sensitivity to dasatinib has had a durable response with this agent with respect to neutrophilia as well as the underlying plasma cell neoplasm. There may be a unique pattern of recurrent cytogenetic abnormalities in true reactive neutrophilia that is associated with higher levels of G-CSF and IL-6. The possibility exists that neutrophilia associated with plasma cell neoplasms may sometimes be reactive and may sometimes be due to a secondary clonal process.

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Figure 1.

Ex-vivo screening reveals sensitivity to dasatinib in select patients. Each sample was screened for kinase inhibitor sensitivity including three replicates of dasatinib. Average half maximal inhibitory concentrations (IC50s) are plotted for each patient sample. (Patient 4 did not reach an IC50, so the highest drug concentration in the inhibitor assay is plotted).

Figure 2.

Impact of treatment on clinical parameters for patients 1 and 2. (A) White blood cell count ($\times 10^9/\text{L}$) at various time points during treatment for patient 1. (B) White blood cell count, absolute neutrophil count and plasma cells in the bone marrow during treatment for patient 2. Bone marrow core biopsy with H&E stain and CD138 immunohistochemical stain (inset) at $200\times$ for patient 2 at baseline (C) and after 12 months of treatment (D) with dasatinib.

Figure 3.
Forward and reverse traces reveal mutations in SETBP1 for patients 3 and 5.

Figure 4.

Chromosomal abnormalities are present in the plasma cell population exclusively. Patient 4 dual color enumeration FISH assay for 1p32 (p18, CDKN2C) in green and 1q21 (CKS1B) in red reveals a normal signal pattern in a segmented cell (panel A) and trisomy/tetrasomy 1q in two plasma cells (panel B). Of 200 plasma cells scored, 10.5% had a gain of 1q; 0/200 segmented cells scored had a gain of 1q.

Figure 5. Serum IL-6 (A) and G-CSF (B) levels are elevated in the SETBP1 wild-type patients with neutrophilia compared to those with SETBP1 mutations and matched non-neutrophilic MGUS controls.

Table 1

Baseline features of patients with neutrophilia and concomitant clonal plasma cell dyscrasias.

Patient #	Sex	Age	WBC (ANC)	Hgb	Plts	SPIE and κ or λ	$K:\lambda$ FLC ratio	BM Bx cellularity	BM blasts	BM plasma cells	Clinical MM subtype	Cytogenetics/ FISH	Jak2	BCR-ABL	c-Kit	CSF3R	SETBP1
1	M	52	27.6 (23.7)	13.2	164	0.6 IgG κ	65.8	99%	1%	23.5%	Smoldering	Trisomy 1q21, trisomy 11q23, trisomy 17p	WT	-	WT	WT	WT
2	M	58	99.4 (74.5)	11.4	104	0.9 IgA κ	1.3	95%	6.5%	15%	Smoldering	Trisomy 1q21, trisomy 11q23	WT	-	WT	WT	WT
3	M	69	77.2 (71.1)	12.3	1621	0.3 IgA κ	2.14	90%	3%	7%	MGUS	46 XY	WT	-	NR	WT	D868N
4	F	69	69.1 (58.3)	13.0	173	SPIE N/A; λ	<0.0002	95%	1%	22%	Multiple myeloma (active)	Trisomy 1q21, del13q14, del1p32	WT	-	WT	WT	WT
5	M	70	79.4 (60.3)	16.2	183	N/A	N/A	95%	5%	3%	MGUS	46 XY	WT	-	N/A	WT	G870S

WBC, white blood cell count; ANC, absolute neutrophil count; Hgb, hemoglobin; Plts, platelets; SPIE, serum protein immunoelectrophoresis; FLC, free light chain; BM Bx, bone marrow biopsy; MM, multiple myeloma; FISH, fluorescent in situ hybridization; N/A, not performed