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RESEARCH REPORT

Identification of novel *MECOM* gene fusion and personalized therapeutic targets through integrative clinical sequencing in secondary acute myeloid leukemia in a patient with severe congenital neutropenia: a case report and literature review

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Abstract Severe congenital neutropenia (SCN) is a rare hematologic disorder characterized by defective myelopoiesis and a high incidence of malignant transformation to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). SCN patients who develop MDS/AML have excessive toxicities to traditional chemotherapy, and safer therapies are needed to improve overall survival in this population. In this report, we outline the use of a prospective integrative clinical sequencing trial (PEDS-MIONCOSEQ) in a patient with SCN and AML to help identify oncogenic targets for less toxic agents. Integrative sequencing identified two somatic *cis*-mutations in the colony stimulating factor 3 receptor (*CSF3R*) gene, a p.T640N mutation in the transmembrane region and a p.Q768* truncation mutation in the cytoplasmic domain. A somatic mutation p.H105Y, in the runt homology domain (RHD) of runt-related transcription factor 1 (*RUNX1*), was also identified. In addition, sequencing discovered a unique in-frame *EIF4A2-MECOM* (*MDS1* and ectopic viral integration site 1 complex) chromosomal translocation with high *MECOM* expression. His mutations in *CSF3R* served as potential targets for tyrosine kinase inhibition and therefore provided an avenue to avoid more harmful therapy. This study highlights the utility of integrative clinical sequencing in SCN patients who develop leukemia and outlines a strategy on how to approach these patients in a future clinical sequencing trial to improve historically poor outcomes. A thorough review of leukemia in SCN and the role of *CSF3R* mutations in oncologic therapy are provided to support a new strategy on how to approach MDS/AML in SCN.

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INTRODUCTION

Severe congenital neutropenia (SCN) is a rare hematologic disorder characterized by peripheral blood absolute neutrophil counts of <500 cells/μl, maturation arrest at the promyelocyte/myelocyte stage in the bone marrow (Boxer and Dale 2002) and recurrent bacterial infections (Boxer 2006). SCN is genetically heterogeneous with autosomal dominant mutations in the *ELANE* gene, which encodes the serine protease neutrophil elastase, accounting for 50%–60% of cases (Dale and Link 2009). Treatment of SCN patients with granulocyte colony stimulating factor (G-CSF) can restore neutrophil counts in most patients, but a minority of patients will develop myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) with a cumulative incidence of 22% at 15 yr (Rosenberg et al. 2010).

Treatment of AML in non-SCN patients consists of intensive induction chemotherapy followed by consolidation chemotherapy in low to intermediate risk patients and hematopoietic cell transplant (HCT) for high-risk individuals. For SCN patients, HCT is considered necessary for cure secondary to the concurrent underlying hematopoietic defect. However, cytoreductive induction therapy is difficult in SCN patients as they develop significant toxicity secondary to an inability to recover neutrophils necessary for tissue repair and infection eradication (Choi et al. 2005). Therefore, clinicians are faced with the decision to administer full or reduced dose induction regimens and risk excessive toxicity or proceed directly to transplant conditioning therapy with a substantial leukemic burden.

A better approach would be identification of nontoxic therapies. Personalized sequencing can identify oncogenic mutations that may be amenable to safer treatment with small molecule inhibitors. In this case report, we detail a pediatric patient with *ELANE*-associated SCN with refractory AML who underwent exome and transcriptome sequencing. Sequencing data identified potential oncogenic targetable mutations in the colony stimulating factor 3 receptor (*CSF3R*) gene and a previously unreported translocation involving the *MDS1* and ectopic viral integration site 1 (*EVI1*) complex (*MECOM* locus) and eukaryotic initiation factor 4A (*EIF4A*). This report highlights the importance of personalized medicine for the SCN population, discusses unique treatment options upon malignant transformation, and provides a thorough review of cancer in SCN and the current state of knowledge of *CSF3R* mutations in leukemia.

RESULTS

Clinical Presentation and Family History

A 13-yr-old Caucasian male was referred to our institution for management of SCN-related AML. He was identified to have an *ELANE* p.P205R mutation after presenting with severe neutropenia, otitis media, and pneumonia at 3 mo of age and was managed with high-dose G-CSF therapy to maintain a normal neutrophil count. Family history was limited secondary to prior adoption, but biologic parents, three full siblings, and one maternal half-sibling were reportedly healthy. At 13 yr 5 mo of age his G-CSF was increased to 480 mcg/day because of persistent neutropenia. Two months later, he complained of fatigue and bruising; peripheral blood counts revealed a white blood count of 14,000 cells/μl with 12% myeloid blasts, hemoglobin 7.9 g/dl, and platelets 20,000/μl. G-CSF was discontinued but peripheral blood counts 2 wk later demonstrated 52% circulating blasts. Bone marrow biopsy demonstrated 90% cellularity with 23% blasts, dysplastic changes in the erythroid and myeloid series, and absent megakaryocytes. Tumor karyotype identified trisomy 21 and +7q21, and FISH additionally identified −7q31. He was diagnosed with AML with MDS-associated features.

Because of his complex social situation, he initially received only supportive care with transfusions, intermittent G-CSF, and infection prophylaxis. He was transferred to our institution at age 13 yr 11 mo. Repeat bone marrow evaluation showed progression of disease with 71% blasts. His blasts were noted to have CD19 coexpression with myeloid markers (CD11c, CD13, CD33, CD117). Tumor karyotype continued to demonstrate trisomy 21 and +7q21. Targeted molecular analysis for *BCR-ABL*, *MPL*, *FLT3*-ITD, and *JAK2* mutations was negative. Over concern for excessive toxicity from traditional AML induction therapy, he was initiated on azacitidine with only a transient response. He therefore transitioned to a reduced dosed induction regimen of Ara-C, daunorubicin, and thioguanine. His course was complicated by fungal pneumonia, and despite concerns for driving leukemic proliferation, re-initiation of G-CSF to treat his infection was required.

A follow-up bone marrow test demonstrated a reduction in his blast count to 30%. Given his previous fungal infection and goal to avoid further toxicity from cytoreductive therapy, he was taken to a 10/10 matched related donor HCT with persistent disease at age 14 yr 1 mo. His preparative regimen consisted of clofarabine and myeloablative busulfan. His early transplant course was uncomplicated, but his day +30 bone marrow demonstrated persistent disease with 8% blasts. His immune suppression was rapidly tapered to induce a graft-versus-leukemia effect and he was restarted on azacitidine. He then developed several complications including Gram-negative sepsis, bone marrow aplasia, immune-mediated hemolytic anemia, and EBV viremia with splenomegaly while on prednisone for his hemolytic anemia. His lymphoproliferative disease was treated with prednisone discontinuation and administration of Rituximab.

With persistent disease post-HCT, a bone marrow sample was collected for personalized sequencing. As his AML was progressing, additional treatment was required while awaiting sequencing results. Treatment included (1) Ara-C and L-asparaginase; (2) 6-mercaptopurine; and (3) Ara-C, daunorubicin, and etoposide. The patient had poor response and was transitioned to arsenic trioxide palliative therapy. He unfortunately died as a result of progressive AML before targeted therapies based on sequencing could be attempted.

Genomic Analyses

Whole-exome (germline/reference and leukemia) and transcriptome (leukemia) sequence analysis were performed on the patient's bone marrow (leukemia) and buccal swab (germline/reference). The experimental procedure is outlined in the Methods section. Details of sequencing depth and quality are presented in Table 1. The tumor content of the collected marrow specimen was 38%. The mean sequencing depth of the tumor and reference DNA were 175× and 141×, respectively. Pathogenicity of somatic variants was determined through a review of the published literature and public databases. Clinical relevance of somatic variants was investigated using an integrated approach incorporating technical considerations (e.g., recurrence, variant allele fraction, expression levels, and predictive algorithms for pathogenicity), variant-specific information (i.e., ClinVar, published literature, and curated gene resources), and published correlations of drug and variant sensitivity profiles. Considerations of tumor heterogeneity, including clonal versus subclonal mutation, were addressed by comparing variant allele fractions and copy-number estimates for each of the mutations to postsequencing estimates of tumor content derived from single-nucleotide variation and copy-number analyses. To eliminate variants contributed by donor cells in the sample, mutation candidates were further filtered by their occurrence frequency in the Exome Aggregation Consortium (ExAC) and by variant allelic fraction. Variants that had a dbSNP entry with concordant high frequency in ExAC and low allelic fraction in the patient were most likely derived from the donor and hence excluded. Results of this analysis were

Table 1. Sequencing details of normal and tumor exome and tumor transcriptome

Exomes								Transcriptomes	
No. of clusters		Alignment (%)		Mean coverage (×)		PCR duplication percentage		Tumor	
Tumor	Reference	Tumor	Reference	Tumor	Reference	Tumor	Reference	# of clusters	Alignment (%)
106,146,826	83,659,569	94.1	94.0	175	141	4.0	5.6	47,608,723	91.5

PCR, polymerase chain reaction.

then presented during a multidisciplinary tumor board meeting to discuss the potential impact of identified mutations as well as potential clinical ramifications.

For this patient, somatic mutations of pathogenic and/or clinical importance were identified and summarized in Table 2. Additional discovered somatic mutations can be found in Supplemental Table 1. Copy-number analysis identified previously known trisomy 21 and 7q-, as well as a new copy gain of 3q (Fig. 1). Sequencing identified a somatic mutation in runt homology domain (RHD) of runt-related transcription factor 1 (*RUNX1*): p.H105Y, as well as a unique in-frame *EIF4A2-MECOM* chromosomal translocation. The fusion was predicted to contain the first two exons of *EIF4A2* and exons 3–16 of *MECOM* (NM_004991) with resultant chimera protein (Fig. 2A). There were 416 spanning reads and 149 encompassing read pairs that support this fusion identification and the translocation resulted in high *MECOM* expression (Fig. 2B). Two somatic *cis*-mutations in *CSF3R*, a p.T640N mutation in the transmembrane region and a p.Q768* truncation mutation in the cytoplasmic domain, were also identified (Fig. 3).

Table 2. Highlighted somatic variants identified in the patient

Gene	Genomic location and reference>variant	HGVS protein reference	Variant type	Predicted effect	dbSNP	Genotype	Allelic frequency
Single-nucleotide variants							
CSF3R	Chr 1: 36932248 G>A	NP_000751.1: p.(Q741*)	Substitution	Nonsense	N/A	Heterozygous	48%
CSF3R	Chr 1: 36933198 G>T	NP_000751.1: p.(T640N)	Substitution	Missense	rs121918426	Heterozygous	46%
RUNX1	Chr 21: 36259178 G>A	NP_001001890.1: p.(H78Y)	Substitution	Missense	N/A	Heterozygous	60%
Copy-number alterations							
3q	Chr 3	N/A	Copy gain	Gain of 3q	N/A	N/A	N/A
21	Chr 21	N/A	Copy gain	Gain of 21	N/A	N/A	N/A
7q	Chr 7	N/A	Copy loss	Loss of 7q	N/A	N/A	N/A
Rearrangement analysis							
EIF4A2-MECOM	Chr 3	N/A	Inversion	EIF4A2-MECOM in-frame fusion	N/A	N/A	N/A

HGVS, Human Genome Variation Society; dbSNP, The Single Nucleotide Polymorphism database; N/A, not available or not applicable.

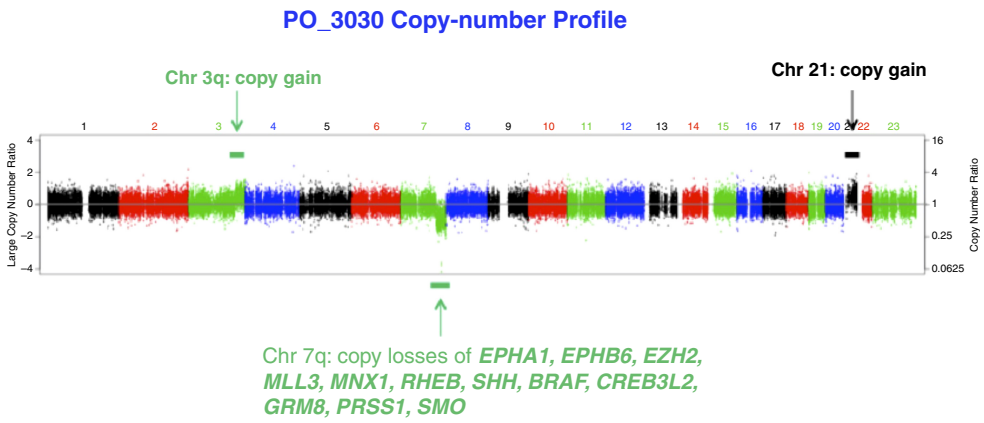

Figure 1. Copy-number profile of described patient (PO_3030) including specific genomic losses secondary to Chromsome 7q deletion.

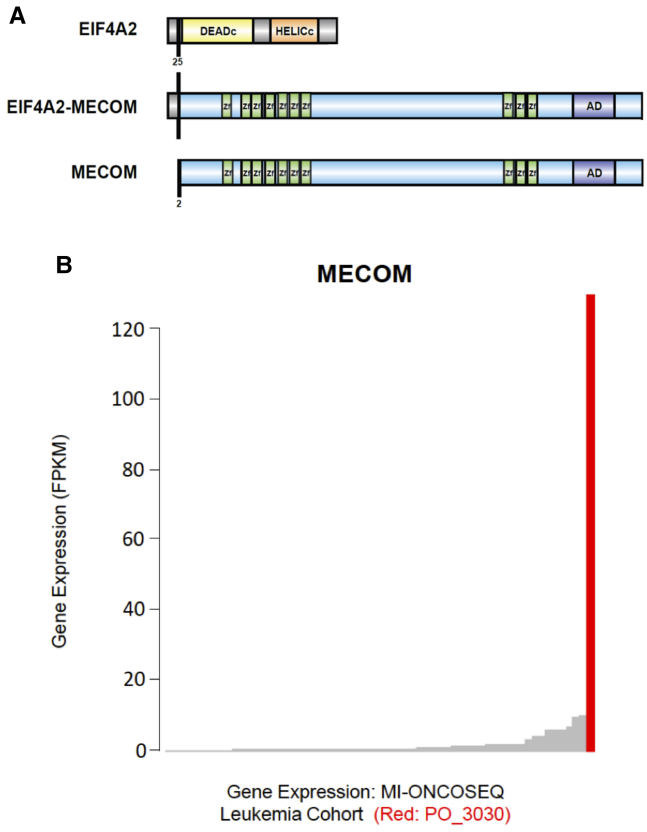

Figure 2. (*A*) Schematic representation of novel in-frame EIF4A2-MECOM fusion in the described patient. The protein domains encoded by the 5′ and 3′ genes of the fusion, with the predicted fusion protein in between are illustrated. (*B*) RNA transcriptome expression of *MECOM* (*MDS1-EVI1*) in FPKM (fragments per kilobase of exon per million fragments mapped) of the described patient (PO_3030) in red versus tumor bank of known cancers in gray.

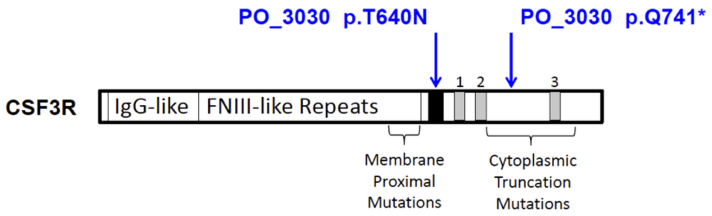

Figure 3. Schematic of *CSF3R* gene with identified mutations of the described patient (PO_3030) in blue in the transmembrane (p.T640N) and cytoplasmic (p.Q741*) domains. The transmembrane region (black rectangle), box 1–3 sequences (gray rectangles), and locations of membrane proximal and cytoplasmic truncation mutations identified in myeloid malignancies are also depicted.

DISCUSSION

This case report describes the first published patient with SCN who underwent personalized integrative sequencing to identify targetable mutations with intent to treat his refractory AML. His sequencing identified a novel translocation between *MECOM* and *EIF4A*, as well as somatic mutations in *RUNX1* and *CSF3R*. The acquisition sequence of his somatic mutations is unknown as his bone marrow was only sequenced at a single time throughout his clinical course. Testing at different time points would facilitate better understanding of his leukemogenesis, particularly how *ELANE* may cause and/or cooperate with acquired mutations to create disease. *ELANE* mutations are known to lead to apoptosis of myeloid precursors. Misfolding of neutrophil elastase and subsequent unfolded protein response is likely the cause of premature death (Grenda et al. 2007), but there is also evidence that intracellular mislocalization of the defective protein contributes to myeloid apoptosis (Nayak et al. 2015). It is currently debated whether patients with *ELANE* mutations are at higher risk of leukemia compared to SCN with wild-type (wt) *ELANE*. Two separate studies reported that patients with *ELANE* mutations had an increased risk of MDS/AML compared to wt *ELANE* SCN patients (Bellanne-Chantelot et al. 2004, Germeshausen et al. 2013). However, a multicenter study in North American and Australia did not find a difference in malignant transformation rates between mutant wt *ELANE* SCN patients (Rosenberg et al. 2008). Although some reports identify particularly high-risk mutations, including p.C151Y, p.G214R, and defects in the 5′ UTR through exon 2 (Rosenberg et al. 2008; Makaryan et al. 2015), other investigations have reported no association between *ELANE* genotype and disease phenotype (Thusberg and Vihinen 2006; Germeshausen et al. 2013). Still, even if patients with particular *ELANE* mutations associate with higher leukemia risk, it is uncertain if abnormal *ELANE* contributes directly to leukemogenesis or, more likely, indirectly through a greater degree of impaired myelopoiesis driving stress induced changes in hematopoietic stem cells (HSCs).

Although the contribution of *ELANE* mutations remains unclear, significant progress has been made in the role of *CSF3R* mutations and cancer. CSF3R is the receptor for G-CSF, and ligation upon myeloid progenitor cells leads to proliferation, survival, and differentiation into neutrophils (Touw and van de Geijn 2007). CSF3R is a single transmembrane protein and the intracellular domain contains two motifs in the membrane proximal region, termed box 1 and box 2, that are necessary for transduction of proliferative signals, and a distal membrane region important for differentiation of myeloid progenitor lines and transduction of effector signals in mature neutrophils (Touw and van de Geijn 2007). Ligation to CSF3R activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT), phosphoinositide-3 kinase/Akt, and $p21^{RAS}$/mitogen-activated protein kinase (MAPK) pathways (Touw et al. 2013). Negative regulation of G-CSF/CSF3R includes internalization of CSF3R that is

accelerated by G-CSF, dephosphorylation of CSF3R, and inhibition by suppressor of cytokine (SOCS) proteins (Touw and van de Geijn 2007).

Mutations in *CSF3R* were first detected in SCN patients in the 1990s (Dong et al. 1994). The majority of *CSF3R* mutations in SCN are nonsense or frameshift mutations located in the cytoplasmic region resulting in receptor truncation (Klimiankou et al. 2016b). Truncation of the protein has multiple dysregulatory effects including reduced internalization of the receptor, sustained activation of STAT5 signaling, lack of binding of inhibitory SOCS proteins, and increased production of reactive oxygen species (ROS). In essence, loss of the carboxy-terminal fragment hampers the differentiation signals but amplifies the proliferative signals.

It is now believed that these mutations represent premalignant events that are not necessary for leukemia in SCN patients, but that are predictive and likely increase the risk of malignant transformation. This was demonstrated in a survey of SCN patients in which 78% of patients with malignant transformation carried a somatic truncation *CSF3R* mutation compared to 34% without MDS/AML (Germeshausen et al. 2007). The time between acquisition of a *CSF3R* mutation and leukemia development is also variable, from a few months to years later, indicating that the mutation is also not sufficient for leukemia development. In addition, SCN patients can harbor small populations of hematopoietic progenitors with different *CSF3R* mutations, and these mutations can disappear and subsequently reappear over time (Beekman et al. 2012). Two patients with cyclic neutropenia secondary to germline *ELANE* mutations and somatic *CSF3R* mutations have also been identified (Klimiankou et al. 2016a). Although AML/MDS had not been reported in cyclic neutropenia previously, one of these two patients developed AML, suggesting a role of the receptor mutation in cancer formation.

A leading hypothesis is that development of a *CSF3R* mutation provides a growth advantage to HSCs and the acquisition of cooperating mutations, perhaps from elevated ROS production, leads to a dominant *CSF3R*-mutated clone and eventual leukemic population (Touw and Beekman 2013). A common acquired mutation in SCN patients is *RUNX1*. In a study of 31 SCN patients, 20 (65%) patients with MDS/AML had *RUNX1* mutations and 17/20 also had *CSF3R* mutations (Skokowa et al. 2014). In patients who had available historic DNA, 6/10 developed *CSF3R* mutations before *RUNX1*, two patients had *RUNX1* and *CSF3R* mutations detected simultaneously at the earliest available DNA, and two patients only had an acquired *RUNX1* mutation. This report would support the hypothesis that *CSF3R* mutations lead to development of cooperating mutations, in this case *RUNX1* defects that are known to impair hematopoietic differentiation and increase G-CSF sensitivity (Sakurai et al. 2014; Chin et al. 2016).

Unlike SCN, *CSF3R* mutations are uncommon in AML. In pediatric patients, only 10 out of 522 AML patients were found to have *CSF3R* mutations, and no patients with MDS, JMML, or essential thrombocythemia were found to have this defect (Sano et al. 2015). This is also a rare finding in adult AML with a frequency of ~1% (Maxson et al. 2013). Interestingly, *CSF3R* mutations had a high association with CCAAT/enhancer binding protein α (*CEBPA*) mutations in adults (4/14 patients) and *CEBPA* and core binding factor mutations in children (18/28 patients) (Sano et al. 2015; Lavallee et al. 2016; Maxson et al. 2016b). Because of this association with known favorable prognostic factors, patients with AML and *CSF3R* mutations have improved outcomes. This may not be true for chronic myelomonocytic leukemia as one study demonstrated inferior overall survival for patients with somatic *CSF3R* mutations (50% vs. 83% in patients with wt *CSF3R*) (Kosmider et al. 2013).

The cancers most commonly associated with *CSF3R* somatic mutations are chronic neutrophillic leukemia (CNL) at ~80% and atypical chronic myeloid leukemia (aCML) at 40% of cases (Gotlib et al. 2013; Maxson et al. 2013, 2016a). Unlike SCN, the majority of CNL and aCML patients harbor a point mutation on the extracellular surface close to the plasma membrane (termed "membrane proximal mutation"), the most common being p.T618I (Gotlib

et al. 2013). Membrane proximal mutations lead to constitutive receptor activation by stabilizing CSF3R dimers through helix–helix interactions (Liongue and Ward 2014) with a dominant mode of signaling through the JAK/STAT pathway (Maxson et al. 2013). In vitro studies also identified activation of the SRC family kinases (SFKs) and tyrosine kinase nonreceptor 2 (TNK2) in patients with CNL and *CSF3R* truncation mutations. White blood cells from patients with *CSF3R* mutations demonstrated enhanced in vitro sensitivity to tyrosine kinase inhibitors (TKIs), but the degree of inhibition was dependent on the location of the mutation. Both *CSF3R* membrane proximal and truncation mutations were sensitive to JAK kinase inhibitors while only truncation mutations were sensitive to SFK-TNK2 kinase inhibitors. Furthermore, a CNL patient with a p.T618I mutation had a significant decline in white count to JAK kinase inhibition with ruxolitinib, and samples from four patients with *CEBPA*-mutated AML and membrane proximal *CSF3R* mutations showed heightened ruxolitinib sensitivity (Maxson et al. 2013; Lavallee et al. 2016).

Our patient demonstrated a truncation mutation in the cytoplasmic domain of CSF3R as well as two missense mutations in the *RUNX1* gene. He also had a missense p.T640N mutation in the transmembrane region of CSF3R. The finding of a mutation outside the cytoplasmic region and having two mutations on the same allele is a very unique finding in SCN as *CSF3R* mutations are almost exclusively found on the cytoplasmic region and without a second mutation (Klimiankou et al. 2016b). There has been one previous publication detailing a patient with SCN and AML with leukemic clones harboring both a *CSF3R* truncation mutation and a membrane proximal p.T618I mutation (Beekman et al. 2012). Introduction of the p.T618I mutation into murine bone marrow progenitor cells bestowed G-CSF-independent growth and addition of the truncation mutation conferred a higher proliferative capacity (Beekman et al. 2012). The authors hypothesized that the heightened, autonomous signaling through the mutated *CSF3R* was critically important for leukemia formation in this patient. The p.T640N mutation in our patient has been identified in rare AML patients (Forbes et al. 2002) but no SCN patients to date. Interestingly, the p.T640N mutation has also been reported to confer G-CSF independence in murine bone marrow colony assays (Plo et al. 2009) and therefore could have similarly cooperated with our patient's truncation mutation to drive leukemogenesis through hyperactive signaling.

In addition to previous work demonstrating *CSF3R* truncation mutations having JAK kinase inhibitor sensitivity (Maxson et al. 2013; Lavallee et al. 2016), murine experiments have also shown that the p.T640N mutation is sensitive to this class of TKIs (Forbes et al. 2002; Maxson et al. 2016a). The importance of identifying these targetable mutations in SCN patients with MDS/AML is critical given the intolerability to chemotherapy and poor historic transplant outcomes. A recent review of published literature reported survival in only 8/18 patients (44%) (Connelly et al. 2012) and a retrospective analysis of the European Society for Blood and Marrow Transplantation demonstrated only 64% event-free survival (EFS) at 3 yr in transplant recipients (Fioredda et al. 2015). The European branch of the Severe Chronic Neutropenia International Registry (SCNIR) compared transplant outcomes both before and after 2001 and reported an improved survival rate from 27.3% to 83.3% (Zeidler et al. 2013), likely attributed to newer protocols that avoid traditional, toxic chemotherapy agents for cytoreduction prior to conditioning therapy.

Tyrosine kinase inhibition has not been reported in SCN patients who develop *CSF3R* mutations. However, a word of caution is necessary in considering TKIs in SCN. The first concern is that *ELANE*-mutated HSCs may rely on the same pathways as malignant cells for neutrophil production and treatment with kinase inhibitors prior to HCT could result in significant neutropenia. Perhaps a more appropriate setting would be to include kinase inhibitor therapy posttransplant, where donor cells can better support myelopoiesis, and this therapy has already been shown to be safe and effective in post-HCT patients with AML, CML, and Ph+ acute lymphoblastic leukemia (Bar and Radich 2013; Ribera 2013; Schiller et al. 2016). As the

number of patients with SCN who transform to MDS/AML per year in the United States is small with only nine patients reported to the North American SCNIR over the past 4 yr (A Bolyard, pers. comm.), inclusion of these few patients into clinical trials is absolutely necessary to advance our understanding of leukemia and potential benefit and side effects of TKIs in this population.

A second caveat is polymorphisms in *CSF3R* with unknown impact on CSF3R function (Bilbao-Sieyro et al. 2015). This is less likely to occur in the SCN population as the majority of mutations are truncation defects. However, our patient also had a point mutation in the transmembrane region, and without prior publications indicating transforming ability and kinase sensitivity, it would have been difficult to determine the effect of this nucleotide change. This case also demonstrates the importance of sequencing the entire *CSF3R* gene as mutations outside the cytoplasmic domain, although rare in SCN, may be amenable to targeted inhibition. The third point is that cooperating mutations in leukemia cells may render kinase inhibition ineffective.

For our patient, sequencing also identified a novel fusion protein, EIF4A2–MECOM with high *MECOM* gene expression. The unique fusion partner, EIF4A, is a helicase important for mRNA unwinding and initiation of transcription (Raza et al. 2015). Rearrangements of the *MECOM* locus frequently lead to *EVI1* overexpression (Goyama and Kurokawa 2009; Balgobind et al. 2010), although *EVI1* expression in our patient was notably not elevated (data not shown). EVI1 is considered a marker of "stemness," typically restricted to embryonic and adult HSCs. Overexpression is a poor prognostic factor in adult myeloid leukemias, and when identified in pediatric AML, is predominantly found in subtypes with intermediate or poor prognosis (Balgobind et al. 2010; Sato et al. 2014). High *EVI1* expression and possibly high *MECOM* expression have also been associated with TKI resistance in CML (Shimada et al. 2012; Sato et al. 2014). Because *EIF4A-MECOM* is a novel gene fusion, the clinical relevance of high expression of *MECOM* but not *EVI1* in refractory AML with this fusion remains unclear.

Unfortunately, our patient died before we were able to attempt the use of JAK kinase inhibitors. This was similar to the experience of some of our other high-risk patients sequenced in our trial, primarily because of prolonged turnaround time of ~6–7 wk in the early phase of our clinical study (Mody et al. 2015). We recognize this as a limitation to our approach, and this unfortunate outcome highlights the need to sequence these cases early in their clinical course and to reduce the turnaround time for sequencing results. In fact, during the last 12 mo, we have reduced this time interval to <3 wk based on additional computational help from commercial vendors such as Amazon Cloud and additional bioinformatics support. We have also recently established a tumor bio-repository, so in the future when a patient is identified to have actionable findings, we will be able to carry out real-time in vitro testing of potential therapeutic targeted agents in the patient's primary cell line, before attempting it clinically.

A further improvement in our clinical approach to SCN patients would be to incorporate up-front comprehensive sequencing in all patients with newly discovered malignant transformation. Because of the intolerability of traditional chemotherapy agents, attempting to identify targetable susceptible variants by whole-exome sequencing should be applied to all SCN patients and not only those that present with relapsed disease. An alternative approach would be to use targeted sequencing of the *CSF3R* gene only as this is commonly mutated in SCN cancers and likely targetable with TKI. This approach would be an efficient use of sequencing given the high probability of actionable findings. But several disadvantages to this limited approach include exclusion of identifying other possible mutations that may lead to treatment options, providing no additional treatment options in the minority (22%) of SCN patients who do not harbor *CSF3R* mutations, and limiting our ability to accumulate additional knowledge on leukemogenesis in this unique patient population.

In conclusion, our case demonstrates the importance of personalized sequencing in a patient with SCN and AML. He was found to have a transmembrane and truncation mutation in *CSF3R*, a missense mutation in *RUNX1*, and a novel gene fusion, *EIF4A2-MECOM*. His mutations in *CSF3R* are known to up-regulate signaling pathways, in particular the JAK/STAT pathway that may have been amenable to JAK kinase inhibition. However, the impact of an additional *EIF4A2-MECOM* fusion on his leukemia biology and TKI sensitivity remains unclear. Given the intolerability to traditional chemotherapy agents in SCN patients with leukemia, identification of novel oncogenic targets with reduced toxicity agents is critical for improving patient outcomes. To this end, we plan to screen other SCN patients with MDS/AML with integrative clinical sequencing and perform in vitro and in vivo studies to better understand the biology of leukemic mutations and the impact of TKI therapy in a clinical trial in order to improve historically poor outcomes to traditional treatment strategies.

METHODS

Patient History

The patient was cared for by the University of Michigan C.S. Mott Children's Hospital. Details of his clinical course were extracted from his medical records for this report. After enrollment on a prospective integrative clinical sequencing trial (PEDS-MIONCOSEQ), the patient underwent paired tumor/normal whole-exome sequencing and tumor transcriptome sequencing (RNA-seq).

Tissue Acquisition and Integrative Clinical Sequencing

Specifics of the sequencing procedure and bioinformatics analyses have been described previously (Robinson et al. 2013; Wu et al. 2013; Mody et al. 2015). In brief, fresh tumor tissue was obtained during standard of care diagnostic core needle bone marrow biopsy. Histologic sections were evaluated by board-certified pathologists for estimation of tumor content prior to submission of bone marrow samples for sequencing. Nucleic acid preparation and high-throughput sequencing were performed using standard protocols, which adheres to the Clinical Laboratory Improvement Amendments (CLIA). Paired-end whole-exome libraries from bone marrow samples that were matched with normal DNA and with transcriptome libraries either from polyadenylated tumor RNA (Poly(A)$^+$ transcriptome) or from total RNA captured by human all-exon probes (capture transcriptome) were prepared and sequenced using the Illumina HiSeq 2000 and 2500 (Illumina). Aligned exome and transcriptome sequences were analyzed to detect putative somatic mutations, insertions and deletions, copy-number alterations, gene fusions, and gene expression as described previously (Robinson et al. 2013; Wu et al. 2013; Mody et al. 2015).

Extraction of DNA and RNA

Genomic DNA from frozen buccal swabs and paraffin embedded formalin fixed bone marrow sections were isolated using the QIAGEN DNeasy Blood and Tissue Kit. Genomic DNA and total RNA were extracted from the frozen needle bone marrow biopsy using the AllPrep DNA/RNA/miRNA kit (QIAGEN) with disruption using a 5-mm bead on a Tissuelyser II (QIAGEN). RNA was extracted from paraffin embedded formalin fixed tissue sections using the miRNeasy protocol (QIAGEN). RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA Nano reagents (Agilent Technologies).

Preparation of Next-Generation Sequencing Libraries

Transcriptome libraries were prepared using 1–2 µg of total RNA. Poly(A)$^+$ RNA was isolated using Sera-Mag oligo(dT) beads (Thermo Scientific) and fragmented with the Ambion Fragmentation Reagents kit (Ambion). cDNA synthesis, end-repair, A-base addition, and ligation of the Illumina indexed adapters were performed according to Illumina's TruSeq RNA protocol (Illumina). Libraries were size-selected for 250–300 bp cDNA fragments on a 3% Nusieve 3:1 (Lonza) agarose gel, recovered using QIAEX II gel extraction reagents (QIAGEN), and PCR-amplified using PhusionDNA polymerase (New England Biolabs). The amplified libraries were purified using AMPure XP beads (Beckman Coulter). Total transcriptome libraries were prepared as above, omitting the poly(A) selection step and captured using Agilent SureSelect Human All Exon V4 reagents and protocols. Library quality was measured on an Agilent 2100 Bioanalyzer for product size and concentration. Paired-end libraries were sequenced with the Illumina HiSeq 2500 (2 × 100 nt read length). Reads that passed the chastity filter of Illumina BaseCall software were used for subsequent analysis. Exome libraries of matched pairs of tumor/normal genomic DNAs were generated using the Illumina TruSeq DNA Sample Prep Kit. In brief, 1–3 µg of each genomic DNA was sheared using a Covaris S2 to a peak target size of 250 bp. Fragmented DNA was concentrated using AMPure XP beads, followed by end-repair, A-base addition, and ligation of the Illumina indexed adapters. The adapter-ligated libraries were electrophoresed on 3% Nusieve agarose gels and fragments between 300 and 350 bp were recovered using QIAEX II gel extraction reagents. Recovered DNA was amplified using Illumina index primers for eight cycles, purified using AMPure XP beads, and the DNA concentration was determined using a Nanodrop spectrophotometer. One microgram of the library was hybridized to the Agilent SureSelect Human All Exon V4 at 65°C for 60 h following the manufacturer's protocol (Agilent Technologies). The targeted exon fragments were captured on Dynal M-280 streptavidin beads (Invitrogen), and enriched by amplification with the Illumina index primers for nine additional PCR cycles. PCR products were purified with AMPure XP beads and analyzed for quality and quantity using an Agilent 2100 Bioanalyzer and DNA 1000 reagents. We used the publicly available software FastQC to assess sequencing quality. For each lane, we examine per-base quality scores across the length of the reads. Lanes were deemed passing if the per-base quality score boxplot indicated that >75% of the reads had >Q20 for bases 1–80. In addition to the raw sequence quality, we also assess alignment quality using the Picard package.

Gene Fusion Detection

Paired-end transcriptome sequencing reads were aligned to the human reference genome (GRCh37/hg19) using a RNA-seq spliced read mapper TopHat244 (TopHat 2.0.4), with "–fusion-search" option turned on to detect potential gene fusion transcripts. In the initial process, TopHat2 internally deploys an ultrafast short read alignment tool Bowtie (Version 0.12.8) to map the transcriptome data. Potential false-positive fusion candidates were filtered out using "TopHat-Post-Fusion" module. Further, the fusion candidates were manual examined for annotation and ligation artifacts. Junction reads supporting the fusion candidates were realigned using an alignment tool BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) to reconfirm the fusion breakpoint. A full-length sequence of the fusion gene was constructed based on supporting junction reads and evaluated for potential open reading frames (ORFs) using an ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Further, the gene fusions with robust ORFs, the amino acid sequences of the fused proteins, were explored using the Simple Modular Architecture Research Tool (SMART) to examine the gain or loss of known functional domains in the fusion proteins.

Gene Expression

The BAM file "accepted_hits.bam" which was generated by the TopHat mapping module, was utilized to quantify the expression data, through Cufflinks (Trapnell et al. 2012) (Version 2.0.2), an isoform assembly and RNA-seq quantitation package. Structural features of 56,369 transcripts from the Ensemble resource (Ensemble66) was used as an annotation reference for quantifying expression of individual transcripts/isoforms. The "Max Bundle Length" parameter was set to "10000000" and "multi-read-correct" is flagged on to perform an initial estimation procedure to more accurately weight reads mapping to multiple locations in the genome.

Mutation Analysis

Whole-exome sequencing was performed on Illumina HiSeq 2000 in paired-end mode and the primary base call files were converted into FASTQ sequence files using the bcl2fastq converter tool bcl2fastq-1.8.4 in the CASAVA 1.8 pipeline. The FASTQ sequence files generated were then processed through an in-house pipeline constructed for whole-exome sequence analyses of paired cancer genomes. The sequencing reads were aligned to the reference genome build hg19, GRCh37 using Novoalign Multithreaded (Version2.08.02) (Novocraft) and converted into BAM files using SAMtools (Version 0.1.18) (Li et al. 2009). Sorting and indexing of BAM files utilized Novosort threaded (Version 1.00.01) and duplicates reads were removed using Picard (Version 1.74). Mutation analysis was performed using VarScan2 algorithms (Version2.3.2) (Koboldt et al. 2012) utilizing the pileup files created by SAMtools mpileup for tumor and matched normal samples, simultaneously performing the pairwise comparisons of base call and normalized sequence depth at each position. For single-nucleotide variant detection, filtering parameters including coverage, variant read support, variant frequency, *P* value, base quality, homopolymer, and strandedness are applied. For indels analysis, Pindel (Version 0.2.4) was used on tumor and matched normal samples and indels common in both samples were classified as germline and indels present in tumor but not in normal were classified as somatic. Finally, the list of candidate indels as well as somatic and/or germline mutations was generated by excluding synonymous SNVs. ANNOVAR48 was used to functionally annotate the detected genetic variants and positions are based on Ensemble66 transcript sequences. Tumor content for each tumor exome library was estimated from the sequence data by fitting a binomial mixture model with two components to the set of most likely SNV candidates on two-copy genomic regions. The set of candidates used for estimation consisted of coding variants that (1) exhibited at least three variant fragments in the tumor sample, (2) exhibited zero variant fragments in the matched benign sample with at least 16 fragments of coverage, (3) were not present in dbSNP, (4) were within a targeted exon or within 100 bp of a targeted exon, (5) were not in homopolymer runs of four or more bases, and (6) exhibited no evidence of amplification or deletion. In order to filter out regions of possible amplification or deletion, we used exon coverage ratios to infer copy-number changes, as described below. Resulting SNV candidates were not used for estimation of tumor content if the segmented log-ratio exceeded 0.2 in absolute value. Candidates on the Y chromosome were also eliminated because they were unlikely to exist in 2-copy genomic regions. Using this set of candidates, we fit a binomial mixture model with two components using the *R* package flexmix, version 2.3–8. One component consisted of SNV candidates with very low variant fractions, presumably resulting from recurrent sequencing errors and other artifacts. The other component, consisting of the likely set of true SNVs, was informative of tumor content in the tumor sample. Specifically, under the assumption that most or all of the observed SNV candidates in this component are heterozygous SNVs, we expect the estimated binomial proportion of this component to represent one-half of the proportion of tumor cells in the sample. Thus, the estimated binomial proportion

as obtained from the mixture model was doubled to obtain an estimate of tumor content. Copy-number aberrations were quantified and reported for each gene as the segmented normalized $\log_2$-transformed exon coverage ratios between each tumor sample and matched normal sample (Lonigro et al. 2011). To account for observed associations between coverage ratios and variation in GC content across the genome, lowess normalization was used to correct per-exon coverage ratios prior to segmentation analysis. Specifically, mean GC percentage was computed for each targeted region, and a lowess curve was fit to the scatterplot of $\log_2$-coverage ratios versus mean GC content across the targeted exome using the lowess function in *R* (version 2.13.1) with smoothing parameter $f = 0.05$. Partially redundant sequencing of areas of the genome affords the ability for cross validation of findings. We cross-validated exome-based point mutation calls by manually examining the genomic and transcriptomic reads covering the mutation using the UCSC Genome Browser. Likewise, gene fusion calls from the transcriptome data can be further supported by structural variant detection in the genomic sequence data, as well as copy-number information derived from the genome and exome sequencing.

ADDITIONAL INFORMATION

Data Deposition and Access

All somatic mutation information generated from this study has been uploaded to dbGaP (https://www.ncbi.nlm.nih.gov/gap) under accession number phs000673.v2.p1. The patient's germline ELANE variant has been uploaded to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) under accession number SCV000693655.

Ethics Statement

The parents provided informed consent, the patient provided written assent, and the family received mandatory pre-enrollment genetic counseling. The patient was enrolled on a prospective integrative clinical sequencing trial (PEDS-MIONCOSEQ) approved by the University of Michigan Medical Institutional Review Board (IRBMED) (Mody et al. 2015).

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Author Contributions

R.J.M. developed the study concept and design; R.J.M. analyzed and interpreted the data; E.R. was the study coordinator; D.R.R. and Y.-M.W performed clinical sequencing and the precision tumor board; R.J.L. and P.V. performed bioinformatics analysis for sequencing; B.A. developed the figures; J.A.C. and K.W. cared for the patient; J.A.C., K.W., and R.J.M. drafted the manuscript; and all authors assisted with editing of the manuscript.

Competing Interest Statement

The authors have declared no competing interest.

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