HHS Public Access

Author manuscript

Leukemia. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

Leukemia. 2015 October ; 29(10): 2113–2116. doi:10.1038/leu.2015.81.

Next generation sequencing reveals clinically actionable molecular markers in myeloid sarcoma

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Letter to the Editor

Myeloid sarcoma (MS) is a rare hematological neoplasm characterized by the extramedullary proliferation of myeloid blasts disrupting the normal architecture of the affected tissue[1]. It can occur in any organ, but is particularly common in the skin, gastrointestinal tract, lymph nodes, and bone. MS is most often found in patients with previously or recently recognized acute myeloid leukemia (AML) but may occasionally present in the absence of detectable peripheral blood (PB) or bone marrow (BM) involvement (isolated MS). Aberrant tropism of leukemic blasts for extramedullary tissues is poorly understood. Homing of tumor cells is determined by the complex interplay of factors including the expression of chemokine receptors and adhesion molecules, which are themselves controlled by genetic and epigenetic mechanisms. One may hypothesize that MS may differ in its genetic profile from typical AML, and that specific genetic abnormalities increase the tendency of MS cells to home outside of the BM.

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AUTHOR CONTRIBUTIONS

ZL, MS, MKM, KLY and OB performed the experiments. FS, RL, WS and KO contributed patient samples and clinical data. ZL, MS, FS, KO, LJ and GR analyzed and interpreted the data. ZL, FS, KO and GR wrote the paper. GR designed and coordinated the research study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary information is available on Leukemia's website.

Conventional cytogenetic analysis is rarely performed for MS, since it is frequently mistaken for a solid tumor at the time of diagnosis, and samples appropriate for cytogenetic analysis are not collected. Molecular testing thus may be especially important for the diagnosis and treatment of MS. Still, no comprehensive mutation analysis has been conducted in MS, and mutations have only been studied in a limited number of cases, and in a few selected genes such as *FLT3* and *NPM1* [2-3].

Formalin-fixed-paraffin-embedded (FFPE) tissue is frequently the only sample type available from MS, but performing extensive mutation screening using DNA from FFPE MS tissue is technically challenging, and has not been previously attempted. The lack of information about molecular pathogenesis is particularly evident for MS which presents as an isolated lesion, so that concurrent or subsequent BM and PB leukemia cannot be sampled for cytogenetic and molecular studies. We performed next-generation sequencing (NGS)-based mutation analysis in 6 cases of isolated MS, to test the feasibility of systematic, comprehensive mutation testing using DNA from FFPE MS tissue, and to evaluate cases of isolated MS for the presence of mutations implicated in typical AML. Our analysis revealed the presence of mutations in a broad spectrum of AML and myelodysplastic syndrome (MDS)-associated genes and pathways in isolated MS.

Diagnostic FFPE tumor samples were obtained from six patients with isolated MS seen at the University of Chicago Hospital (UCH) or the Universitätsklinikum Carl Gustav Carus Dresden. At both institutions these studies were approved by their respective institutional review boards. Tumor DNA was extracted from FFPE MS tissue, while germline DNA was obtained from remission BM samples. DNA isolation was performed using the Qiagen DNA extraction kits (Qiagen Inc Valencia, CA), according to the manufacturer's instructions. NGS was performed using the Ion Torrent platform (Life Technologies), according to the manufacturer's specifications. Tumor DNA was sequenced with a custom design panel that targets 21 AML and MDS-associated genes, *DNMT3A*, *PDGFRA*, *KIT*, *NPM1*, *WT1*, *FLT3*, *TP53*, *RUNX1*, *MPL*, *SF3B1*, *IDH1*, *GATA2*, *TET2*, *EZH2*, *JAK2*, *CBL*, *ETV6*, *IDH2*, *ASXL1*, *ZRSR2* and *UTX*. The variants detected by NGS were confirmed by Sanger sequencing (primers available in the Supplementary table 2), using the BigDye® Terminator v3.1 Cycle Sequencing chemistry together with automated capillary gel electrophoresis on the ABI 3730xl instrument (Life Technologies, Carlsbad, CA). The available clinical and pathology data for the six studied cases are summarized in Table 1. Cytogenetic information was available for only two cases. Molecular testing for *FLT3*-ITD and *NPM1* mutations was performed previously for four patients, and Case 1 was found to be positive for mutations in both genes (Table 1, Supplemental Figure S1). NGS of 21 genes frequently mutated in AML and MDS identified a total of 12 non-synonymous sequence variants in 8 genes (Table 2, Supplemental Table S1 and Supplemental Figure S2). Among these variants, eight have been reported in the Catalogue Of Somatic Mutations in Cancer database (COSMIC)[4], while others have not been described previously. Testing of DNA from BM samples obtained at the time of remission confirmed the absence of the previously unreported variants in the patients' germline DNA, confirming that they were acquired mutations in leukemia cells (Table 2, Supplemental Figure S3).

Our study validated the reported high frequency of *FLT3* and *NPM1* mutations in MS [2-3]. We confirmed the presence of *FLT3*-ITD and *NPM1* mutations detected by routine clinical laboratory testing in Case 1. In addition, an *NPM1* mutation together with the *FLT3* D835H (*FLT3*-TKD) mutation was detected in Case 5, while Case 4 presented with a three nucleotide insertion in *FLT3*, but was negative for an *NPM1* mutation. *NPM1* and *FLT3* mutations have clear prognostic significance in AML, and their detection at the time of diagnosis is critical for proper risk stratification and clinical management[5].

A missense variant in the *KIT* gene, resulting in a replacement of the amino acid leucine at codon 541 with a methionine (M541L), was found in 4 out of 6 patients (Table 2). This variant is reported as a benign polymorphism in dbSNP with a frequency of 6.4% in general population (rs3822214), but has also been described as a somatic mutation in COSMIC (COSM28026), in association with a variety of tumors including aggressive fibromatosis[6], meningiomas[7], and chronic myeloid leukemia[8]. In all four patients sequence analysis revealed that the M541L variant was present in the germline (Table 2, Supplementary Figure S4). Although it is likely that this polymorphism is benign, some studies suggest that the *KIT* M541L variant may confer increased risk for hematologic malignancies[9]. The high frequency of the M541L allele in our cohort may suggest that this variant warrants further investigation in MS.

We detected mutations that affect several molecular pathways implicated in AML pathogenesis, including epigenetic regulation and RNA splicing.

Two patients had mutations in epigenetic-modifying genes (*TET2*, *ASXL1* and *EZH2*) (Table 2, Supplemental Table S1). Case 6 had mutations in both *TET2* and *ASXL1*. *TET2* alters the epigenome through modulation of hydroxymethylation on DNA, while *ASXL1* functions as an ubiquitinase component of the polycomb repressive complex 2 (PRC2) which initiates dimethylation and trimethylation of lysine 27 of histone H3 (H3K27)[10]. *ASXL1*and *TET2* mutations have been frequently observed in MDS, AML and other hematological malignancies, and have been associated with unfavorable outcome[10]. Case 3 had two novel heterozygous missense mutations, R298H and C571R, in the *EZH2* gene. EZH2 is a histone methyltransferase and constitutes a catalytic unit of the PRC2[10]. The two detected mutations are located in the highly conserved domain II and the cysteine-rich domain (CXC) of the protein, which is required for histone methyl transferase (HMT) activity[10]. Monoallelic or biallelic loss of function mutations in *EZH2* have been described in myeloid malignancies, most commonly in MDS, CMML, primary myelofibrosis (PMF) and AML[10]. Correlative studies have demonstrated that *EZH2* mutations associate with adverse outcome in MDS, PMF and AML[10].

Case 2 carried a mutation in the *SF3B1* gene. *SF3B1* is one of the most commonly mutated genes in MDS, particularly in Refractory anemia with ring sideroblasts (RARS)[11]. However, mutations in *SF3B1* and other genes involved in regulation of splicing have also been implicated in AML pathogenesis[11-12]. A novel frameshift mutation in the *WT1* tumor suppressor gene was observed in Case 1, which also presented with *FLT3* and *NPM1* mutations. The *WT1* frameshift mutation leads to the formation of a premature stop codon and a truncated protein lacking the C-terminal zinc fingers. *WT1* mutations have been

reported in 10–22 % of cases of cytogenetically normal AML[13], and are known to co-occur with *FLT3* and *NPM1* mutations[13]. Recent studies have shown promising responses in patients with AML by using a WT1 peptide vaccine to induce WT1-specific immune responses[14].

In summary, we successfully performed NGS of 21 AML-associated genes using DNA from 6 FFPE MS samples, thus demonstrating the feasibility of performing comprehensive mutation analysis for MS in research and clinical settings. Our study confirmed the previously reported frequent occurrence of *FLT3* and *NPM1* mutations in MS, and identified mutations in a broad spectrum of other AML associated genes. The mutated genes belong to several functional categories shown previously to be significant in AML, including tyrosine kinases (*FLT3* and *KIT*), tumor suppressors (*WT1*), epigenetic modifiers (*TET2, ASXL1* and *EZH2*) and spliceosome proteins (*SF3B1*). Multiple mutations were observed in the same patients, consistent with the notion that a single mutation is not sufficient to engender malignant transformation. The identification of mutations in the genes with a variety of cellular functions in MS patients provides novel insight into molecular pathogenesis of MS, which appears to overlap with pathogenesis of typical AML arising in the bone marrow. As the molecular diagnostics of AML moves towards comprehensive NGS-based testing for prognostically important or targetable genetic abnormalities in large panels of genes, our results suggest that the same approach may be feasible and warranted for MS. In addition, the unusual tropism of the MS blasts for extramedullary tissues may be based on unique subsets of genetic abnormalities that distinguish MS from AML with classical presentation. Future whole exome and whole genome sequencing studies to identify mutations specific for MS may be helpful in elucidating molecular mechanisms that underlie homing and proliferation of leukemia cells outside of the tissue of origin, resulting in systemic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the American Cancer Society Institutional Research Grant (#IRG-58-004) and the University of Chicago Comprehensive Cancer Center Support Grant (#P30 CA14599).

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Table 1

Summary of available demographic, pathology and laboratory data for six cases of MS without PB and BM involvement.

Case	Age/Sex	Tumor location	Histopathology, flow cytometry and immunohistochemistry of the mass	Conventional cytogenetics	Clinically tested molecular mutations
1	61/F	left breast lump	Sheets of blasts with high N:C ratio, vesicular chromatin, and prominent nucleoli in a background of geographic necrosis. Consistent with MS with monocytic/myelomonocytic differentiation. CD45 (dim), CD34 (partial), CD13, CD33+, CD117+, CD15+, CD7+, and HLA-DR+. Strong immunoreactivity for CD68 and lysozyme with focal positivity for CD117 and CD34. Scattered MPO positivity. CD20 and CD3 negative.	not available	FLT3-ITD present; NPM1 mutation present
2	40/F	clavicle	Sheets of blasts with vesicular chromatin. CD45+, CD33+, CD43+, MPO+, CD68+, CD34-, CD56-, CD14-. Negative for cytokeratin, AE1/AE3, CAM 5.2, EMA, S100, TLE1, PAX5, CD20, CD3 and CD5.	not available	No FLT3-ITD; No NPM1 mutation
3	24/M	mediastinal mass	Consistent with MS. Strong aberrant CD56 and CD7 expression.	not available	No FLT3-ITD; No NPM1 mutation
4	38/F	small bowel	Extensive infiltration of small bowel by intermediate to large sized blast cells with variably prominent nucleoli, extending from the mucosal to the serosal surface. Eosinophilic myelocytes noted throughout the biopsies, consistent with MS, FAB M4eo. Positive for CD45 (leukocyte common antigen), CD34 and CD33. Strong expression of MPO, CD43 and CD117. Scattered TdT positive cells, no expression of B- or T-cell markers.	46, XX, inv(16)(p13.1q22)[7]/47, idem, +22[7]/46, XX[7]	No FLT3-ITD
5	73/M	scrotum	Diffuse proliferation of medium sized cells with a high mitotic rate and blast-like appearance with fine chromatin; findings consistent with MS with expression of monocytic markers. Blasts with a monocytic profile: CD15+, CD11b+, CD13+, CD33+, HLA-DR+, CD38+, CD56+; 20% of cells coexpressed CD15 and CD34 by flow cytometry. The cells revealed immunoreactivity for CD43 and vimentin, lysozyme, CD14 (weak) and CD68, and were negative for B- and T-cell markers, CD117 and MPO.	47, XY, +8[20]	NT
6	76/M	testis	Infiltration by blastoid cells of the testis, epididymis, rete testis and proximal spermatic cord into the tunica albuginea, with a negative tumor margin in the spermatic cord. Positive for CD33 and CD68 and negative for CD20 and CD79a.	not available	NT

N:C- nuclear-cytoplasmic; FISH- Fluorescence in Situ Hybridization; CN-LOH-copy-neutral loss of heterozygosity; ITD-internal tandem duplication; NT-not tested

Table 2

Summary of gene mutations identified by next generation sequencing

Patient ID	Gene	Sequence variant	Protein alteration	Variant frequency	Known mutation	Absent in germline DNA
Case 1	FLT3	c.1805_1806ins25	p.K602Nfs*5	40.71	Yes	-
	NPM1	c.860_863dup	p.Y288Cfs*12	25.99	Yes	-
	WT1	c.1137dup	p.R380Tfs*5	35.72	No	Yes
Case 2	SF3B1	c.1868A>G	p. Y623C	41.13	Yes	-
	KIT	c.1621A>C	p. M541L	53.15	Yes	No
Case 3	EZH2	c.1711T>C	p. C571R	26.9	No	Yes
	EZH2	c.893G>A	p. R298H	20.89	No	Yes
	KIT	c.1621A>C	p. M541L	54.87	Yes	No
Case 4	FLT3	c.1737_1738insAGG	p.V579_Q580insR	37.79	No	Yes
	KIT	c.1621A>C	p. M541L	47.07	Yes	No
Case 5	FLT3	c.2503G>C	p. D835H	42.86	Yes	-
	NPM1	c.863_864insCAGG	p.W288Cfs*12	35.03	Yes	-
Case 6	ASXL1	c.1816C>T	p. R606W	52.05	Yes	-
	TET2	c.4879C>T	p. Q1627*	47.8	Yes	-
	KIT	c.1621A>C	p. M541L	50.55	Yes	No