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Utility of Multiplex Mutation Analysis in the Diagnosis of Chronic Myelomonocytic Leukemia

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Abstract

Chronic myelomonocytic leukemia (CMML) is a myeloid neoplasm characterized by both myeloproliferative and myelodysplastic features in addition to persistent peripheral blood monocytosis (>1×10^9/L) that is required for the diagnosis. Clonal cytogenetic abnormalities are identified in only 20%-30% of CMML patients and it can be diagnostically challenging to exclude reactive monocytosis in some cases. Several gene mutations have recently been implicated in the pathogenesis of CMML that involve tyrosine kinase-signaling pathways, transcriptional regulation, metabolism, splicing, and epigenetic regulatory mechanisms. This study was designed to assess recurrent mutations in CMML using a multiplex mass spectrometry based approach, and to determine the utility of mutation screening in CMML, particularly in cytogenetically normal cases. The Oregon Health and Science University (OHSU) surgical pathology database was searched from 2010-2012 to identify consecutive CMML cases fulfilling WHO diagnostic criteria. Cytogenetic analyses and molecular studies were performed on the diagnostic bone marrow specimens. DNA extracts were screened for point mutations using a multiplex PCR panel with mass-spectroscopy read out that covers 370 point mutations across 31 genes associated with leukemia. Of the 48 CMML cases identified in the OHSU files, 43 had available cytogenetic studies. Of these, 10/43 cases (23%) had cytogenetic abnormalities including: trisomy 8 (n=4), trisomy 21 (n=2), deletion 7q (n=1), del 13q (n=1), complex karyotype (n=1) and t (3;3) (n=1). Of the cases with cytogenetic data, 22 had available DNA for mutation analysis, and 11 of these genotyped cases (50%) had detectable mutations in the following genes: CBL (n=3), CKIT, JAK2, KRAS (n=2), NRAS (n=3) and NPM1. Nine cases with detected mutations had normal cytogenetics. Concomitant molecular and cytogenetic abnormalities were seen in 2 cases: one case with trisomy 8 and CBL C384Y and one case with trisomy 21 and JAK2 V617F. In the 22 cases with available cytogenetic and molecular data, performing routine multiplex molecular testing in addition to cytogenetic studies in CMML patients increased the detection of genetic abnormalities from 23% (5/22) to 64% (14/22), with frequent CBL and RAS mutations in our cohort. This study confirms that gene mutations are common events in CMML, and multiplex mutation analysis can be applied in the clinical setting to assist in diagnosis and may identify actionable mutations for targeted therapy.

Keywords: Chronic myelomonocytic leukemia; Clonal cytogenetic abnormalities; Multiplex mutation analysis

Introduction

Chronic myelomonocytic leukemia (CMML) is a clonal hematopoietic stem cell disorder characterized by both myelodysplastic and myeloproliferative features and is defined by the presence of an absolute monocytosis (>1×10^9 L^{-1}) according to the 2008 World Health Organization (WHO) classification [1]. CMML is subclassified as CMML-1 with <10% bone marrow and ≤ 5% peripheral blasts, and CMML-2 with 10-19% bone marrow and/or 5-19% peripheral blasts or when Auer rods are identified. This subdivision has been shown to confer a prognostically significant survival difference, with a median survival of 20 months for CMML-1 and 15 months for CMML-2 (p<0.005) [2-4]. The same study showed an increased cumulative risk of evolution to AML in CMML-2 (p<0.001) [4].

There are recurring cytogenetic abnormalities reported in CMML which include: monosomy 7, trisomy 8, complex karyotype involving ≥ 3 abnormalities, trisomy 21, isochromosome 17, deletion 5q, and deletion 20q [5]. Such et al. [6] has previously shown cytogenetic abnormalities to be prognostic in CMML [6]. Based on their survival analysis, they defined three cytogenetic risk categories: low risk (normal karyotype and loss of Y chromosome as a single anomaly), high risk (trisomy 8 alone or with one additional abnormality, abnormalities of chromosome 7 alone or with one additional abnormality and complex karyotype), and intermediate risk (all other single or double abnormalities) [6]. However, none of these cytogenetic findings are specific for CMML and the overall incidence of chromosomal abnormalities is approximately 20-30% [1]. A significant majority of CMML cases are diagnosed without a cytogenetic abnormality to support the diagnosis or allow risk stratification.

Several gene mutations have recently been implicated in the pathogenesis of CMML and involve tyrosine kinase-signaling pathways, transcriptional regulation, epigenetic regulatory mechanisms, and genes involved in the splicing machinery [7-15,16]. In this study, we evaluated the frequency of cytogenetic abnormalities in CMML and report our single institution experience of mutational analysis with a multiplex mass spectrometry based approach.

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Material and Methods

Cases

After Institutional Review Board approval, pathology files of Oregon Health & Science University were searched for peripheral blood and bone marrow specimens with a diagnosis of CMML; 48 cases were identified (2010-2012). Clinical data were collected including age, sex, white blood cell count, absolute monocyte count, and CMML sub classification according to WHO 2008 criteria.

Multiplex mutation screening

DNA was extracted and purified from bone marrow aspirate specimens, or formalin-fixed, paraffin-embedded tissue using standard protocols (Qiagen Qiamp Mini kit, Valencia, CA) in 22 CMML cases. DNA extracts were screened for mutations in genes known to be associated with leukemia (Table 1) using a multiplex PCR panel with mass spectroscopy readout (Sequenom Mass Array) as previously described [17]. The mutation panel covers 370 point mutations across 31 genes encoding for the following: receptor tyrosine kinases (FLT3, KIT, FMS, PDGFRB, FGFR4, NTRK1, MET), cytoplasmic-tyrosine kinases (JAK1, JAK2, JAK3, FES, ABL1), signaling molecules (CBL, CBLB, NRAS, KRAS, HRAS, SOS1), serine/threonine kinases (AKT1, AKT2, AKT3, BRAF), receptors (MPL, NOTCH1), phosphatase (PTPN11), metabolic pathway genes (IDH1, IDH2), tumor suppressor (FBXW7), and transcription factors (GATA1, NPM1, PAX5). Point mutations identified by multiplex PCR/mass spectrometry were confirmed by direct DNA sequencing on an ABI3130 sequencer using the Big Dye terminator method.

Cytogenetics

Standard cytogenetic karyotype analysis on the diagnostic bone marrow aspirate material was performed in 35 out of 48 cases of CMML. In eight cases only fluorescent in situ hybridization (FISH) analysis was performed. The specimen was cultured for 24 to 48 hours in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA). Cells were harvested, and slides were prepared according to standard laboratory protocol. Slides were treated with 10% trypsin (Invitrogen) for 40 to 55 seconds followed by Wright stain (Sigma, St Louis, MO) for 2 minutes and 30 seconds. These Trypsin Writght (GTW)-banded preparations were analyzed on a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY) with Applied Imaging CytoVysion software (Genetix, San Jose, CA). When possible, at least 20 metaphase cells were examined for each case. In eight cases, only fluorescent in situ hybridization (FISH) analysis was performed using the following probes: 5q EGR1 (5q31), D7S486 (7q31), CEP 8 (SA), D20S108 (20q12), MLL (11q23) break-apart, TP53 (17p13.1).

Statistics

Statistical analysis was performed using Chi-square to compare the detection rate of genetic abnormalities by cytogenetic testing alone vs. combined cytogenetic and molecular testing. A p value of <0.05 was considered statistically significant.

Results

Patient demographics

Our cohort consisted of 48 patients with CMML, 17 (35%) females and 31 (65%) males. The median age was 66 (range 42-88). The white blood cell count ranged from 1.5 to 96×10^9 L^{-1} with a median value of 19×10^9 L^{-1}. The median absolute monocyte count was 3.3×10^9 L^{-1} (range 1 to 40×10^9 L^{-1}). Based on 2008 WHO classification criteria, 35 (73%) patients were diagnosed with CMML-1, and 13 (27%) with CMML-2 (Table 2). Six of 48 patients transformed to AML.

Frequency of chromosomal abnormalities

In the 43 of 48 cases of CMML that had an available specimen for cytogenetic/FISH studies, the karyotype was normal in 33 (77%) and abnormal in 10 (23%) cases. The most frequent cytogenetic abnormalities were trisomy 8 (n=4) and trisomy 21 (n=2). Other cytogenetic abnormalities included: deletion 7q, deletion 13q, complex karyotype, and t (3;3). If classified according to the cytogenetic risk stratification established by Such et al. [6] more than half of the cases with abnormal cytogenetic analysis (6 out of 10 cases) harbored high-risk cytogenetic abnormalities including 4 cases of trisomy 8, 1 case with deletion 7q, and 1 case with complex karyotype. Cytogenetic analysis was performed in all six cases which transformed to AML. Trisomy 8 was detected in one case and trisomy 21 identified in another; the remaining cases had a normal karyotype (Table 3).

Results of multiplex mutation screening

22 cases had available DNA for multiplex mutation screening. Of these, 11 cases (50%) had mutations in the following genes: CBL (n=3), NRAS (n=3), KRAS (n=2), CKIT, JAK2 and NPM1. Of the 11 cases with mutations, nine had normal cytogenetics and two had concomitant cytogenetic abnormalities. RAS mutations were the most commonly detected (23%, 5/22); they were all identified in cytogenetically normal cases and distributed equally amongst CMML-1 and CMML-2. None of the cases with RAS mutations transformed to AML.

Combining multiplex mutation screening and cytogenetic analysis: utility of mutation screening in cytogenetically normal CMML

There were 22 cases of CMML in which both cytogenetic analysis and multiplex mutation screening were performed (Figure 1). Of these

R-tyrosine kinase	FLT3	KIT	FMS	PDGFRB	FGFR4	NTRK1	MET
C-tyrosine kinase	JAK1	JAK2	JAK3	FES	ABL1		
Signaling molecule	CBL	CBLB	NRAS	KRAS	HRAS	SOS1	
Serine/threonine kinase	AKT1	AKT2	AKT3	BRAF			
Cytokine receptor	MPL						
Receptor	NOTCH1						
Phosphatase	PTPN11						
Metabolic pathway	IDH1	IDH2					
Tumor suppressor	FBXW7						
Transcriptional factor	PAX5	NPM1	GATA1				

Table 1: Genes tested using multiplex mass spectrometry based approach.

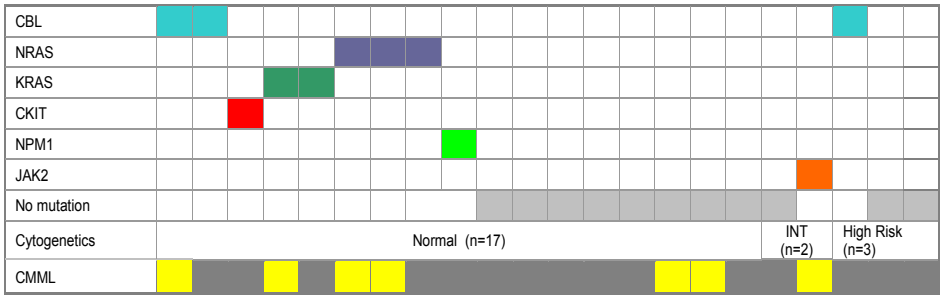
Case	Age	Sex	Diagnosis	WBC	AMC*	Mutated Gene	Codon	Cytogenetics
1	70	F	CMML-2	38	16	*CBL*	C404Y	Normal
2	72	M	CMML-1	36.8	40	*CBL*	R420P	Normal
3	66	F	CMML-1	53	7.9	*CKIT*	D816V	Normal
4	57	M	CMML-2	29.1	8.1	*KRAS*	A146T	Normal
5	79	M	CMML-1	16	22	NEG		Normal
6	66	M	CMML-1	39.8	14	NEG		Normal
7	67	M	CMML-1	28.5	10.3	NEG		Normal
8	72	M	CMML-1	7.2	3.6	NEG		Normal
9	NA	M	CMML-2	NA	NA	NEG		Normal
10	75	M	CMML-2	65	30	NEG		Normal
11	52	M	CMML-1	30.3	1.8	NEG		Normal
12	61	F	CMML-1	20.4	3.3	NEG		Normal
13	60	M	CMML-1	2.1	0.2	*NPM*	W288fs*12	Normal
14	56	F	CMML-1	62.5	14.4	*NRAS*	G12D	Normal
15	65	M	CMML-2	26	NA	*NRAS*	G12S	Normal
16	66	M	CMML-2	4.4	1.5	*NRAS*	G12V	Normal
17	60	M	CMML-1	8.9	1.5	*KRAS*	G13C	Normal
18	58	F	CMML-1	7.7	1.6	NEG		t(3;3)
19	67	M	CMML-2	10.9	16	*JAK2*	V617F	TRISOMY21
20	76	F	CMML-1	11.6	2.7	NEG		TRISOMY 8
21	77	M	CMML-1	1.5	0.8	*CBL*	C384Y	TRISOMY 8
22	68	F	CMML-1	23.3	7.9	NEG		COMPLEX

AMC = absolute monocyte count

Table 2: Clinical characteristics, molecular mutations and cytogenetic findings in 22 CMML patients with parallel cytogenetic and mutation testing performed.

Diagnosis	Mutation Screening	Cytogenetics
CMML-2/AML	Negative	Normal
CMML-2/AML	Not done	Normal
CMML-1/AML	CBL C384Y	Trisomy 8
CMML-1/AML	CKIT D816V	Normal
CMML-2/AML	JAK2 V617F	Trisomy 21
CMML-1/AML	Not done	Normal

Table 3: Mutation screening and cytogenetic analysis in cases with transformation to AML.



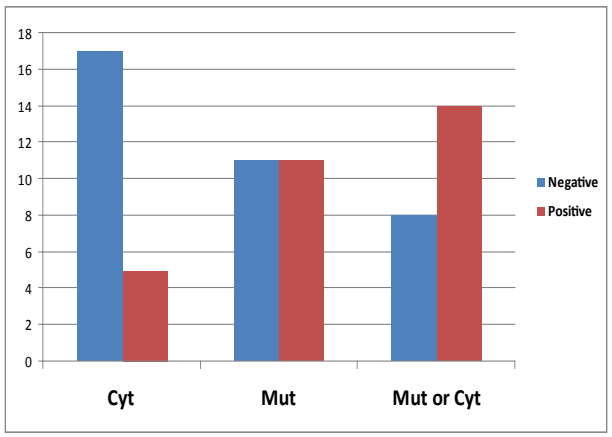
Cytogenetic risk classification based on Such et al. prognostic risk stratification. INT = Intermediate risk cytogenetics Yellow: CMML-2, Grey: CMML-1

Figure 1: Distribution of mutations in CMML across cytogenetic groups.

cases, cytogenetic analysis alone demonstrated clonal abnormalities in 23% (5/22). In comparison to cytogenetic analysis, gene mutation screening demonstrated a higher mutation frequency (11/22, 50%). Two cases had concomitant gene mutations and cytogenetic abnormalities including one case with trisomy 8 and CBL C384Y and one case with trisomy 21 and JAK2 V617F. On the other hand, 9 of 11 (22%) cases with gene mutations had normal cytogenetics. In these cases, identification of a gene mutation provided strong support for a diagnosis of CMML where otherwise no clonal abnormalities were detected by conventional cytogenetic analysis. Parallel cytogenetic and molecular testing in CMML resulted in a combined detection rate of genetic abnormalities of 64% (14/22), which was significantly higher than that identified with cytogenetic analysis alone ($p < .05$) (Figure 2).

Discussion

CMML is a heterogeneous neoplasm characterized by variable clinical presentations, disease course, hematologic findings, and genetic abnormalities [18]. The degree of heterogeneity can pose diagnostic challenges, and in some cases exclusion of reactive causes of monocytosis is difficult. Cytogenetic studies can help identify clonal abnormalities although the karyotype is frequently normal. In a study of 414 CMML patients, 27% of CMML cases harbored cytogenetic



Detection rate of clonal/genetic abnormalities in 22 CMML cases, by cytogenetic testing alone (Cyt), multiplex mutation testing (Mut), and combined cytogenetic and multiplex mutation testing (Mut or Cyt)

Figure 2: Detection rate of clonal/genetic abnormalities in 22 CMML cases.

abnormalities while 73% had normal cytogenetics [6]. In this study, a three tiered cytogenetic risk stratification is described having independent prognostic significance in multivariate analysis with a five year overall survival of 35%, 26% and 4% for low, intermediate, and high risk cytogenetic groups, respectively. Similarly, in our cohort, cytogenetic analysis of 43 CMML patients detected a clonal abnormality in only 23% of cases.

Recently, a large number of recurrent gene mutations in CMML have been reported. Detection of a characteristic gene mutation may be very informative, particularly in cases where the diagnosis is not clear and there is a normal karyotype by cytogenetic analysis. In this study we genotyped CMML using a multiplex mass spectrometry based approach, and identified gene mutations in 50% of our CMML cohort. This overall incidence is lower than previously reported (75%), although prior studies also tested for genes involved in epigenetic regulation (TET2, ASXL1, EZH2) and genes involved in the splicing machinery (SRSF2), which were not analyzed in this study [9,19,20].

The incidence of specific mutations in our study, including *CBL, KRAS, NRAS, NPM, and JAK2*, is in agreement with those that have been reported in previous studies [9,19,20]. In 9 of 11 cases with gene mutations, cytogenetic analysis was normal, and in several of these cases, the initial diagnosis of CMML was difficult. As an example, one patient in our cohort was diagnosed with a peripheral blood monocytosis (3.1 K/cu mm) with a normal total white blood cell count (8.2 K/cu mm). The monocytes exhibited mature morphology and there were no distinctive dysplastic features. There was a mild anemia, but otherwise neutrophil and platelet counts were normal and cytogenetic analysis was normal. A reactive process was suspected; however gene mutation screening performed on the peripheral blood identified a CBL mutation (known to be associated with CMML) and the diagnosis was thus revised as CMML. This illustrates the diagnostic utility of mutation analysis in CMML, especially in cases with normal cytogenetics, which comprise approximately 69-73% of CMML cases [6]. Moreover, certain mutations, such as *RAS* mutations, may predict sensitivity of these tumors to drugs targeting the *RAS/RAF/MAPK* pathway [21,22]. To date, the prognostic significance of mutations in CMML is unknown, and further studies are needed.

The prognosis of CMML is still poor with only a few therapeutic options including hydroxyurea to control for myeloproliferation and hypomethylating agents to delay disease progression [23,24]. The only potentially curative therapeutic option for CMML is allogeneic stem cell transplantation, although most patients will be excluded by age and comorbidities [25]. In 2 of our CMML cases with CBL C404Y and KRAS A146T, we were able to assess the molecular status after treatment. Both patients received several cycles of a hypomethylating agent azacitidine. In both post-treatment cases, we identified the presence of the same genetic mutation, indicating the persistence of the clone after hypomethylating agent therapy. Thus, another potential utility of knowing the molecular status in CMML patients would be to monitor response to therapy and early detection of relapse in the post-transplant setting – although this indication would likely require genotyping assays with well-defined quantitative linearity and limits of detection.

Our comprehensive analysis confirms that gene mutations are common events in CMML, and revealed the presence of mutations in 50% of patients with CMML. This study shows that multiplex mutation analysis can be applied in the clinical setting and can aid in establishing a somatic genetic abnormality to confirm and monitor a diagnosis of CMML, particularly in cases with normal cytogenetics. Although our current approach using a multiplex PCR and mass-spectrometry based analysis did not include a number of recently reported mutations in epigenetic (ASXL1, TET2, EZH2) and splicing genes (SRSF2, SF3B1, U2AF35) [9,19,20], we have recently developed and validated a larger mutation panel including these (and other) genes using a next-generation sequencing approach and are now using this panel for all newly diagnosed leukemias as well as myelodysplastic syndromes. This should further improve our diagnostic sensitivity in CMML, support the development of targeted clinical trials for select patients, and help monitor disease progression and/or evolution during treatment.

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