Spliceosomal gene mutations are frequent events in the diverse mutational spectrum of chronic myelomonocytic leukemia but largely absent in juvenile myelomonocytic leukemia

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ABSTRACT

Chronic myelomonocytic leukemia is a heterogeneous disease with multifactorial molecular pathogenesis. Various recurrent somatic mutations have been detected alone or in combination in chronic myelomonocytic leukemia. Recently, recurrent mutations in spliceosomal genes have been discovered. We investigated the contribution of *U2AF1*, *SRSF2* and *SF3B1* mutations in the pathogenesis of chronic myelomonocytic leukemia and closely related diseases. We genotyped a cohort of patients with chronic myelomonocytic leukemia, secondary acute myeloid leukemia derived from chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia for somatic mutations in *U2AF1*, *SRSF2*, *SF3B1* and in the other 12 most frequently affected genes in these conditions. Chromosomal abnormalities were assessed by nucleotide polymorphism array-based karyotyping. The presence of molecular lesions was correlated with clinical endpoints. Mutations in *SRSF2*, *U2AF1* and *SF3B1* were found in 32%, 13% and 6% of cases of chronic myelomonocytic leukemia, secondary acute myeloid leukemia derived from chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia, respectively. Spliceosomal genes were affected in various combinations with other mutations, including *TET2*, *ASXL1*, *CBL*, *EZH2*, *RAS*, *IDH1/2*, *DNMT3A*, *TP53*, *UTX* and *RUNX1*. Worse overall survival was associated with mutations in *U2AF1* (P=0.047) and *DNMT3A* (P=0.015). *RAS* mutations had an impact on overall survival in secondary acute myeloid leukemia (P=0.0456). By comparison, our screening of juvenile myelomonocytic leukemia cases showed mutations in *ASXL1* (4%), *CBL* (10%), and *RAS* (6%) but not in *IDH1/2*, *TET2*, *EZH2*, *DNMT3A* or the three spliceosomal genes. *SRSF2* and *U2AF1* along with *TET2* (48%) and *ASXL1* (38%) are frequently affected by somatic mutations in chronic myelomonocytic leukemia, quite distinctly from the profile seen in juvenile myelomonocytic leukemia. Our data also suggest that spliceosomal mutations are of ancestral origin.

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Introduction

Chronic myelomonocytic leukemia (CMML) is a clonal stem cell disorder characterized by absolute monocytosis with myeloproliferative and myelodysplastic features. Despite pathomorphological similarities among cases, the molecular pathogenesis may be different and involves diverse somatic mutations occurring at varying frequencies along with predominating unbalanced chromosomal abnormalities.[1-3] Juvenile myelomonocytic leukemia (JMML) is pathophysiologically related to CMML but is associated with a different spectrum of molecular lesions. Recurrently mutated genes in CMML include *CBL*, *RAS*, *DNMT3A*, *IDH1/2*, *EZH2*, *RUNX1*, *TET2*, *ASXL1* and *UTX* in various combinations.[1-3] Many of these mutations are probably secondary events and a clear set of ancestral genetic events for CMML has not been established. It is worth noting that no pathogenic molecular lesions can be found in some cases, suggesting that additional, as yet unidentified defects may exist.

Recently, we and others described mutations in genes involved in spliceosomal function.[4,5] Among them, in preliminary screens *U2AF1* and *SRSF2* mutations appeared to be associated with CMML, while *SF3B1* mutations were most commonly encountered in lower risk myelodysplastic syndromes, in particular refractory anemia and cytopenias with ring sideroblasts (RARS, RCMD-RS).[4-7] *SF3B1* mutations were also detected in chronic lymphocyte leukemia.[8-10] *U2AF1* is a splicing factor belonging to the SR (serine-arginine rich) fami-

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ly and encodes the small subunit of U2 auxiliary factor, required for the U2-snRNP to bind to pre-RNA.[11,12] *SF3B1* is one subunit of the 3B complex which is part of the snRNP that anchors U2 snRNP to pre-RNA.[13] *SRSF2* is a member of the SR family of pre-mRNA splicing factors, which constitute part of the spliceosome.[14] It contains an RNA recognition motif (RRM) for binding RNA and an RS domain for binding other proteins. The RS domain facilitates interaction among different SR splicing factors. In addition to being critical for mRNA splicing, the SR proteins are involved in mRNA export from the nucleus and in translation.[14]

In the current study, we aimed to determine the frequency, clinical features and genotypic associations of somatic mutations of the three most frequently recurring, splicing-related genes in CMML and JMML.

Design and Methods

Patients

We assessed 87 patients with a diagnosis of CMML or secondary acute myeloid leukemia (sAML) derived from CMML and 49 cases of JMML. The study was approved by the Institutional Review Boards of Cleveland Clinic (5024 approval date: 3/22/2011) and Nayoga University (issue # 328,328-2 issue date: 1/27/2006). Written informed consent was obtained according to institutional protocols. Patients were selected based on clinical and pathomorphological criteria, according to the 2008 World Health Organization classification.[15] Clinical data were collected at the time of sampling and a survival analysis was calculated from the sampling date. Prognosis was assessed according to the MD Anderson algorithm for CMML.[16] Previously, we reported genotyping results for *UTX, CBL, EZH2, ASXL1, RAS, TET2, DNMT3A,* and *IDH1/2* for 72 of these patients[2]. This original cohort has been expanded by an additional 15 patients and we have also typed both the original cohort and the new patients for the presence of *TP53* and *RUNX1* mutations. Spliceosomal mutations have been reported for 44 of the patients included in a previous study[6] but these patients were not investigated for other mutations, including the newly added *RUNX1* and *TP53*. Most importantly, we screened these patients for the presence of the three most common spliceosomal mutations. Our cohort included 48 patients with CMML-1, 16 with CMML-2 and 23 with sAML with a clear history of antecedent CMML (Table 1). The JMML cases were screened for mutations in *ASXL1, CBL, RAS, TET2, DNMT3A, EZH2, IDH1/2* and the three spliceosomal genes. The diagnosis of JMML was based on internationally accepted criteria[17] and excluded patients with Noonan syndrome.

Single nucleotide polymorphism array karyotyping analysis

Metaphase cytogenetic and genome-wide Human SNP array 6.0 were used for karyotyping according to previously described protocols.[2] A rational algorithm[18,19] was applied to allow for recognition of somatic *versus* germ line lesions. Defects not previously described as recurrent and those that were not excluded by the above mentioned algorithms were confirmed by analysis of germ line DNA derived from $CD3^+$ lymphocytes separated by magnetic beads.

Mutational screening

Mutations were detected following polymerase chain reaction-based amplifications of DNA derived from bone marrow as previously described.[2] Amplification included exons of *EZH2* (all exons), *TET2* (all exons), *ASXL1* (exon 12), *UTX* (all exons), *TP53* (all exons), *RUNX1* (all exons), *DNMT3A* (exons 18-23), *NRAS* (exons 1 and 2), *KRAS* (exons 1 and 2), *SRSF2* (exons 1 and 2), *SF3B1* (exons 13-16) and *U2AF1* (exons 2 and 6). Sequencing was carried out using standard techniques on an AB1 3730x1 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). All mutations found and confirmed as somatic in other sources, including the Wellcome trust Sanger database (*http://www.sanger.ac.uk*) and in previous studies, were not further confirmed by the analysis of germ line DNA. Likewise, the confirmatory analysis was not conducted for many of the invariant, well-known mutations (e.g., *JAK2, TP53* or *RAS*). All the new putatively somatic lesions were confirmed by sequencing germ line DNA derived from paired $CD3^+$ cells. A list of primers for spliceosomal genes is given in *Online Supplementary Table S1*.

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess overall survival in subgroups of patients; the results were compared using the log-rank test. For comparison of the frequencies of mutations between disease groups, categorical variables were analyzed using Fisher's exact test. A two-sided *P* value of less than 0.05 was considered to be statistically significant. Response criteria suggested by the International Working Group[20] were used to describe the effects of treatment with a hypomethylating agent.

Results

Clinical features of the patients with chronic myelomonocytic leukemia

We studied a cohort of patients with current or antecedent CMML (Table 1). The median follow-up period was 9.2 months (range, 0-67 months). Most patients (77%) had abnormal cytogenetics by both metaphase cytogenetics and single nucleotide polymorphism array-based karyotyping with a similar rate of abnormal cytogenetics in sAML (87%) and CMML-1 (69%; *P*=0.23) or CMML-2 (81%; *P*=0.67). A total of 43% of patients had uniparental disomy (UPD) (sAML 70%, CMML-2 56% and CMML-1 23%) including: UPD7q in nine patients, UPD4q in seven, UPD11q in five, UPD2p in four, UPD1p or UPD21q in three patients each, and UPD6p in two patients. Single cases of UPD were found for 3p, 5q, 9p, 13, 13q, 14q, 16p, 17p and 17q. Trisomy 8 and del 7/7q were found in seven patients each (Table 2). In 21 patients, less frequently recurrent chromosomal abnormalities were detected (*Online Supplementary Table S2*). According to the modified MD Anderson risk stratification for CMML, 24,

Table 1. Characteristics of the CMML patients participating in the study.

Diagnosis	Number	Age(year) Range	Sex M/F	Cytogenetics Abnormal/Normal
CMML-1	48	38-89	32/16	32/15 (68%)
CMML-2	16	53-81	11/5	13/3 (81%)
sAML	23	45-82	15/8	20/3 (87%)
Total	87	38-89	55/28	62/19 (77%)
JMML	49	1-75 months	32/15	27/21 (56%)

CMML: chronic myelomonocytic leukemia; sAML: secondary acute myeloid leukemia derived through progression of primary CMML; JMML: juvenile myelomonocytic leukemia.

6, and 13 patients belonged to low, intermediate-1 and intermediate-2 risk groups, respectively. For comparison, we also studied 49 patients with JMML; 55% had abnormal cytogenetics by single nucleotide polymorphism array and/or metaphase cytogenetics with UPD11q and UPD17q in four cases and one case, respectively. Del7/7q was found in 16% of JMML cases; 26 patients showed other miscellaneous chromosomal abnormalities (gains of 1p, 1q, 7p, 7q, 10p, 13, 1, 5q, 21, X; losses of 1q, 2p, 5q, 6q, 8p, 12p, 17q,18q, 19p, 20 and Y; Table 2).

Mutations in spliceosomal genes

Earlier studies[5,6] indicated that mutations in *SF3B1*, *U2AF1* and *SRSF2* were frequent in myeloid malignancies. In our CMML cohort, *SF3B1* mutations were found in 6% of patients (Figure 1A). All mutations were heterozygous and located on exons 13-16. The mutation for *SF3B1* exon 15 (K700), which is most common in myelodysplastic syndromes, was not found in our CMML patients, but, of course unlike *U2AF1* and *SRSF2*, *SF3B1* mutations are quite rare in CMML. There were four missense mutations, including K666N; c.1998 G>T in two patients, K666R; c.1997 A>G, E622D; c1866 G>T and R625L; c.1874 G>T in one patient each (*Online Supplementary Table S2*). In support of recent data,[4,7] prominent ring sideroblasts were found on examination of bone marrow in one of the mutant cases.

U2AF1 mutations were found in 13% of patients (Figure 1A, *Online Supplementary Table S2*) and were evenly distributed among all subcategories (4, 3 and 4 in CMML-1, CMML-2 and sAML, respectively); six of the patients had a missense mutation c.470A>C p.Q157P, of which all were heterozygous. In four cases, a heterozygous mutation at c.101C>T p.S34F was found. A missense heterozygous mutation at c.467A>G p.R156Q was found in one patient (*Online Supplementary Table S2*). Mutations were located in both zinc finger domains surrounding the *U2AF1* homology motif (UHM) motif. Of note, five out of 11 mutant cases have concomitant LOH7q (3 del7/7q and 2 UPD7q).

SRSF2 mutations were found in 32% of patients (Figure 1A, *Online Supplementary Table S2*), including four missense mutations located in exon 1, specifically in a region between the RRM and the SR region. A total of 16 patients had a mutation at c.284 C>A, p. P95H, five had a missense mutation at c.284 C>G, p.P95R, three at c.284 C>T, p. P95L and two at c.283 C>G, p.P95A. Interestingly, among the 26 mutant cases, five were associated with abnormalities on chromosome 7, and two had trisomy 8. We found no frame shifts in *SRSF2* near codon 95, previously reported to be frequent types of *SRSF2* lesions.[21]

Genotypic associations with other mutations

In addition to genotyping genes frequently mutated in CMML, we detected *TET2*, *ASXL1*, *CBL*, *EZH2*, *RAS*, *IDH1/2*, *DNMT3A* and *UTX* mutations in 48%, 38%, 15%, 6%, 9%, 3%, 9% and 7% of patients, respectively. We also found *TP53* and *RUNX1* mutations in 5% and 17% of patients, respectively (Figure 1). The *JAK2* V617F mutation was present in one case of seemingly typical CMML.

Spliceosomal gene mutations seem to be mutually exclusive from each other but were frequently associated with other non-spliceosomal gene mutations that we screened for. Within the cohort of 26 *SRSF2* mutant cases, 16 had coexisting *TET2* mutations, 12 had *ASXL1* mutations, seven had *RUNX1* and five had *CBL* mutations. Among 11 *U2AF1* mutant cases, three, five and three had *TET2*, *ASXL1* and *RUNX1* mutations, respectively. The five *SF3B1* mutant cases were associated with *TET2*, *ASXL1*, *RUNX1*, *UTX* and *DNMT3A* mutations. In general, *TET2*, *RUNX1* and *ASXL1* mutations seem to be most frequently associated with spliceosomal mutations. The number of patients harboring more than two mutations was significantly higher in the group of patients with *SRSF2* mutations than in the group with the wild-type gene (P=0.001), while *SF3B1* and *U2AF1* mutations were not associated with a higher mutational burden. It is of note that only *CBL* mutations were found to be significantly linked to abnormal karyotype (P=0.05; *Online Supplementary Table S3*).

Impact of mutations on clinical features and outcomes

Cross-sectional analysis demonstrated that more advanced stages of disease (i.e. CMML-1 *versus* CMML-2 *versus* sAML) were not associated with a higher mutational burden. Individually, *DNMT3A*, *TP53* and *IDH1/2* mutations were more prevalent in sAML than in CMML-1 (26%, 13%, 9%, respectively, in sAML *versus* 2%, 0%, 0%, respectively, in CMML-1) (Figure 1B). *CBL* and *TET2* mutations seemed to be evenly distributed in cases of CMML-1, CMML-2 and sAML (17%, 13%, 13%, respectively, for *CBL* and 50%, 38%, 52%, respectively for *TET2*). *SF3B1*, *RAS* and *U2AF1* seemed more prevalent in advanced stages of disease, although the association was not statistically significant. Similarly, the *SRSF2* mutational rate did not increase with advanced stage of disease. Four patients had coexistent mastocytosis, three of whom had spliceosomal mutations (2 in *U2AF1* and 1 in *SRSF2*). In addition, two of these patients had *TET2* mutations and three had *ASXL1* mutations (Figure 1B).

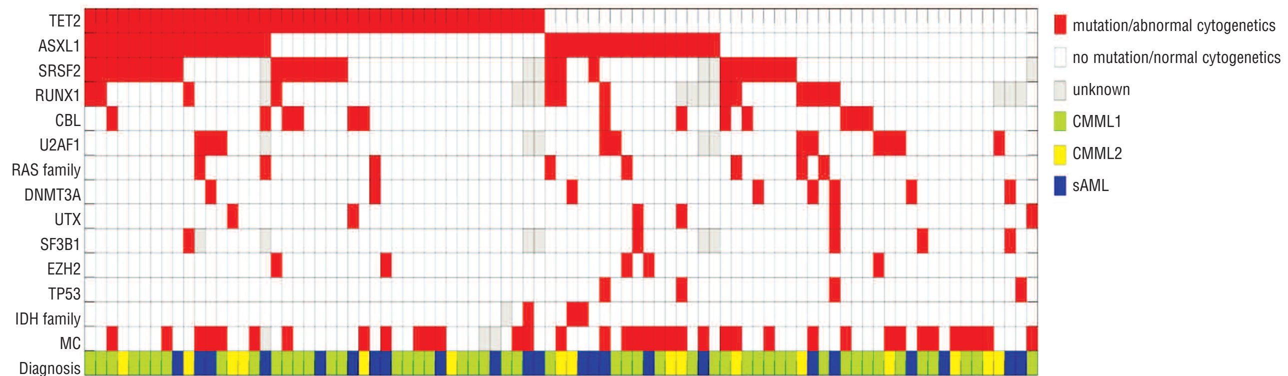
Among patients who received hypomethylating agents, 21 were evaluable for response; six (30%) responded, with three patients achieving an overall complete response, one patient experiencing overall partial remission, one patient having complete remission in the bone marrow and one patient having partial hematologic remission. Stable disease was observed in six patients while nine patients were

Table 2. Karyotype of patients detected by single nucleotide polymorphism array A karyotyping and/or metaphase cytogenetics.

	CMML1/2 & sAML	CMML-1	CMML-2	sAML	JMML
Normal	23%	31%	19%	13%	43%
Abnormal					
Total	77%	69%	81%	87%	55%
Gain	31%	25%	6%	52%	33%
Loss	38%	40%	31%	39%	58%
UPD*	43%	23%	56%	70%	8%
Y loss	5	4	0	1	1
Trisomy 8	7	2	2	2	1
Mono7 / del7q	7	4	2	3	10
Others**	21	10	4	6	22

*CMML: chronic myelomonocytic leukemia; sAML: secondary acute myeloid leukemia derived through progression of primary CMML; JMML: juvenile myelomonocytic leukemia. *In CMML: uniparental disomy (UPD) 7q in nine cases, UPD 4q in seven cases, UPD 11q in five cases, UPD 2p in four cases, UPD 1p in three cases, UPD 21q in three cases, UPD 6p in two cases, UPD13/13q in two cases, UPD 3p/5q/9p/13/13q/14q/16p/17p/17q in one case each. In JMML: UPD 11q in four cases and UPD 17q in one case. **Deletion on Ch 5, Ch11, Ch12, Ch13, Ch16, Ch17 and Ch20 / Inversion on Ch1 and Ch3 / Trisomy on Ch1, Ch8, Ch13, Ch19 and Ch21.*

A



B

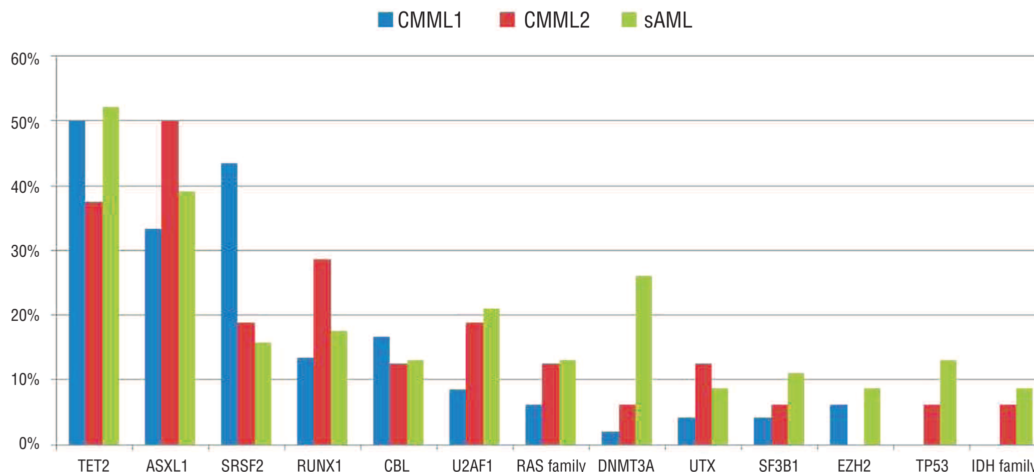


Figure 1. Prevalence of mutations in CMML. (A) The distribution of gene mutations in the CMML cohort. Each column represents one patient. Each row corresponds to one gene. The color of the squares represents the status of the gene; red mutated, white wild type. Gray squares not done. The last row indicates the diagnosis; green CMML-1, yellow CMML-2 and blue sAML from primary CMML. The row named as metaphase cytogenetics (MC) represents the karyotypic status (normal; white / abnormal; red) of each patient determined by MC. (B) The frequency of mutant cases of each gene according to disease category. Blue, red and green indicate CMML-1, CMML-2 and sAML, respectively.

refractory to therapy. *SRSF2*, *SF3B1*, *U2AF1*, *RUNX1* and *CBL* mutational rates were similar in responders and non-responders. *TET2* and *ASXL1* seem to be mutated more frequently in responders than in non-responders, but the difference was not statistically significant because of the low numbers. No *DNMT3A* mutations were found in either group. No *RAS* or *IDH1/2* mutants were identified among responders and none of the refractory cases harbored *EZH2* or *TP53* mutations.[22]

The association between mutational status and overall survival of patients was assessed using Kaplan-Meier statistics. While all possible permutations were tested, we point out here only significant positive and relevant negative results. When the whole cohort was analyzed, the presence of *U2AF1* or *DNMT3A* mutations conferred a worse overall survival (P=0.0473 and P=0.0154, respectively) (Figure 2A, B). Comparing a less advanced group, comprising CMML-1/CMML-2 *versus* sAML patients, RAS mutations correlated with worse survival in sAML (P=0.0456) (Figure 2C). When patients were sub-grouped into lower (CMML-1) and higher risk disease (CMML-2 and sAML), the prognostic significance of molecular lesions was weaker in each of the individual cohorts tested. The median overall survival of the patients in the CMML-1 group was 16 months, that in the CMML-2 group was 12 months while that in the sAML group was 4 months.

Serial analysis

We also analyzed the mutational status over time in eight patients studied serially at time points corresponding to various stages of the disease. Six had a diagnosis of CMML-1 for the initial sample and two had CMML-2. Mutations in the splicing machinery, if present, were found in the earliest samples. Four patients had mutations in *SRSF2* and three had mutations in *U2AF1* initially. Progression samples had the same genotype as the initial samples. One patient had wild-type genes in the initial sample and did not develop any mutations with progression (*Online Supplementary Table S4*).

Comparison of molecular defects between chronic and juvenile myelomonocytic leukemia

JMML and CMML showed very different mutational patterns with regards to the mutations frequently encountered in CMML. While *CBL* and *RAS* were mutated at the same rate in CMML and JMML cases (15% *versus* 10% for *CBL*; 9% *versus* 6% for *RAS*, respectively), *ASXL1* was mutated in 4% of JMML compared with 38% of CMML cases (P<0.001). *TET2* was the most frequently mutated gene in CMML, but mutations in this gene were not encountered in JMML (48% *versus* 0%; P=0.01). No mutations in *DNMT3A*, *IDH1/2* and *EZH2* were found in JMML whereas they were found in, respectively, 9% (P=0.025), 3% (P=0.292) and 6% (P=0.295) of cases of CMML. Similarly, none of the JMML patients harbored mutations in *SF3B1*, *U2AF1* or *SRSF2*, while in CMML these mutations were found in 6% (P=0.082), 13% (P=0.007) and 32% (P=0.001) of patients, respectively. Abnormal chromosomes were found in 55% of cases of JMML *versus* 77% of cases of CMML (P=0.02). Trisomy 8 was found in seven patients with CMML while only one patient with JMML harbored this lesion. Finally, 16% of patients with JMML had del7/7q, while only 8% of CMML cases had this lesion.

Discussion

The systematic application of molecular screens and the identification of new genomic lesions have led to better insights into the complexity of CMML that could not be discerned through traditional pathomorphological approaches, which identify this entity based on the presence of monoblasts and dysplastic monocytes. In this respect, the comparison of CMML and JMML is illustrative, as on purely morphological grounds these conditions are similar. The molecular heterogeneity is reflected not only in diverse types of somatic mutations and chromosomal aberrations but also in the recognition that CMML is most often driven by a combination of molecular defects, as might be expected in a disease that occurs primarily in a population of older adults.

The goals of this study were to include newly discovered spliceosomal mutations[4,6,7,23,24] in the context of previously known molecular lesions to enrich the previous catalogue of somatic mutations characterizing CMML, as demonstrated through screens by our group[2] and others.[3,25] In addition to the inclusion of *U2AF1*, *SRSF2* and *SF3B1* in the spectrum of previously genotyped mutations (*CBL*, *EZH2*, *UTX*, *TET2*, *ASXL1*, *DNMT3* and *RAS*), we also sequenced *TP53* and *RUNX1* and found mutations.

We found that *SRSF2* and *U2AF1* are among the most frequent somatic mutations in CMML and are typically mutually exclusive. In contrast, the presence of *SF3B1* mutations is a rare event and is associated with ring sideroblasts, showing that the latter is the clinical phenotype associated with *SF3B1* mutations rather than a feature of low-risk myelodysplastic syndromes such as RARS or RCMD. Overall, spliceosomal mutations were present in 58% of CMML patients, which is comparable to the frequency of mutations in *TET2* and *ASXL1*. While it is not clear whether these defects represent ancestral events, preliminary evidence stemming from serial studies shows that *U2AF1* and *SF3B1* are present at the earliest presentation of the disease and that their frequency does not increase significantly either serially or cross-sectionally when different stages of the disease are compared. Clinically, the presence of *U2AF1* and *SRSF2* mutations was not associated with distinct clinical and phenotypic features; only patients with *U2AF1* mutations showed shorter survival. Malcovati *et al.*, in a recent study assessing the mutational rate of *SF3B1* in patients with myelodysplastic syndromes and myelodysplastic/myeloproliferative disorders, found that mutations were present in 6.5% of all cases of CMML,[26] a result similar to that of

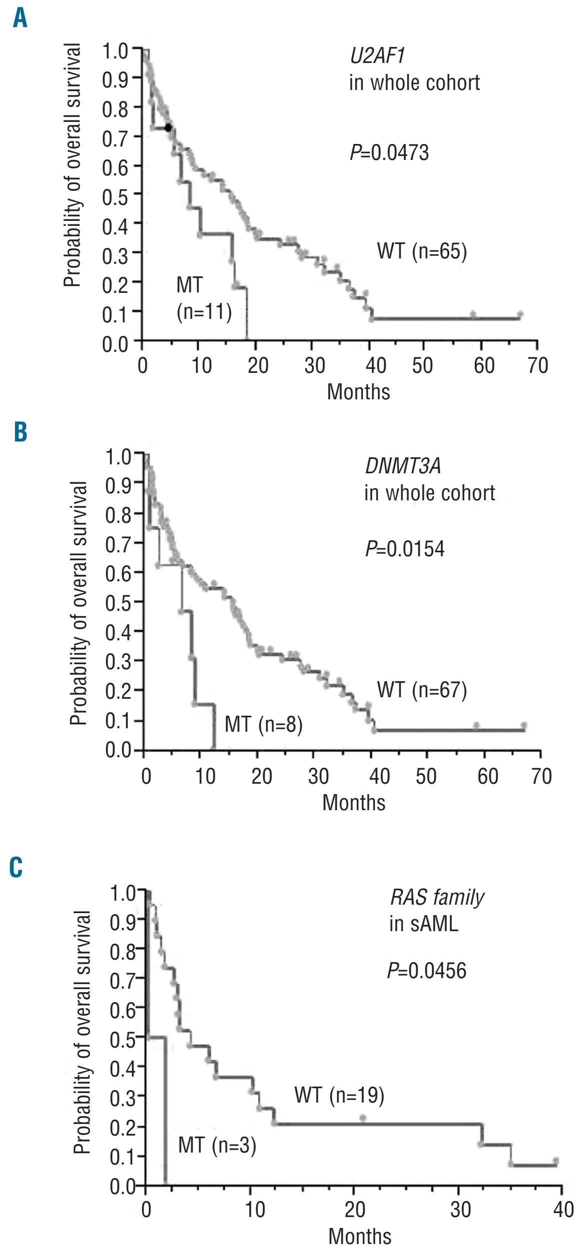


Figure 2. Survival analysis using the Kaplan-Meier method was performed to assess the association of gene mutations with overall survival. When the cohort was considered all together, mutations in *U2AF1* (A) and *DNMT3A* (B) were significantly associated with lower overall survival (P=0.0473 and 0.0154, respectively). When the patients were categorized into low-risk (comprising CMML-1 and CMML-2 patients) and high-risk groups (comprising sAML patients), *RAS* mutations in sAML predicted worse survival (P=0.0456) (C).

our study. Yoshida *et al.* assessed the frequency of mutations in spliceosomal proteins in patients with myelodysplastic syndromes (n=228) and CMML (n=88) and found that the latter had a high rate of *SRSF2* mutations, reaching 28%, while mutational frequencies were 8% for *U2AF1* and 4.5% for *SF3B1*.[5] Splicing genes mutations seem to be uniformly present across populations despite ethnic differences. *SF3B1* was shown to be strongly associated with RARS and RCMD, which usually follow a benign clinical course with high platelet and neutrophil counts, low blast count and longer event-free survival,[7] but overall survival seems not to be affected by the mutational status of the gene according to our findings and other reports.[27] *U2AF1* mutations, although present in only around 10% of the cohort, tend to affect survival more strongly than the more frequent *SRSF2* mutations.

While mutations in diverse genes such as *TET2*, *ASXL1* and others have been found to affect survival,[28,29] in our group only *DNMT3A* and *U2AF1* were associated with shorter survival. However, we acknowledge that the rarity of various other mutations precluded more conclusive statistics. For instance, *RAS* and *TP53* are invariably associated with poor prognosis in various myeloid neoplasms, with a particularly strong correlation of *TP53* with LOH17p del(7/7q) and del(5q).[30,31] In larger cohorts of patients with myeloid malignancies, *EZH2*[32-34] and *CBL*[32,35] mutations have been associated with poor survival; the difference within the current study may be attributed to the overall less favorable prognosis of CMML patients as compared with, for example, patients with low-risk myelodysplastic syndromes. It is noteworthy that not only the presence of individual mutations but also the mutational burden (not detectable by the Sanger sequencing used in our study) may have prognostic significance, as recently shown through the deep sequencing of *TP53* in myelodysplastic syndromes.[36]

Alternative splicing may be associated with the molecular pathogenesis of cancer.[37] Although quality control mechanisms such as nonsense-mediated mRNA decay can help to eliminate aberrantly spliced variants, some can escape surveillance and be translated into proteins that are structurally aberrant, mislocalized, or less susceptible to degradation, all of which contribute to the development of disease.[38] The usefulness of cell type-related variability in splicing for the diagnosis and possible treatment of certain cancer types has been investigated. For instance, head and neck squamous cell carcinoma cells can be targeted by antibodies against exon 6 of CD44, which was found to be expressed in those tumor cells specifically and not in normal cells.[39] It is possible that individual splicing lesions in CMML will also correspond to distinct biological features.

The study of genotypic associations showed that the tested spliceosomal mutations are most frequently associated with *TET2* and *ASXL1* mutations, but various combinations are possible. Concomitant *SRSF2* and *RUNX1* mutations did not lead a better overall survival. At least theoretically, spliceosomal mutations can effectively substitute for inactivating mutations in other genes by leading to dysfunctional splicing of genes and ultimately defects in protein translation. For instance, it is possible that functional haploinsufficiency of *EZH2*, *TET2* or *RUNX1* can be a result of spliceosomal mutations, which effectively produce a phenocopy of mutations found in these genes. Such a mechanism would explain how molecular heterogeneity can produce convergent dysfunctional pathways and, thereby, similar phenotypes.

In contrast to phenotypic convergence, the mutational spectra of two diseases can be very divergent, even if morphological features are similar. For instance, mutations in splicing machinery-related genes are frequent in CMML patients but are absent in JMML patients. This finding is consistent with those described by others in JMML and pediatric myelodysplastic syndromes.[40] Similarly, *TET2* and *EZH2* mutations are absent in JMML, which most likely utilizes other molecular pathways including mutations in *PTPN11* or *NF1*. In contrast to these genes, CMML and JMML share mutations in *RUNX1* and, most significantly, *CBL*.

In conclusion, our results demonstrate a high frequency of spliceosomal mutations in CMML and their coexistence with other mutations. In addition to loss of function or hypomorphic mutations, deletion and promoter silencing, it is likely that splicing defects may lead to functional haploinsufficiency and have leukemogenic consequences. Thus, spliceosomal mutations may have a clinical impact indistinguishable from that of other mutations. Moreover, the presence of multiple mutations may hinder assessment of the presence of individual mutations on clinical outcomes or phenotypic features.

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