TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia

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ABSTRACT

Background

Acquired somatic deletions and loss-of-function mutations in one or several codons of the *TET2* (*Ten-Eleven Translocation-2*) gene were recently identified in hematopoietic cells from patients with myeloid malignancies, including myeloproliferative disorders and myelodysplastic syndromes. The present study was designed to determine the prevalence of *TET2* gene alterations in chronic myelomonocytic leukemias.

Design and Methods

Blood and bone marrow cells were collected from 88 patients with chronic phase chronic myelomonocytic leukemia and from 14 with acute transformation of a previously identified disease. Polymerase chain reaction analysis and direct sequencing were used to sequence exons 3 to 11 of the *TET2* gene. Annotated single nucleotide polymorphisms were excluded. Survival curves were constructed by the Kaplan-Meier method.

Results

We detected *TET2* mutations in 44 of 88 (50%) patients with chronic myelomonocytic leukemia, which suggests that *TET2* gene mutations are especially frequent in this myeloid disease. A *TET2* gene alteration was identified in 18 of the 43 patients studied at diagnosis and was associated with a trend to a lower overall survival rate; confining the analysis to the 29 patients with chronic myelomonocytic leukemia-1, according to the WHO classification, the difference in overall survival between patients with or without *TET2* gene mutations became statistically significant.

Conclusions

TET2 gene alterations are more frequent in chronic myelomonocytic leukemia than in other subgroups of hematopoietic diseases studied so far and could negatively affect the patients' outcome. The striking association between *TET2* gene alterations and monocytosis, already observed in patients with systemic mastocytosis, could indicate a negative role of *TET2* in the control of monocytic lineage determination.

Key words: *TET2* gene, mutation, chronic myelomonocytic leukemia.

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Introduction

Chronic myelomonocytic leukemia (CMML) is a clonal myelodysplastic/myeloproliferative disorder observed in the elderly.[1] The most frequent genetic abnormalities identified in this disease include mutations in *RUNX1*[2,3] and in the polycomb-associated gene *ASXL1*.[4] A copy-neutral uniparental disomy is also frequent and can be associated with mutations of the *CBL* gene.[5,6] *RAS* gene mutations are observed in one third of CMML,[7] while other acquired genetic abnormalities are limited to small subgroups.[8] It remains difficult to distinguish among these somatic events those that drive the disease pathogenesis from those that are acquired as a consequence of disease progression. Using various genetic approaches, acquired somatic mutations (deletions, insertions, nonsense and missense point mutations) in the coding sequence of *TET2* (*Ten-Eleven Translocation-2*) gene were recently identified in hematopoietic cells from patients with myeloid malignancies, including myeloproliferative disorders and myelodysplastic syndromes.[9-17] Colony studies in informative cases of myeloproliferative disorders suggested that *TET2* mutations could precede the *JAK*^{V617F} mutation and may endow cells with an increased ability to repopulate the bone marrow of NOD/SCID mice, with respect to *TET2* wild-type hematopoietic stem cells.[14] The incidence of *TET2* gene alterations in various myeloid diseases has been suggested to range between 10 and 25%.[9-17] In patients with systemic mastocytosis, a significantly higher incidence of *TET2* mutations was associated with monocytosis.[10] The aim of this study was to determine the prevalence and prognostic impact of *TET2* gene mutations in CMML.

Design and methods

Samples

Peripheral blood and/or bone marrow cells were collected between February 2005 and October 2008 in Dijon (Inserm UMR866) and Marseille (IPC) from 88 patients with CMML1 (n=70) or CMML2 (n=18) according to the World Health Organization (WHO) criteria[1] and from 14 patients with acute blastic transformation of a previously identified CMML. Patients signed informed consent to participation in the study in accordance with current ethical regulations. Six of the nine CMML patients included in a previous study[14] are part of the present series (the other three were excluded because of lack of sufficient clinical or biological information). We also included 46 patients in whom the *ALXL1* sequence had been previously examined.[4] None of the other cases has ever been reported. Samples were collected from consecutive patients seen in the different centers, pending the collection of sufficient biological material and annotations. Patients with CMML in chronic phase were either newly diagnosed (n=43) or known to have this hematopoietic disease and were being followed up every 3 months in the absence of active therapy, while receiving supportive care or during cytotoxic treatment (n=45), in most cases with hydroxyurea. Acute transformation was considered to have occurred when the blastic phase was identified. The main characteristics of the patients studied are summarized in Table 1.

Nucleic acid methods

Blood and bone marrow samples were collected on EDTA and mononuclear cells were selected by Ficoll Hypaque. DNA was extracted using commercial kits (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) analysis and direct sequencing were performed using standard conditions with gene-specific primers designed to amplify coding sequences spanning from exon 3 to exon 11 of the *TET2* gene, as described elsewhere.[14,17] For each PCR reaction, 20 ng of genomic DNA were used for the PCR amplification followed by magnetic bead purification and bidirectional sequencing using ABI 3300 capillary sequencers (Agencourt Bioscience, Beverly, MA, USA). Mutation Surveyor

Table 1. Characteristics of the studied patients according to the pressence or absence of *TET2* mutations. The only significant difference (higher count of peripheral blood monocytes in the *TET2* mutated group) was not confirmed when analysis was limited to patients at diagnosis.

Parameter	All patients				Patients studied at diagnosis			
	All	Non mutated *TET2*	Mutated *TET2*	*p* value	All	Non mutated *TET2*	Mutated *TET2*	*p* value
Number of patients	88	44	44		43	25	18	
Median age years (range)	76 (54-93)	74 (54-87)	76 (61-93)	0.07	73.5 (54-87)	73 (54-86)	73.5 (61-87)	0.11
Males (%)	61 (69%)	30 (68%)	31 (70%)	0.82	27 (63%)	17 (68%)	10 (56%)	0.40
Hemoglobin level (g/L)	118 (78-164)	111 (90-150)	123 (78-164)	0.10	107 (89-150)	103 (90-150)	123 (89-140)	0.47
Leukocyte count ($\times 10^9$/L)	12.0 (3.8-112.0)	16.0 (3.8-112.0)	11.9 (4.0-106.0)	0.08	18 (4.0-105)	18 (4.5-55.0)	19 (4.0-105.0)	0.95
Platelet count ($\times 10^9$/L)	145 (9-664)	163 (16-664)	104 (9-652)	0.06	184 (11-664)	184 (11-664)	136 (11-652)	0.26
Monocyte count ($\times 10^9$/L)	2.1 (1.0-26.0)	1.3 (1.0-26.0)	2.4 (1.0-20.8)	0.02	1.7 (1.0-5.7)	1.0 (1.0-1.9)	2.1 (1.0-5.7)	0.10
Bone marrow blast cells (%)	6 (0-18)	6 (0-18)	6 (0-17)	0.69	6 (0-18)	6 (0-18)	6 (1-16)	0.93
Abnormal /done karyotype	20/77 (26%)	10/38 (26%)	7/34 (21%)	0.57	8/33	5/21	3/11	0.90
CMML1/CMML2 (WHO)	70/18	36/8	34/10	0.60	34/10	19/6	13/5	0.78
Myeloproliferative/ myelodysplastic (FAB)	41/52	16/28	23/21	0.13	19/23	11/14	10/8	0.45

Table 2. List of the 68 *TET2* mutations identified in 46 CMML patients, of whom 44 were in chronic phase and 2 in blastic transformation. UPN are arbitrary numbers given to each patient.

UPN	Age	Sex	At diagnosis	WHO	FAB	Karyotype	Exon	Nucleotide change	Consequence	Type of mutation	N. of anomalies
1	85	M	Yes	CMML1	MP	46, XY [20]	10 & 11	c.5043n-1G>A; Dup 6575_6579 (GAGCA)	Mutation splice receptor site exon ex10; M1907FS	Mutation in splice site + frameshift	2
2	70	M	No	CMML1	MP	46, XY [20]	5	c.4453G>A	W1198X	Nonsense	1
3	87	M	Yes	CMML2	MP	46, XY [20]	5	c.4431C>T	Q1191X	Nonsense	1
4	79	F	No	CMML1	MP	46, XX [20]	10 & 11	c.5214C>T; Ins 5537 (A)	R1452X ; Y1560FS	Nonsense + frameshift	2
5	76	M	No	CMML1	MP	46, XY [20]	9	c.4942G>A	G1361S	Missense	1
6	74	F	Yes	CMML2	MD	47, XX, +8 [20]	10	c.5070C>T	R1404X	Nonsense	1
7	72	F	Yes	CMML1	MD	47, XX, +8 [20]	5	c.4439T>G	C1193W	Missense	1
8	65	M	Yes	CMML1	MP	46, XY [20]	7	c.4726G>T	C1289F	Missense	1
9	82	F	Yes	CMML1	MD	46, XX [20]	10	c.5100C>T	Q1414X	Nonsense	1
10	69	F	Yes	CMML2	MD	46, XX [20]	5	Del 2655_2658 (CAAA)	N598FS	Frameshift	1
11	71	F	Yes	CMML1	MD	46, XX [20]	11	Del 6023 (G)	L1721FS	Frameshift	1
12	70	F	Yes	CMML1	MD	46, XX [20]	3	Del 1921 (C)	S354X	Nonsense	1
13	88	M	Yes	CMML2	MP	47, XY, +21 [20]	11	Ins 5602_5606 (TCCAA)	S1582FS	Frameshift	1
14	73	M	No	AT	AT	46, XY, ?del(20)(q11q13) or -20, + mar [18] /46, XY [4]	3	c.3235C>A	S792X	Nonsense	1
15	87	M	No	CMML1	MP	46, XY [20]	6 & 10	c.4500C>A; Del 5118_21 (TTAT)	R1214W; L1420FS	Missense + frameshift	2
16	68	M	Yes	CMML1	MP	46, XY [20]	8 & 10	c.4827G>T; Ins 5178 (A)	E1323X; R1440FS	Nonsense + frameshift	2
17	87	M	No	CMML2	MP	45, X, -Y	3	c.2814C>T	Q652X	Nonsense	1
18	73	M	No	CMML1	MD	46, XY [20]	3 & 9	delT 4172; c.5011A>T	F1104FS, D1384V	Frameshift + missense	2
19	84	M	No	CMML1	MP	46, XY [20]	10 & 11	del 5362_5365; c.6441G>A	G1501FS; G1860R	Frameshift + missense	2
20	69	M	No	CMML1	MD	46, XY [20]	3	c.2631C>T	Q591X	Nonsense	1
21	81	M	No	CMML1	MD	NA	11	Del 6507 (A)	T1883FS	Frameshift	1
22	72	M	No	CMML1	MD	NA	3	c.2961C>T	Q701X	Nonsense	1
23	82	M	No	CMML1	MD	46, XY [20]	3 & 9	c.1818G>T; c.4936G>A	E320X; R1359H	Nonsense + missense	2
24	69	M	No	CMML1	MP	46, XY [20]	6	c.4515C>T	H1219Y	missense	1
25	65	M	No	CMML1	MP	46, XY [15]/ 46, XY, del20q [5]	6 & 11	c.4663n+1 G>A; Del 6424_33	Mutation splice donor site exon 6 + L1855FS	Mutation in splice site +frameshift	2
26	70	M	No	CMML1	MP	46, XY [20]	3	ins 2468_9 (AA)	K536FS	Frameshift	1
27	76	F	Yes	CMML1	MP	46, XX [20]	3 & 10	insG 2703; ins 5125_26 (AA)	L615FS; K1422FS	Frameshift x2	2
28	81	F	No	CMML1	MD	NA	3 & 8	c.1272C>A; c.4814n-1 G>A	Q138X , Mutation splice receptor site exon 8	Nonsense + mutation in splice site	2
29	81	F	No	AT	AT	46, XX [20]	3 & 10	c.2490C>T; Del 5334 (G)	R544X; E1492FS	Nonsense + frameshift	2
30	78	M	No	CMML2	MD	45, X, -Y	4 & 11	Ins 4293 (A); c.6510A>G	G1145FS; T1884A	Frameshift + missense	2
31	93	M	No	CMML1	MD	NA	3 & 5	Ins 3151 (C); c.4390T>G	Q764FS; I1175S	Frameshift + missense	2
32	77	M	No	CMML1	MD	NA	3	c.3675C>T	Q939X	Nonsense	1
33	84	F	Yes	CMML1	MP	46, XX [20]	3	Ins of 2951_86 (dup)	L718FS	Frameshift	1
34	73	M	No	CMML2	MD	NA	4 & 11	delT 4277; c.6598G>T	I1139FS; G1913V	Frameshift + missense	2
35	64	F	No	CMML1	MD	46, XX [20]	7 & 11	delG 4754; dup 6569_6573 (GAGA)	K1298FS; M1570FS	Frameshift x2	2
36	67	M	Yes	CMML1	MD	46, XY [20]	5 & 8	c.4638G>A; c.4825T>C	C1193Y; L1322P	Missense x2	2
37	61	M	Yes	CMML1	MD	46, XY [20]	11 & 11	c.6414C>T; c.6496A>C	Q1852X; E1879A	Nonsense + missense	2
38	82	F	No	CMML2	MP	46, XX [20]	9	c.4936G>C	R1359S	Missense	1
39	72	M	No	CMML1	MP	46, XY [20]	3 & 8	delA 3874; del 4830_31 (TC)	K1008FS; S1324FS	Frameshift x2	2
40	74	M	No	CMML1	MD	46, XY [20]	3 & 4	c.2208A>T; del 4347 (A)	K450X; I1163FS	Nonsense + frameshift	2
41	73	M	No	CMML1	MP	46, XY [20]	11	c.6478T>C	I1873T	Missense	2
42	77	F	No	CMML1	NA	46, XX [20]	3 & 3	ins 1921 (A); ins 2703 (G)	S354FS; L615FS	Frameshift x2	2
43	77	M	Yes	CMML1	MP	46, XY [20]	3	del 3859 (A)	N1000FS	Frameshift	1
44	76	M	No	CMML1	MD	46, XY [20]	3 & 3	ins 3995 (T); c.4059 A>T	E846FS; R1067X	Frameshift + nonsense	2
45	71	M	Yes	CMML2	MP	46, XY [20]	3 & 10	c.2784 C>T; c.5253 C>T	Q642X; R1465X	Nonsense x2	2
46	87	M	Yes	CMML1	MP	46, XY [20]	3	del 1264_66 (AAA)	E135FS	Frameshift	1

Age in years. M: Male; F: Female. CMLL1 and 2 were classified according to the WHO classification, myloproliferative (MP) and myelodysplastic (MD) according to the FAB classification and based on the leukocyte count. AT indicates acute transformation.

(Softgenetics, Inc., Stat College, PA, USA) was used to detect nonsense and missense mutations located in conserved regions spanning from 1134-1444 and 1842-1921 and sequences were reviewed manually to detect frameshift mutations. *TET2* abnormalities were numbered according to the FM 992369 EMBL nucleotide sequence database. Previously annotated single nucleotide polymorphisms (*http//www.hapmap.org*) were not considered pathogenic. The *ASXL1* sequence was determined in 49 samples, as described previously.[4]

Comparative genomic hybridization arrays

Comparative genomic hybridization (CGH) was performed using 244K CGH Microarrays (Hu-244A, Agilent Technologies, Massy, France) with a resolution up to 6 Kb. Scanning was done with an Agilent Autofocus Dynamic Scanner (G2565BA, Agilent Technologies).[2,4] Copy number changes in the 4q24 region between 104,680 and 106,960 according to *http//www.genome.ucsc.edu* were characterized.

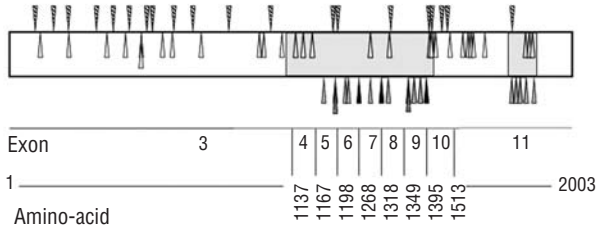

Figure 1. Schematic representation of the location of the 68 mutations identified in 46 patients with CMLL patients, of whom 44 in chronic phase CMML and 2 in blastic transformation. Two distinct mutations were identified in 19 of these patients (see Table 2); 29 frameshifts (white triangles); 20 nonsense mutations (hatched triangles), 16 missense mutations (gray triangles) and 3 mutations in splice sites (black triangles) are indicated. Gray bars indicate conserved domains (AA 1134-1444 and 1842-1921). *TET2* abnormalities were numbered according to the FM 992369 EMBL nucleotide sequence database. Previously annotated single nucleotide polymorphisms were not considered pathologic.

Statistical analysis

Statistical analyses were performed using Stata 10™. All p values were two-tailed and the threshold of statistical significance was p less than 0.05. Clinical and biological parameters were recorded at the time of diagnosis or referral to the medical center. Categorical variables are reported as counts and relative frequencies (%) and compared between groups by χ^2 or exact Fisher's statistics. Continuous variables are indicated as medians and ranges. We used the Mann-Whitney U test to compare continuous variables. Survival curves were constructed by the Kaplan-Meier method using the interval from the date of diagnosis to the date of last contact or death and compared using the log-rank test. A multivariable Cox model was fitted in order to take age at diagnosis into account.

Results

The nature and frequency of somatic mutations affecting the *TET2* coding sequences were studied in bone marrow or peripheral blood collected from 88 patients with chronic phase CMML according to the WHO criteria. A mutation of the *TET2* gene was detected in 44 out of these 88 (50%) patients. The *TET2* gene was mutated in 18 (42%) of the 43 patients studied at diagnosis, and in 26 of the 45 patients (58%) studied during the course of their disease. These results suggest that the prevalence of *TET2* mutations is higher in CMML than in any other myeloid disease studied.[9-17] The broad range of myeloid disorders in which mutations in the *TET2* gene have been identified suggests

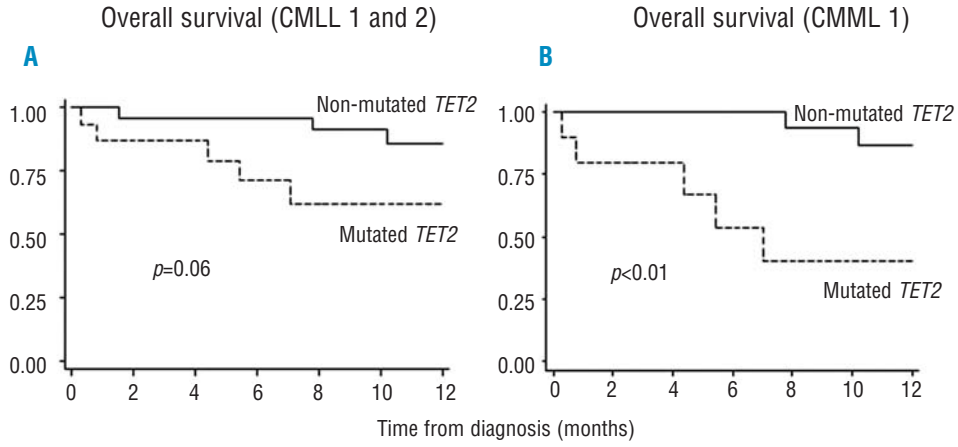

Figure 2. Kaplan-Meier analysis of overall survival in patients in whom the *TET2* mutation was analyzed at diagnosis. (A) Overall survival of 43 patients with either CMML1 or CMML2 according to the WHO classification (mutated *TET2*: 18 cases; non-mutated *TET2*: 25 cases). (B) Overall survival of the 29 patients with CMML1 in chronic phase according to the WHO classification (mutated *TET2*: 10 cases; non-mutated *TET2*: 19 cases).

that the gene has a pleiotropic role in these diseases. It remains to be established what role the mutation plays in the phenotype of the disorders. The striking association between the presence of a *TET2* mutation and monocytosis in patients with systemic mastocytosis[10] and the very high incidence of *TET2* mutations in CMML1 found in this and other studies[1-14] lends support to a phenotypic association.

Among an additional series of 14 CMML patients who had 20% or more blast cells in the bone marrow, indicating blastic transformation, a *TET2* mutation was identified in two patients (14%) (Table 2). It was shown recently that the *JAK2*^{V617F} mutation is frequently absent in leukemic blast cells from patients with transformed *JAK2*^{V617F}-positive myeloproliferative disorders. In these patients, leukemic transformation could arise from a *JAK2*^{V617F}-negative ancestor cell.[18] Larger series and follow-up of individual cases will be needed to determine whether the *TET2* mutation, when present at diagnosis, can be lost upon leukemic transformation.

Two distinct mutations in the sequence of the *TET2* gene were identified in 23 out of the 44 (52%) *TET2*-mutated patients with chronic phase CMML, including 6 out of the 18 (33%) patients whose mutations were identified at diagnosis, and 16 out of the 26 (61%) patients whose mutations were identified during the course of their disease. Two distinct mutations were also identified in one of the patients in blastic transformation of CMML. Altogether, 68 mutations were identified, including 29 frameshift mutations, 20 nonsense mutations, 16 missense mutations and 3 mutations targeting a splice site. The mutations most frequently involved exon 3 (22 events), exon 10 (9 events) and exon 11 (13 events). Missense mutations were considered if located in conserved domains, most of them leading to modifications in potentially important amino-acids in the protein[19] (Table 2, Figure 1).

Conventional cytogenetic analysis of bone marrow was performed in 72 of the 88 patients with chronic phase CMML and detected abnormalities in 17 cases but never identified any deletion of the 4q24 band, in either the mutated or the non-mutated group of patients. Genome-wide high-density arrays (CGH) compared the leukemic cell profile to normal DNA in 28 of the 88 cases and detected a *TET2* deletion (according to the *http://genome.uscs.edu*) in one of the ten studied patients with a mutated *TET2* copy. Thus, copy number alterations and deletion of the wild-type *TET2* copy in *TET2*-mutant CMML cases appears to be uncommon, although this remains to be proven in larger series.[20] It is too early to determine whether the numbers and types of mutation (point mutation or frameshift) and the gene dosage (loss of one or two copies) differ among the various myeloid disorders and contribute to the disease phenotype. When the *ASXL1* gene sequence could be analyzed simultaneously (n=49), a mutation was found in patients with wild-type as well as mutated *TET2* (7 of 14 samples and 14 of 35 respectively; p=ns).

The clinical and biological features of the 88 patients with chronic phase CMML are presented in Table 1. The presence of a *TET2* mutation was associated with a trend towards higher monocyte and lower platelet counts. Analysis of overall survival was performed in the 43 patients whose *TET2* status was determined at diagnosis and indicated a lower 1-year overall survival rate in the 18 patients of this cohort with a *TET2* mutation, but the difference was not statistically significant (Figure 2A). When the overall survival analysis was limited to the 29 patients with CMML1, according to the WHO classification, and a follow-up of at least 2 months, the difference between those with and without *TET2* mutations became significant (p<0.01 Figure 2B). The survival of CMML1 and CMML2 patients was not significantly different at 12 months but all patients with CMML2 died within 28 months of diagnosis whereas half of the CMML1 patients were still alive (*data not shown*). The survival of patients with secondary acute myeloid leukemia was significantly shorter than that of those with CMML1 or CMML2 (p<0.04, *data not shown*). None of the other tested parameters, including age, sex and FAB classification, affected survival. Given the low number of patients in each group, only age was introduced in the Cox model and did not affect the trend for a negative effect of *TET2* mutation on survival (p=0.08).

Discussion

The present study indicates that, in addition to *RUNX1*,[2,3] *ASXL1*,[4] and *RAS* genes,[6] *TET2* is a commonly mutated gene in patients with CMML. Half of the patients with mutated *TET2* had two distinct gene alterations, suggesting that the two gene copies were affected. In four cases, sequencing results showed a 100% mutant sequence, which could indicate a combination of *TET2* mutation and either uniparental disomy or 4q24 deletion or a homozygous mutation. We did not collect enough biological material to perform CGH, which would have been able to identify 4q24 deletions involving *TET2*, and single nucleotide polymorphism analysis, which would have been able to detect segmental acquired uniparental disomy resulting in loss of heterozygosity.[5] Based on the series published to date, the frequency of *TET2* mutations in chronic phase CMML ranges between 35 and 42%.[11-13] *TET2* gene mutations were associated with increased monocytosis, as observed in mastocytosis.[10] We also noted a higher number of immature dysplastic granulocytes in the peripheral blood of CMLL patients with mutated *TET2* gene (*data not shown*). Identification of other surrogate markers of the *TET2* mutation may be useful as the mutations in this gene as well as in other genes recently identified to be mutated in CMML, such as *ASXL1*,[4] are spread over the full length of the genes.

The *TET* family includes three genes (*TET1*, *TET2* and *TET3*) with highly conserved regions.[13] *TET1*, standing for *ten-eleven translocation 1*, is also known as *CXX6* or *LCX* and was identified as a fusion partner of the *MLL* gene in the acute myeloid leukemia-associated translocation t(10;11)(q22;q23).[21,22] The *MLL-TET1* fusion gene was also detected in two adults with CD10-negative B-cell precursor acute lymphoblastic leukemia[23] and a single nucleotide polymorphism in the *TET1* gene coding region has been associated with late-onset Alzheimer's

disease.[24] Recent evidence indicates that *TET1*, and possibly other proteins of the family, encodes an enzyme responsible for the conversion of 5-methylcytosine to 5-hydroxymethylcytosine,[19] thus having potential roles in CpG methylation pattern and epigenetic regulation. *TET2* was suggested to have tumor suppressor function[14] but the physiological functions of the TET2 protein in hematopoiesis are yet to be identified, for example, to determine whether TET2 could negatively regulate monocyte lineage determination, and to analyze how the loss of TET2 protein through a variety of genetic mechanisms leads to myeloid cell proliferation and dysplasia.

The negative prognostic impact of *TET2* mutations in chronic phase CMML needs to be corroborated in a prospective study determining the combined clinical impact of recently identified frequent mutations in *TET2*, *ASXL1*, *RUNX1* and *RAS* genes.[2-6] Such a prospective study has been initiated by the *Groupe Francophone des Myelodysplasies* in the context of an ongoing phase II trial testing decitabine in CMML patients.

Authorship and Disclosures

OK and VGB performed genetic analyses, MC and CR received the samples and sorted the cells, VJ performed statistical analyses, NV, BQ, PF, NJB; OBR, AS, FD, NI, and SdB provided samples, WV and OAB discovered *TET2* mutations, DB, MF and ES designed the study, ES wrote the paper.

The authors reported no potential conflicts of interest.

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