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Role of BCL2L10 methylation and TET2 mutations in higher risk myelodysplastic syndromes treated with 5-Azacytidine

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Epigenetic gene regulation has a critical role during normal development and neoplastic transformation. Several tumor suppressor genes are found to be abnormally methylated and silenced in hematological malignancies, and the distribution of DNA methylation follows specific and distinct patterns in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). However, the mechanisms mediating aberrant methylcytosine patterns in MDS have not been defined.

TET2 is a close relative of TET1 and TET3, a family of enzymes sharing two highly conserved domains, which convert DNA 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). The gene encoding for TET2 resides at chromosome 4q24 and is frequently mutated in myeloid malignancies, including about 25% of MDS, 40–50% chronic myelomonocytic leukemia, 15% myeloproliferative neoplasms, 10–20% of AML, in particular in cases secondary to MDS.[1–3] Most recently, Ko *et al*.[4] showed that TET2 mutations compromise the hydroxymethyl-catalytic activity of the protein, with lower levels of 5-hydroxymethylcytosine in genomic DNA from TET2-mutated samples compared with controls.

We studied the prognostic role of TET2 mutations and methylation profiling in 38 patients treated with 5-azacytidine (Vidaza, Celgene Corp., Summit, NJ, USA) and valproic acid, according to the Gimema multicenter clinical trial MDS0205 (EudraCT number 2005-004811-31). Therapy consisted of valproic acid given orally to reach a plasma concentration above 50 μg/ml and 5-azacytidine (5-AZA) at a standard dose of 75 mg/sqm daily, subcutaneously, for 7 days every 4 weeks. Response rate and survival for the whole patient group have been previously reported.[5] The validation group was composed of a retrospective series of 27 patients treated at the Universita' Cattolica Sacro Cuore (Rome, Italy) between September 2007 and June 2010. Inclusion criteria were: diagnosis of higher-risk MDS and treatment with 5-AZA at 75 mg/sqm daily, subcutaneously, 7 days for a median of 4 cycles (range 2–30 cycles). Clinical characteristics of these patients are described in Table 1. Both patient groups had not received any specific treatment before starting 5-AZA, except for supportive therapy. DNA was extracted from bone marrow mononuclear cells obtained from all patients before 5-AZA exposure. All patients signed informed consent in accordance with the Declaration of Helsinki, following institutional guidelines.

We found that TET2 was mutated in 12/38 (32%) patients with Int-2/high risk MDS included in the Gimema multicenter study MDS0205.[5] Mutational analysis of TET2 coding exons 3–11 performed by PCR-based denaturing high pressure liquid chromatography using a WAVE-MDTMSystem (Transgenomic, Omaha, NE, USA) equipped with a DNASep Cartridge, detected five frameshift, three nonsense, six missense (two recurrent and

Table 1 Characteristics of patients included in the validation group

Patients n = *27*	n
Age (median, range)	
64 years (37–77 years)	
Gender	
Males	17
Females	10
FAB classification	
RAEB	19
RAEB-t	4
CMML[a]	4
IPSS	
Int-2	11
High	16
Blood counts	
Hemoglobin (g/dl)	8.9 (7.7–12.7)
Platelets (10^9/l, median, range)	44 (6–315)
Neutrophil (10^9/l, median, range)	1 (0.2–12.2)
Previous LR-MDS	
Yes	11
No	16
MDS duration before 5-ZA	
Months (median, range)	2 (0.1–45)
Response (n = 23)	
CR, PR	8
Stable, progressive	15

Abbreviations: CMML, chronic myelomonocytic leukemia; CR, complete remission; FAB, French-American-British classification; LR-MDS, low-risk MDS; MDS, myelodysplastic syndromes; PR, partial remission; RAEB, refractory anemia with excess blasts.
[a]CMML-1:1 patient, CMML-2:3 patients.

four putative) mutations (Figure 1a), and two putative polymorphisms not annotated in NCBI single-nucleotide polymorphisms database (c.2599T>C p.Y867H; c.5167C>T p.P1723S).[3] Patient characteristics according to TET2 mutations are given in Table 2. There were no associations with gender, IPSS score or karyotype, but TET2 mutations were more frequent in chronic myelomonocytic leukemia (three of four patients, 75%) versus RAEB/RAEB-t (9 of 34 patients, 27%, $P=0.08$). It is interesting to note that TET2 mutations impair monocyte/macrophage differentiation in culture, indicating a putative role for TET2 during monocyte development.[4] Patients with TET2 mutations tended to have lower platelet counts at diagnosis (median platelet counts: 27×10^9/l, range 11–77, versus 62×10^9/l, range 10–573, $P=0.08$), but there were no differences in the occurrence of NCI grade 3/4 thrombocytopenia during treatment, according to TET2 mutations.

Given the functional role of TET2 as epigenetic enzyme, we were interested in associations between TET2 mutations, methylation profile and response to epigenetic treatment, which has shown efficacy in higher-risk MDS.[5,6] Using Real-Time PCR and the Custom Methyl-Profiler PCR Array (SABiosciences, Frederick, MD, USA; Qiagen, Valencia, CA, USA), which allows for quantification of hypermethylated versus unmethylated promoter sequences, we studied the methylation profile of 22 differentiation, apoptosis and targets of polycomb group proteins genes, known to be frequently mutated or methylated in MDS or induced by hypomethylating treatment (RUNX1, FOXO3, TET2, PTEN, DUSP1, EZH2, DAPK1, TWIST1, HOXA9, PNPLA8, NRCAM, GLCCI1, CDH1, KLF5, OLIG1, OLIG2, BIK, BCL6, BCL2L10, TP53, ASXL1 and SPARC) (Supplementary

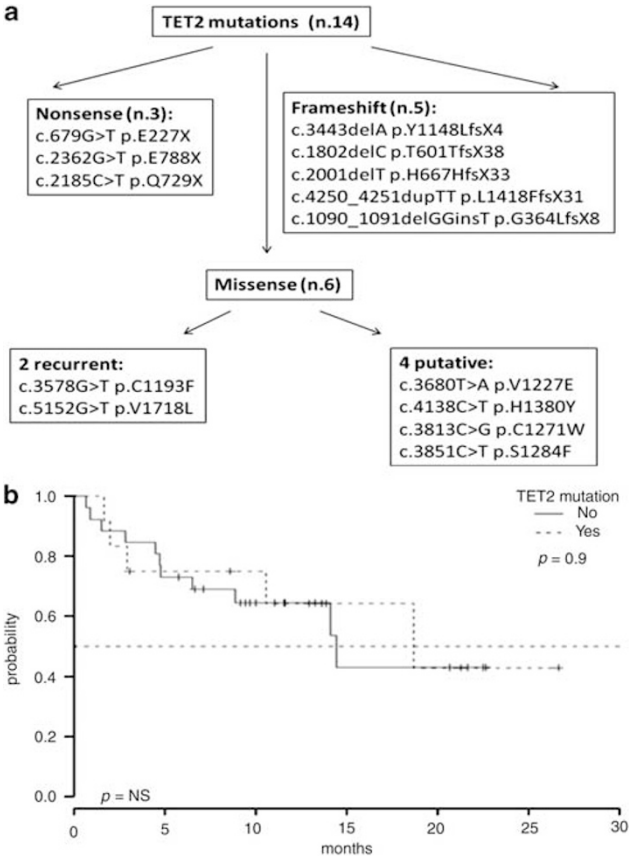

Figure 1 Description of TET2 mutations found in 38 higher-risk MDS patients and their impact on survival. (**a**) Using PCR-based denaturing HPLC, we detected five frameshift, three nonsense and six missense (two recurrent and four putative) TET2 mutations. (**b**) Overall survival of 38 higher-risk MDS patients, treated in the Gimema MDS0205 trial (16), according to TET2 mutational status.

Figures 1 and 2).[7] Statistical analyses were performed using the statistical software environment R (http://www.R-project.org). Principal component analysis was performed to identify methylation patterns. Following factor rotation, we chose four components that could explain about 65% of variance in methylation (screen plot as Supplementary Figure 3). Associations between the four principal components and methylation in the 22 genes are shown in Table 3. TET2 mutations were not associated with any of the components nor with the hypermethylation of any of the genes analyzed. This may be in contrast with the reported correlation between TET2 mutations, low genomic 5hmC content and hypomethylation at differentially methylated CpG sites in patients with myeloid malignancies,[4] and warrants further investigations on the influence of TET2 mutations on DNA methylation patterns.

We then studied the impact of TET2 mutations and methylation profile on outcome of higher-risk MDS patients, treated with epigenetic therapy. Six of 38 patients were not evaluable for treatment response due to early death or premature therapy stop. Five of the eleven TET2-mutated patients (46%) responded to epigenetic treatment, while only five of the 21 patients (24%) with wild-type *TET2* gene responded. Probably due to the relatively small sample size, this difference did not reach statistical significance ($P=0.2$). Duration of response did not differ between the two groups. Median overall survival was 14.4 months, with no differences between TET2-mutated or wild-type patients (log-rank $P=0.9$, Figure 1b). The impact of TET2 mutations on survival is

Table 2 Associations between patients' characteristics and TET2 mutations

Patients n = *38*	*TET2 mutated* n = *12*	*TET2 wild-type* n = *26*	P
Gender			
Males (25)	7	18	0.7
Females (13)	5	8	
FAB classification			
RAEB (22)	6	16	0.08[a]
RAEB-t (12)	3	9	
CMML (4)	3	1	
IPSS			
Int-2 (26)	8	18	1
High (12)	4	8	
Blood counts			
Hemoglobin (g/dl)	10 (8.3–12.2)	9 (5.9–12.2)	0.2
Platelets (10^9/l, median, range)	26.5 (11–77)	61.5 (10–573)	0.08
Neutrophil (10^9/l, median, range)	1.3 (0.2–6.1)	0.9 (0.1–5.3)	0.2
Previous LR-MDS			
Yes (14)	3	11	0.7
No (21)	7	14	
MDS duration before 5-AZA			
Months (median, range)	8.1 (0.9–13.1)	7.3 (0.9–21.4)	0.83
Response			
CR, PR, HI (10)	5	5	0.25
Stable, progressive (22)	6	16	

Abbreviations: CMML, chronic myelomonocytic leukemia; CR, complete remission; FAB, French-American-British classification; HI, hematological improvement; LR-MDS, low-risk MDS; MDS, myelodysplastic syndromes; PR, partial remission; RAEB, refractory anemia with excess blasts.
[a]RAEB/RAEB-t versus CMML (CMML-1:1 patient; CMML2:3 patients).

Table 3 Description of 4 methylation patterns, identified by principal component analysis, able to explain over 65% of sample variance

	Rotated factor pattern			
Gene	*PC1*	*PC2*	*PC3*	*PC4*
TET2	**0.84**			
EZH2	**0.89**			0.26
DAPK1	**0.61**			−0.23
TWIST1	**0.98**			
HOXA9	**0.92**			0.21
ASXL1	**0.9**			
BIK	**0.48**			
CDH1	**0.41**			
RUNX1		**0.91**		
GLCCI1		**0.57**	0.34	0.35
OLIG1		**0.93**		
SPARC		**0.61**		0.33
OLIG2		**0.48**		0.33
DUSP1		**0.26**		
BCL6			**0.82**	
BCL2L10			**0.73**	
TP53	0.25		**0.9**	0.28
NRCAM			**0.38**	0.36
PTEN		0.27		**0.85**
Foxo3		0.28	−0.22	**0.8**
PNPLA8	0.21	0.21		**0.48**
KLF5				**0.24**

Values in bold are those with the strongest association with the principal component (PC).

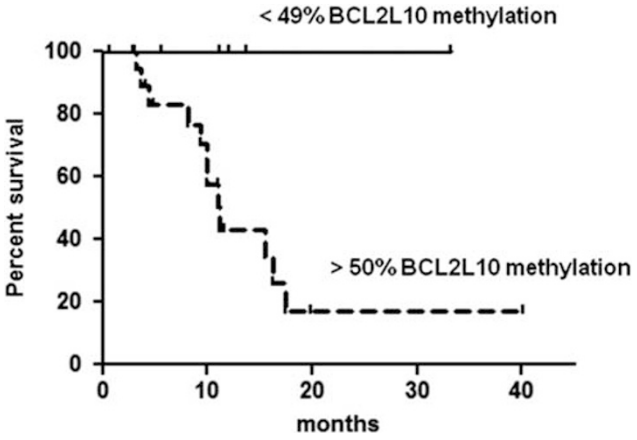

Figure 2 Negative prognostic role of BCL2L10 hypermethylation. The prognostic role of BCL2L10 methylation was confirmed in an independent group of 27 higher-risk MDS patients, treated with 5-AZA at standard doses. Patients with with over 50% BCL2L10 methylation had a significantly lower probability of overall survival ($P = 0.04$).

controversial. Decreased survival in TET2-mutated AML patients has been reported by Abdel-Wahab *et al.*[3] A study on 96 MDS patients, including lower and higher risk MDS, mostly treated with supportive treatment, identified mutant TET2 as a positive prognostic marker, with 77% 5-year overall survival for TET2-mutated patients versus 18% for wild-type patients.[8] On the other hand, TET2 mutations were not associated to IPSS score, karyotype, World Health Organization subtypes, time to AML transformation and overall survival in 320 MDS patients.[9] The GFM recently reported significantly higher response rates to 5-azacytidine in TET2 mutated high-risk MDS patients ($n = 63$ RAEB) and AML with low blast counts ($n = 23$ patients).[10] Similar to our data, no differences for duration of response or survival were reported.[10]

Promoter methylation has been shown to have a significant role in the pathogenesis and progression of myelodysplastic syndromes, but a reliable methylation marker, predictive of treatment response has not been identified yet. Follo *et al.*[11] showed that reduction of phosphoinositide-phospholipase C beta1 methylation correlated with azacytidine response in high-risk MDS, whereas increasing levels of promoter methylation were observed in refractory patients. We then analyzed for associations between methylation patterns and survival. An association became evident between the third component, which strongly impacts on methylation of the genes *NRCAM*, *BCL6*, *TP53* and *BCL2L10*, and overall survival ($P = 0.009$, hazard ratio: 1.89, 95% confidence limits: 1.17–3.06). When the third component was inserted into a multivariate model including the IPSS score (Int-2 versus High), it remained a prognostic factor for overall survival (hazard ratio: 1.63, 95% confidence limits: 10.01–2.64, $P = 0.047$) (Supplementary Figure 4). It is unclear how hypermethylation of the third component influences outcome after epigenetic therapy. High methylation levels of oncosuppressor genes might increase resistance to epigenetic treatment. Among prognostically significant oncosuppressor genes, *p53* methylation is particularly interesting. *p53* inactivation by mutations has been reported to have a significant role in MDS biology, while the role of *p53* methylation in MDS is not well defined. *p53*-deficient mouse embryo fibroblasts, lacking functional *p53* expression, were shown to have low sensitivity to the cytotoxic

function of decitabine.[12] In this line, efficacy of epigenetic therapy may be influenced by *p53* inactivation by methylation.

Among genes included in the third component, a higher methylation rate of the apoptosis gene *BCL2L10* was in particular significantly associated to worse overall survival in patients enrolled into the Gimema MDS0205 multicenter trial. This was confirmed in an independent group of 27 higher-risk MDS patients, treated with 5-AZA at standard doses (Table 1). In both test and validation groups, there were no associations between BCL2L10 methylation, studied as continuous variable, and patient characteristics including age, sex, type and duration of MDS, BM-blast count, WBC, hemoglobin levels, platelet counts and IPSS. Patients with BCL2L10 methylation had a lower probability of achieving response (including complete remission, partial remission and hematological improvement, $P=0.047$). This translated into a significantly lower probability of overall survival for patients with over 50% BCL2L10 methylation ($P=0.04$, Figure 2). At a median follow-up of 10 months (range 0.6–40 months), all seven patients (three with high and four with Int-2 IPSS) with BCL2L10 methylation below 50% were alive, indicating an independent prognostic role of BCL2L10 methylation. These data show that BCL2L10 methylation may predict response to 5-AZA in MDS patients. BCL2L10 is a member of the BCL2 family with contradictory functions in apoptosis. Most recently, a prevalent antiapoptotic function has been described for BCL2L10 protein overexpression in gastric cancer, through activation of the pro-survival activity of the IKK–NF-kB pathway.[13] It is likely that higher methylation levels may be responsible for resistance to demethylation and impair restoration of BCL2L10 expression following epigenetic treatment. In this model, higher rates of BCL2L10 promoter methylation would contrast the apoptotic effect of 5-azacytidine treatment and negatively impact survival. Accordingly, reduced BCL2L10 expression has been shown to function as independent negative prognosticator of gastric carcinoma.[13]

In conclusion, TET2 mutations are a recurrent finding in higher-risk MDS patients, but did not have an impact on the methylation profile of a 22-gene array and on the response to epigenetic treatment in our patients. Methylation profiling, in particular for the *BCL2L10* gene could identify higher-risk MDS patients with worse outcome following epigenetic treatment. Prospective studies on larger patient numbers are warranted to confirm this finding.

Conflict of interest

MTV, PM, CF, VS and GL received 'Honoraria' from Celgene as speakers. The other authors declare no conflict of interest.

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