Leukemia Research 56 (2017) 21–28

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Research paper

TET2 exon 2 skipping is an independent favorable prognostic factor for cytogenetically normal acute myelogenous leukemia (AML) TET2 exon 2 skipping in AML

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 3 January 2017
Accepted 5 January 2017
Available online 16 January 2017

Keywords:
Acute myeloid leukemia mRNA splicing
Intensive chemotherapy TET2
Prognosic

ABSTRACT

In AML, approximately one-third of expressed genes are abnormally spliced, including aberrant TET2 exon 2 expression. In a discovery cohort (n = 99), *TET2* exon 2 skipping (TET2E2S) was found positively associated with a significant reduction in the cumulative incidence of relapse (CIR). Age, cytogenetics, and TET2E2S were independent prognostic factors for disease-free survival (DFS), and favorable effects on outcomes predominated in cytogenetic normal (CN)-AML and younger patients. Using the same cut-off in a validation cohort of 86 CN-AML patients, TET2E2S^{high} patients were found to be younger than TET2^{low} patients without a difference in the rate of complete remission. However, TET2E2S^{high} patients exhibited a significantly lower CIR ($p < 10^{-4}$). TET2E2S and *FLT3-ITD*, but not age or *NPM1* mutation status were independent prognostic factors for DFS and event-free survival (EFS), while TET2E2S was the sole prognostic factor that we identified for overall survival (OS). In both the intermediate-1 and favorable ELN genetic categories, TET2E2S remained significantly associated with prolonged survival. There was no correlation between TET2E2S status and outcomes in 34 additional AML patients who were unfit for IC. Therefore our results suggest that assessments of *TET2* exon 2 splicing status might improve risk stratification in CN-AML patients treated with IC.

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http://dx.doi.org/10.1016/j.leukres.2017.01.012
0145-2126/

1. Introduction

Intensive anthracycline and cytarabine (AraC)-based combination chemotherapy has been used as the backbone of acute myeloid leukemia (AML) treatment for nearly 40 years [1]. Using such a regimen, ~70–80% of patients who are 60 years old or younger with non-promyelocytic AML will achieve complete remission [2,3]; however, most patients will ultimately relapse, and the overall survival of these patients is only 40–45% at 5 years. Among patients who were older than 60 year old, 40–50% of those exhibiting a good performance status can achieve complete remission, but their cure rates are less than 10% and median survival is less than 1 year [4,5]. In addition to patient-dependent factors, pretreatment cytogenetic and molecular abnormalities, such as *NPM1* (nucleophosmin gene 1) and *CEBPA* (CCAAT/enhancer binding protein alpha gene) mutations or *FLT3*-ITD (internal tandem duplications in the fms related tyrosine kinase 3 gene), represent the strongest prognostic factors [6]. Nevertheless, despite several attempts to precisely stratify patients based on these cytogenetic and molecular subcategories, the prognosis of many patients with AML, especially CN-AML, remains uncertain and the optimal post-remission therapy for such patients is unclear. Consequently, some patients remain undertreated and rapidly relapse, whereas others are overtreated and unnecessarily exposed to treatment-related toxicity.

Recently, AML cells have been found to harbor a huge number of alternatively expressed exons [7]. Expression of these exons can be caused by numerous factors, such as spliceosome mutations [8], histone acetylation/methylation, DNA CpG methylation, and WT1 expression [9]. Alternative exon usage (AEU) can also play a role in the plasticity of tumor cells and may thereby influence patient responses to treatment. Previous reports have suggested that certain splicing events, such those involving WT1 [10], TP53 [11], HOXA9 [12], BAALC [13], VEGF [14], or BCL-X [15], might influence disease outcomes.

TET2 is a dioxygenase that catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine and thereby promotes DNA demethylation [16]. *TET2* somatic mutations occur in 7–23% of AML and 25% of myelodysplastic syndrome (MDS) patients, and they are distributed across the entire coding sequence without obvious hot spots [17]. These mutations decrease TET2 enzymatic activity by either truncating the protein or affecting its catalytic activity [16]. *TET2* mutations confer favorable outcomes in high-risk MDS treated with hypomethylating agents [18,19]. In intensively treated AML patients, several studies have investigated the impact of TET2 mutations at the time of diagnosis on outcomes, and have yielded conflicting results as mutations have both been reported to exert unfavorable [20,21] or neutral effects on patient outcomes [22–24]. Recently, fluctuations in TET2 exon 2 expression have been detected in leukemic cells [25]. *TET2* exon 2 skipping (TET2E2S) has been identified in AML cell lines that are resistant to AraC and in fresh diagnosis bone marrow samples deriving from patients subsequently cured with IC [25].

Herein, we hypothesize that TET2E2S may serve as biomarker for outcomes in AML patients who undergo intensive treatment. In 219 AML patients (discovery cohort, 99; validation cohort, 86; AML unsuitable for IC, 34), the TET2E2S status at diagnosis was found to be associated with a reduced relapse rate and prolonged survival in intensively treated patients, independently of routinely used clinical, molecular, and cytogenetic prognostic markers. This positive effect was most robust in CN-AML and younger patients, suggesting that assessments of *TET2* exon 2 splicing status might improve AML risk stratification.

2. Methods

2.1. Patients and sample collection

The medical ethics committee of the Hospices Civils de Lyon (ECHCL) approved this study. Written informed consent, which was approved by the ECHCL, was obtained from each patient and healthy volunteer in accordance with the Declaration of Helsinki and institutional guidelines. The discovery cohort included 99 consecutive non-APL AML patients who were homogeneously treated at Lyon's University Hospital following the French acute leukemia group (ALFA) protocols 9801, 9802, and 9803 between 2001 and 2007 (Table 1). The validation cohort included 86 CN-AML patients who were more recently treated at Lyon and Saint-Etienne's University Hospitals according to either the ALFA 0701, ALFA 0702, or Groupe Ouest Est d'Etude des Leucémies aiguës et Autres Maladies du Sang (GOELAMS) protocols LAM 2006 IR/LAM SA 2007 between 2008 and 2013 (Table 2). In CN-AML from the validation cohort, FLT3–ITD [26] and mutations in NPM1 (exon 12) [27] were assessed as previously described. CEBPA mutations were assessed in CN-AML in the absence of a FLT3-ITD or NPM1 mutation. Patients with double mutant CEBPA were classified in the favorable ELN genetic group.

2.2. RNA purification and reverse transcription

RNA was isolated with TRIzol reagent (Invitrogen). RNA concentrations and purity were determined using UV spectrophotometry (Nanodrop). Prior to carrying out reverse transcription reactions, RNA was treated with DNAase (DNAse-free™, Invitrogen) to prevent DNA contamination. Then, cDNA was synthesized from 1 μg RNA using a random primer (High Capacity cDNA Reverse Transcription Kit, Invitrogen) and Superscript II reverse transcriptase (Invitrogen).

2.3. Quantitative exon-specific polymerase chain reaction (qPCR)

For qPCR, cDNA was amplified in 20 μl reactions using iQ SYBR Green PCR Supermix (BioRad) with 10 μM of each appropriate primer. Thermocycling was carried out using a Bio-Rad Chromo4: CD002161. Expression of a housekeeping gene, *Gus* (NM_000181), was used as an internal control. Splicing events were annotated on the FasterDB database and primers were designed to encompass alternative splicing events using Primer 3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) as previously described [1]. TET2 E2E3, E1E3, and E10 sequences were amplified with TET2E2_3F (5′-CGACTGCAACTGCTGGATT-3′) and TET2 E2_3 R (5′-TGCTTCCAAGCGTTGTAACT-3′) oligonucleotides (ONs), TETE2 E1_3 F (5′-TCTGAGGGCTGGCAAACATT-3′) and TET2 E1_3 R (5′-TCATGTCCTATTGGCTGCTG—3′) ONs, and TETE2 E10F (5′-TGAACACAGAGCACCAGAGTC-3′) and TETE2E10 R (5′-ATGTGCTGCCATTCTGCAT-3′) ONs, respectively. A melting curve (65–92 °C) was generated at the end of each run to verify specificity of the amplicon. The Pfaffl method was used for relative quantification of transcript levels [28]. Primer sequences are available upon request.

2.4. Statistics

Associations between categorical variables were analyzed using Fisher's exact test. Central tendency differences between groups were compared using Mann–Whitney or Kruskal–Wallis tests. Associations between continuous variables were assessed using Spearman's correlation. OS was defined as the time from initiating IC treatment to death from any cause. EFS was defined as the time from diagnosis to either treatment failure, relapse, or death.

Table 1
Characteristics of the 219 patients treated with or without intensive anthracyclin-araC-based chemotherapy.

	Intensively treated AML		P-value	Unfit
	Discovery cohort n = 99	Validation cohort n = 86		n = 34
Age at diagnosis (years)	56	57	ns	74
Gender n (%)			ns	
Male	55 (56%)	45 (52%)		19 (56%)
Female	44 (44%)	41 (48%)		15 (44%)
ELN Cytogenetics n (%)				
Favorable	3 (3%)	–		–
Intermediate	72 (72%)	86 (100%)		34 (100%)
Adverse	24 (24%)	–		–
Molecular features n (%)				
NPM1 mutation	–	58 (67%)		13 (38%)
FLT3-ITD	–	32 (37%)		7 (21%)
Allogeneic Stem Cell Transplantation n (%)	25 (25%)	33 (38%)	ns	0 (0%)
In first CR	13 (13%)	24 (28%)		–
In second CR	10 (10%)	7 (8%)		–
At the time of relapse	2 (2%)	2 (2%)		–
Outcome				
Complete response to induction regimen	76 (77%)	77 (89%)		2 (6%)
Overall survival (5 years, 95% CI)	25% (17–34)	42% (26–57)		0%
Cumulative incidence of relapse (5 years, 95% CI)	53% (42–52)	30% (20–41)		100%

Table 2
Characteristics of the 99 discovery cohort patients according to the level of TET2 exon 2 expression.

	$TET2E2S^{High}$ n = 42	$TET2E2S^{Low}$ n = 57	*P*-value
Age at diagnosis (years)	55	56	ns
Gender n (%)			ns
Male	25 (59%)	30 (53%)	
Female	17 (41%)	27 (47%)	
ELN Cytogenetics n (%)			ns
Favorable	2 (5%)	1 (2%)	
Intermediate	28 (67%)	44 (77%)	
Adverse	12 (28%)	12 (21%)	
Allogeneic Stem Cell Transplantation n (%)	13 (31%)	12 (21%)	ns
Outcome			
Complete response to induction regimen	25 (59%)	51 (89%)	0.001
Cumulative incidence of relapse (5 years, 95% CI)	21% (9.7–35.4)	75% (61.6–84.9)	<0.0001
Disease-free survival (5 years, 95% CI)	52% ± 10%	12% (4.78–22.2)	0.0007
Event-free survival (5 years, 95% CI)	31% (19.8–47.5)	10% (4.28–20.0)	0.21
Overall survival (5 years, 95% CI)	31% (17.3–46.1)	17% (9.03–2.84)	0.37

DFS was defined as the time from achievement of CR to the date of relapse or death. Patients who remained alive were censored at the last follow-up visit. Allograft recipient patients were not censored at the time of transplantation. TET2 exon 2 skipping was quantified by calculating the E1E3/E2E3 ratio [25]. For categorical and survival analyses, the E1E3/E2E3 ratio was dichotomized using a cutoff point that was based using the Maximally Selected Rank Statistics [29] (maxstat R-statistics package, from the R-Project for Statistical Computing: R 3.2.0 software package). Samples with a level of TET2 exon 2 skipping that was higher than that corresponding to the cutoff value were termed $TET2E2S^{high}$, whereas those with a value lower than the cutoff were termed $TET2E2S^{Low}$. Survival rates and DFS were calculated using the Kaplan–Meier method [30]. Log rank tests were used to compare survival rates between groups. To identify prognostic factors for OS, DFS, and EFS, multivariate analyses were performed using a Cox proportional hazards model [31]. A stepwise backwards procedure was used with a threshold significance level of $P < 0.10$ to select factors in the model. All tests were two-sided. The final type I error rate was fixed at 0.05 to identify factors associated with "time to event" outcomes. The cumulative incidence of relapse (CIR) analysis only included patients who achieved a CR, for which the time was calculated from the date of CR until relapse. Patients who remained alive without relapse were censored, whereas those who died without relapse [non-relapse mortality (NRM)] were counted as a competing cause of failure. The mean CIR with standard error (SE) were estimated using the method of Gray [32], and differences between groups were analyzed using a test developed by Gray [33] with the RcmdrPlugin.EZR script from R-software [34].

3. Results

3.1. TET2 exon 2 skipping is an independent favorable prognostic factor among intensely treated AML patients from the discovery cohort

Patient characteristics are summarized in Table 1. We found TET2E2S to be associated with DFS, so we divided the 99 intensely treated patients from the discovery cohort into two categories based on the TET2E2S value. Patients were dichotomized accord-

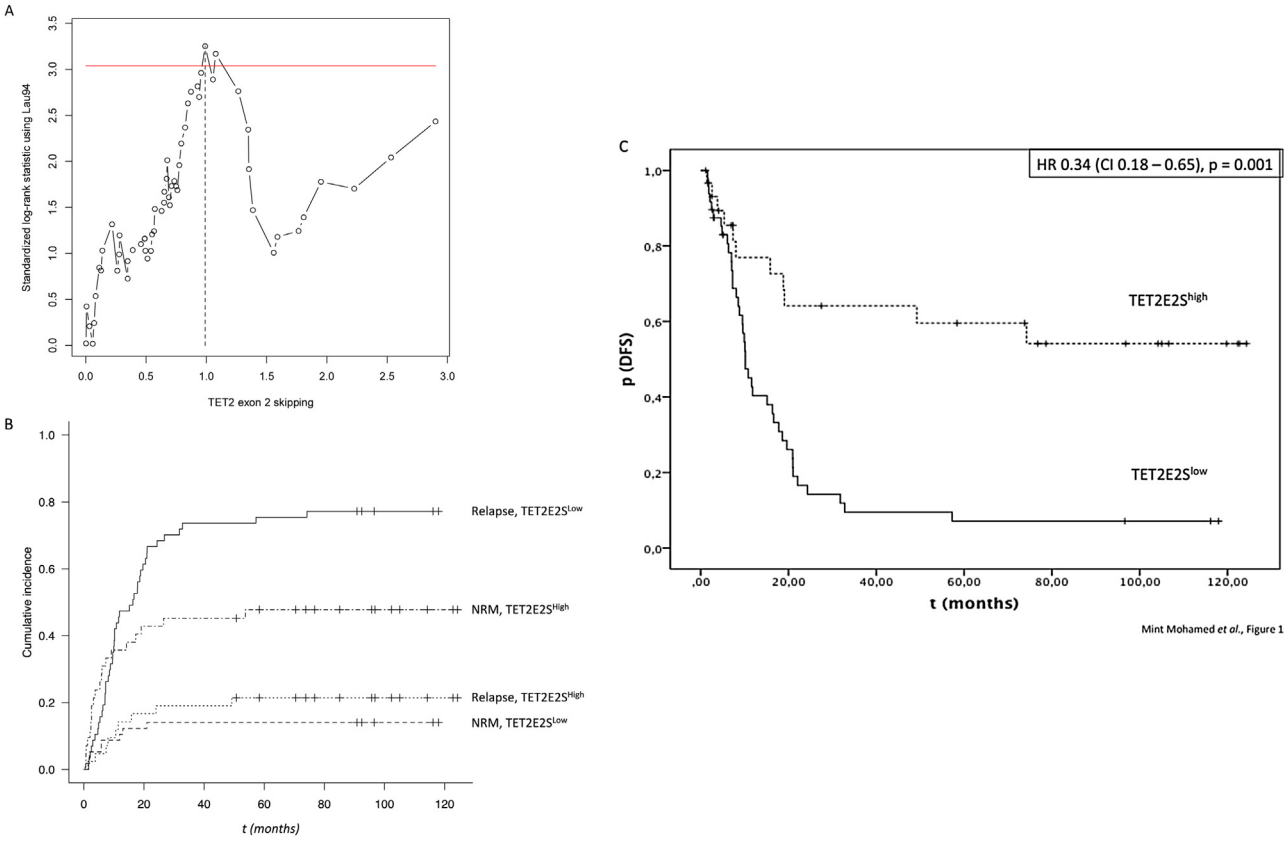

Fig. 1. Effects of TET2 exon 2 skipping on disease-free survival in intensively-treated patients in the discovery cohort. A. Absolute standardized log-rank statistics and significance based on improved Bonferroni inequality. B. Cumulative incidence of relapse and non-relapse mortality. C. Disease-free survival.

Table 3
Multivariate analyses of disease-free survival in intensively treated AML patients from the discovery cohort.

Factor	Disease-free survival		
	Hazard ratio	95% CI	P-value
Age at sampling ≤median vs. >median	2.24	1.29–3.89	0.004
ELN Cytogenetic risk Favorable + Intermediate versus Adverse	2.67	1.40–5.08	0.003
TET2 exon 2 skipping High vs. low expression	0.38	0.20–0.73	0.003

ing to the cutoff determined using maximally selected log-rank statistics (Fig. 1A, Table 2) [29]. The cutoff was 0.99 (Fig. 1A) and TET2E2S^{high} patients were defined as patients with TET2E2S > 0.99, whereas TET2E2S^{low} patients had a TET2E2S value ≤ 0.99. In 4 patients, the level of E2E3 expression was below the sensitivity threshold for the PCR assay; these cases were considered to be TET2E2S^{high}. The two groups were well matched for age, gender, and cytogenetics, while the proportion of allografted patients was not significantly different between TET2E2S^{high} and TET2E2S^{low} patients (Table 2). TET2E2S^{high} patients had a significantly lower CR rate compared with TET2E2^{low} patients (Table 2). However, the CIR was significantly lower in the TET2E2S^{high} than in the TET2E2S^{low} group (Table 2, Fig. 1), while there was no significant difference in the NRM rate between these groups (Fig. 1). The median follow-up duration was 106 months. TETE2S^{high} patients had a significantly longer DFS compared with TET2E2S^{low} patients (Table 2, Fig. 1C). Multivariate analysis showed that age, cytogenetics, and TET2E2S were independent prognostic factors for DFS (Table 3). The overall level of *TET2* expression, as measured based on qRT-PCR amplification of a 10 exon sequence, was not significantly associated with the response rate, relapse rate, CIR, DFS, EFS, or OS (not shown). Furthermore, the overall level of *TET2* expression was not significantly different between TET2E2S^{high} and TET2E2S^{low} patients (3.38 versus 4.78, p = 0.56).

In the 55 patients from the discovery cohort who were younger than 60 years old, the overall response rate was 82% and there was no significant difference between TET2E2S^{high} (18/24, 75%) and TET2E2S^{low} AML patients (27/31, 87%, p = 0.078), while the 5-year CIR was significantly lower in the TET2E2S^{high} group than in the TET2E2S^{low} group (16.7% [95% CI 5–34.3] vs. 71% [95% CI 51–84], p = 0.00011 by the Gray test; Supplementary Fig. 3A). Univariate analysis showed that for patients younger than 60 years old, TET2E2S^{high} patients had significantly longer rates of DFS (log rank test p = 0.00234; Supplementary Fig. 3B), EFS (log rank test p = 0.034; Supplementary Fig. 3C), and OS compared with TET2E2S^{low} patients (log rank test p = 0.039; Supplementary Fig. 3D). In the 44 patients who were greater than 60 years old, the CR rate was 71% and TET2E2S^{high} patients had a significantly lower CR rate than TET2E2^{low} patients (7/18 [39%] vs. 24/26 [92%], $p < 10^{-4}$), whereas the 5-year CIR was significantly lower in the TET2E2S^{high} group than in the TET2E2S^{low} group (27.8 [95% CI 8.4–51.5] vs. 84.6 months [95% CI 58.5–94.9], p = 0.006 by the Gray test; Supplementary Fig. 4). In patients greater than 60 years old, TET2E2S showed no significant impact on OS, DFS, or EFS (not shown).

3.2. *TET2 exon 2 skipping is an independent favorable prognostic factor for CN-AML patients*

The aforementioned results suggested that although TET2E2S is associated with a decreased CR rate, the CR duration is significantly longer in TET2E2S^{high} AML patients, suggesting that TET2E2S

Table 4
Characteristics of the 86 patients from the validation cohort according to TET2 exon 2 skipping.

	TET2E2S^{High} n = 53	TET2E2S^{Low} n = 33	P-value
Age at diagnosis (years)	54	61	0.027
Gender n (%)			ns
Male	28 (53%)	20 (61%)	
Female	25 (47%)	13 (39%)	
Molecular features n (%)			
NPM1 mutation	38 (72%)	20 (61%)	ns
FLT3-ITD	20 (38%)	12 (36%)	ns
CEBPA double-mutation*	3/12 (25%)	0/10 (0%)	ns
ELN genetic group n (%)			ns
Favorable	23 (43%)	11 (33%)	
Intermediate-1	30 (57%)	22 (67%)	
Allogeneic Stem Cell Transplantation n (%)	22 (41%)	11 (33%)	ns
Outcome			
Complete response to induction regimen	46 (87%)	31 (94%)	ns
Cumulative incidence of relapse (2 years, 95% CI)	4% (7–12)	80% (57–92)	<0.0001
Disease-free survival (2 years, 95% CI)	88% (74–95)	19% (7–36)	<0.0001
Event-free survival (2 years, 95% CI)	75% (60–84)	14% (4–29)	<0.0001
Overall survival (2 years, 95% CI)	77% (62–87)	38% (20–56)	0.0002

Table 5
Multivariate analyses of survival in the 86 intensively treated patients with CN-AML from the validation cohort.

Factor	Hazard ratio	Disease-free survival		Event-free survival			Overall survival	
		95% CI	P-value	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
TET2 exon 2 skipping Low vs. high expression	0.088	0.034–0.229	<0.0001	0.226	0.117–0.436	<0.0001	0.277	0.136–0.565	<0.001
FLT3-ITD Present vs. absent	1.949	0.950–3.998	0.069	1.707	0.914–3.187	0.093	–	–	–

has a more favorable effect on the outcome of CN-AML that develops in younger individuals. Accordingly, we next investigated the effects of TET2 exon 2 skipping in a validation cohort of 86 additional consecutive CN-AML patients treated with IC. All patients were diagnosed between 2008 and 2013 and were tested for *FLT3*-ITD and *NPM1* exon 12 mutations; in addition, we searched for *CEBPA* mutations in patients without *FLT3*-ITD or *NPM1* mutations. Patients from the validation cohort were divided into 2 categories according to the TET2E2S cutoff (0.99) that was determined by maximally selected log-rank statistics for the discovery cohort (Table 4). TET2E2S^{high} patients were found to be significantly younger than TET2E2S^{low} patients (Table 4); these two groups of patients were well balanced for sex, ELN genetic group distribution, and *FLT3*-ITD, *NPM1*, and *CEBPA* mutation status (Table 4). The proportion of allograft recipient patients was not significantly different between TET2E2S^{high} and TET2E2S^{low} patients (Table 4). Similarly to CN-AML patients from the discovery cohort, there was no significant difference in the CR rate between TET2E2S^{high} and TET2E2S^{low} patients from the validation cohort (Table 4). The CIR was significantly lower in the TET2E2S^{high} compared with the TET2E2S^{low} patients (Table 4, Fig. 2). The median duration of follow-up was 24 months. Univariate analysis showed that TET2E2S^{high} patients had a significantly longer DFS, EFS, and OS compared with TET2E2S^{low} patients (Table 4, Fig. 2, Supplementary Fig. 5). These differences persisted for DFS, EFS, and OS in the 43 patients who were younger than 60 years old (Supplementary Fig. 6) and for DFS and EFS in the 43 patients who were greater than 60 years old (Supplementary Fig. 7). In both the ELN genetic favorable and intermediate-1 groups, TET2E2S remained a favorable prognostic factor for OS, DFS, and EFS (Supplementary Fig. 8). Differences between the TET2E2S^{high} and TET2E2S^{low} groups were statistically significant for DFS, EFS, and OS in the favorable ELN genetic group. In the intermediate-1 ELN group, differences were statistically significant for DFS and EFS (Supplementary Fig. 8). Multivariate analysis showed that TET2E2S and *FLT3*-ITD, but not age or *NPM1* mutation (with or without *FLT3*-ITD), were independent prognostic factors for DFS and EFS, while TET2E2S was the sole prognostic factor for OS (Table 5).

3.3. Prognostic impact of TET2 exon 2 skipping in AML patients who were unsuitable for intensive chemotherapy

The prognostic effect of TET2E2S was evaluated in an additional series of 34 AML patients who were unsuitable for IC (Table 1) and were instead treated with hydroxyurea (9 patients), azacitidine (5 patients), decitabine (2 patients), low dose AraC (10 patients), or supportive care alone (8 patients). TET2E2S showed no significant impact on the response rate, relapse rate, OS, DFS, or EFS in this cohort (not shown).

4. Discussion

In AML, abnormal alternative splicing involves genes that encode various oncogenes, tumor suppressor proteins, splicing factors, and heterogeneous-nuclear-ribonucleoproteins, which encode proteins involved in apoptosis, cell proliferation, and spliceosome assembly [7]. This suggests the possibility that such deregulated patterns of alternative splicing likely have additional pathogenic, diagnostic, prognostic, and therapeutic implications. This present study was carried out to gain insights into the potential clinical consequences of TET2 exon deregulation in AML. Recently, an analysis of exon arrays permitted TET2E2S to be identified in AraC-resistant cells *in vitro* and allowed for the diagnosis of samples from AML cases who did not relapse with a >4-year follow-up period [25]. Among the 99 intensively treated patients from the discovery cohort, TET2E2S status at the time of diagnosis was associated with a significantly reduced CR rate. This effect appeared to be restricted to older patients and AML patients with an abnormal karyotype, while it was not observed in younger patients from the

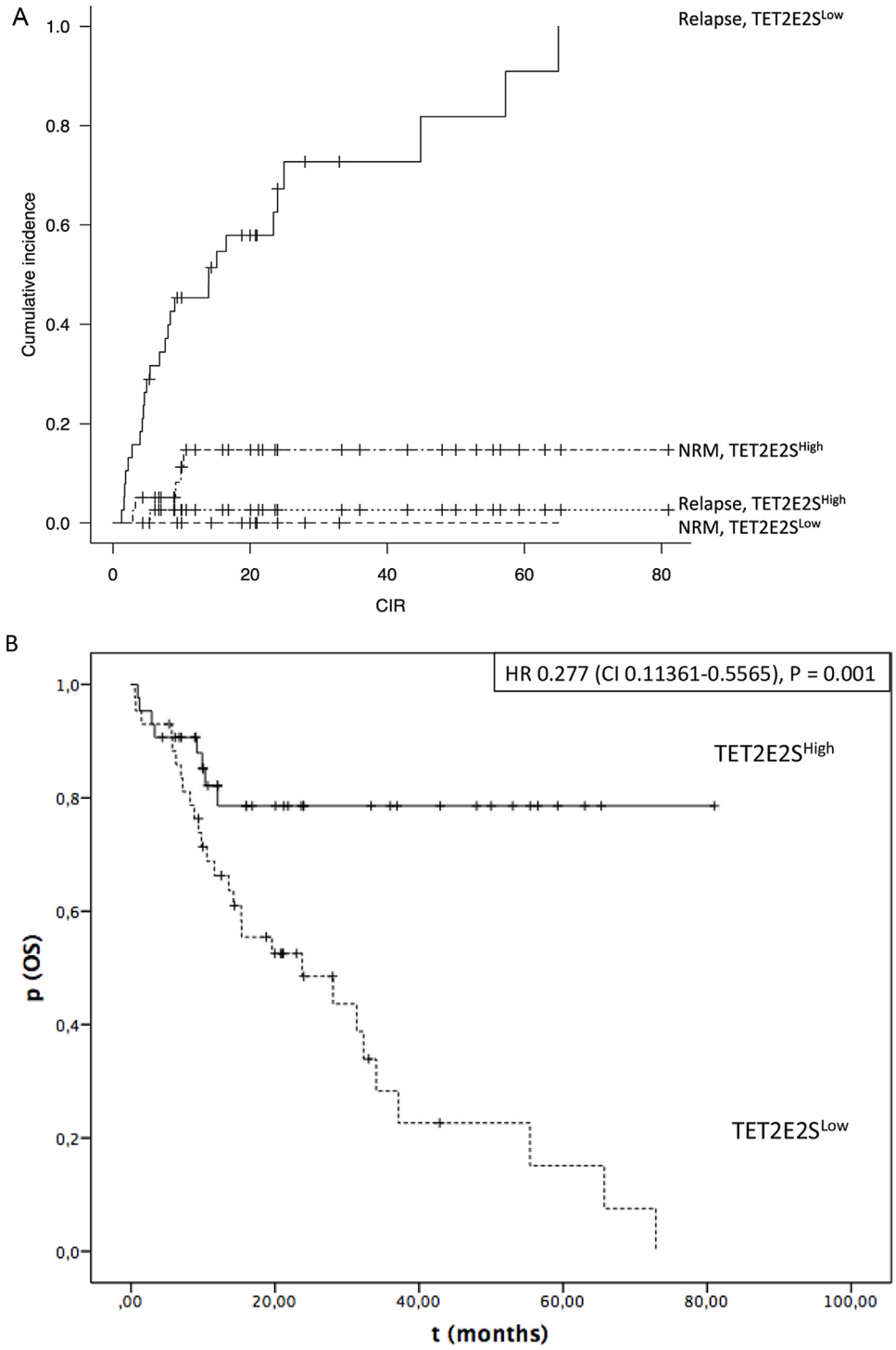

Fig. 2. Effects of TET2 exon 2 skipping on the cumulative incidence of relapse, non-relapse mortality (A) and overall survival (B) in 86 CN-AML patients from the validation cohort.

discovery cohort, including CN-AML patients from both the discovery and validation cohorts. Furthermore, in the discovery cohort, TET2E2S status was associated with a significantly reduced CIR, especially in CN-AML and younger patients. This finding was confirmed in 86 CN-AML patients from the validation cohort in whom the cutoff value of TET2E2S was defined by maximally selected log-rank statistics in the discovery cohort, which allowed the validation cohort to be dichotomized into 2 groups that exhibited clearly distinct outcomes. Accordingly, high levels of TET2E2S were found associated with reduced CIR and prolonged OS, EFS, and DFS, independently of routinely used clinical and molecular prognostic markers. In contrast to intensively treated AML, TET2E2S exhibited no prognostic impact for cases of AML who were unsuitable for IC.

TET2 exon 2 is spliced in a mutually exclusive manner compared with exon 1, and it uses an alternative promoter (https://fasterdb.lyon.unicancer.fr/). However, *TET2* exon 2 is not translated and its role in the regulation of *TET2* expression remains unknown. Present results suggest that *TET2* exon 2 expression does not influence overall TET2 expression in vivo. Using qES-PCR, we have recently shown that levels of TET2E2S are significantly higher in AML samples com-

pared with normal bone marrow mononuclear cells [25]. In accord with *in vitro* findings obtained using AraC-resistant cells, TET2E2S was found to be associated with a significantly lower response rate in intensively treated patients from the discovery cohort. This effect was strongest in older patients and AML patients with an abnormal karyotype, while it was absent in CN-AML and younger patients. TET2E2S did not influence the response rate in the 86 CN-AML patients from the validation cohort irrespective of age. In accord with *in vivo* findings obtained by microarray [25], TET2E2S was found to be associated with a significantly reduced CIR in intensively treated patients from the discovery cohort. This favorable effect was strongest in younger patients and CN-AML patients, and could be confirmed using the same cutoff value in the validation cohort irrespective of the age of the patient. Overall, TET2E2S was found to be independently associated with a favorable outcome in both cohorts. In the discovery cohort, this favorable effect was independent of age and cytogenetics, while it remained strongest in younger and CN-AML patients. In the validation cohort, the prognostic impact of TET2E2S was found to be independent of age, *FLT3*-ITD, and *NPM1* mutation status, and TETE2S remained a favorable prognostic factor for DFS, OS, and EFS in both the ELN favorable and intermediate-1 groups (Supplementary Fig. 1). Together, these results suggest that the favorable prognostic impact of TET2E2S on predicting against relapse. Accordingly, at 5 years 78% ± 6.5% of TET2E2S^{high}/CN-AML patients from the validation cohort were predicted to be alive and in CR compared with only 7.5% ± 6.6% of TET2E2S^{low}/CN-AML patients ($p < 10^{-4}$, Fig. 1). Because this present report is based on a retrospective analysis, further studies will be necessary to confirm these findings.

To date, the prognostic impact of TET2 in hematological malignancies has been assessed at the genomic level. In MDS, *TET2* mutations have been associated with a higher rate of response to hypomethylating agents [18,19]. In AML patients who receive intensive therapy, Metzeler et al. identified *TET2* mutations as a negative prognostic factor restricted to patients with CN-AML and favorable genetic aberrations (*NPM1* mutated/*FLT3* no ITD, or *CEBPA* mutated) [20]. This finding was not confirmed in other studies [17,23,24]. By contrast, Chou et al. (2011) found that *TET2* mutations were a negative prognostic factor for patients with an intermediate cytogenetic risk. However, the presence of *TET2* mutations lost its independent significance if additional genetic aberrations were also considered [35]. To the best of our knowledge, these present findings are the first to link aberrant *TET2* mRNA splicing with clinical outcomes in intensively treated CN-AML patients. As detailed in the Methods section, we measured *TET2* exon 2 skipping using qES-PCR by calculating the ratio E1E3/E2E3, while overall *TET2* expression was measured based on the qRT-PCR amplification of a sequence from exon 10, which is not known to be alternatively spliced (https://fasterdb.lyon.unicancer.fr/). Using these two methods to analyze patient samples, our results indicate that in contrast to TET2E2S, fluctuations in the overall mRNA transcript expression levels of TET2 did not correlate with patient responses to IC, relapse risk, CIR, NRM, DFS, EFS, or OS. This finding demonstrates that aberrant *TET2* splicing, but not transcription influences the outcome of intensively treated AML patients. Accordingly, these present findings suggest that, at least in the case of *TET2*, measurements of AEUs might be more appropriate than measurements of overall gene expression for assessments of disease severity.

The mechanisms that govern TET2 exon 2 expression remain to be elucidated. Recently, we found that WT1 expression influenced the splicing of some ATP-binding transporter mRNAs but not that of TET2 [9]. The mutational landscape of the spliceosome is now available for hematological malignancies and certain mutations have been found linked to specific of splicing patterns [9]. Overall, the proportion of cases carrying spliceosome mutations ranges from <1–90% in MDS, MDS/MPN and secondary AML compared to <1–10.5% in de novo AML [9]. Given the present results, it becomes important to test whether specific spliceosome gene mutations might interfere with TET2 exon 2 expression in de novo AML.

5. Conclusion

In conclusion TET2E2S, but not overall *TET2* gene expression levels, was found to be independently associated with a favorable DFS, EFS, and OS in intensively treated CN-AML patients, but not in AML patients who were unsuitable for IC. The overall impact of TETEE2S as a novel prognostic encouraging biomarker in the AML diagnostic workup will need to be prospectively validated in a larger collective of patients. This finding highlights the need for assessing AEUs rather than overall gene expression levels in prognostic assessments. As they indicate that the level of TET2E2S is significantly associated with survival for currently accepted cytogenetic and molecular prognostic categories, our findings suggest that assessing levels of TET2E2S will permit to better assessments of disease risk, thereby improving patient management and outcomes. Accordingly, our study suggests that TET2 exon 2 skipping should be considered in the diagnostic workup of AML patients.

Acknowledgments

This study was supported by the Agence Nationale pour la Recherche (ANR), Association Laurette Fugain, Association pour la Recherche sur le Cancer (ARC), Fondation de France, Hospices Civils de Lyon, University Lyon-1, Centre National pour la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (Inserm), Association Guillaume Espoir, Amgen, Celgene, Pierre Fabre Medicament, and Novartis. A.M.M. was supported by bursaries from the Islamic Development Bank, the Fondation pour la Recherche Médicale, and the "Les Amis de Rémy" association. F.M. was supported by Inserm and the Hospices Civils de Lyon (AVIESAN CHRT 2010). E.W. was supported by the Hospices Civils de Lyon and Lyon I University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2017.01.012.

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