Adverse prognostic effect of homozygous *TET2* mutation on the relapse risk of acute myeloid leukemia in patients of normal karyotype

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Supplementary methods

The endpoints of response and survival

CR was defined as the presence of a morphologically normal marrow with fewer than 5% blasts, no evidence of extramedullary leukemia, and recovery of peripheral platelet counts to at least 100×10^9/L and of neutrophils to 1.0×10^9/L or more, for at least 4 weeks, in the absence of chemotherapy. The relapse incidence (RI) was defined as the time from attainment of remission to the date of relapse in all patients who achieved CR, with consideration of the competing risk of death without relapse. Non-relapse mortality was defined as death occurring in the absence of relapse. Event-free survival (EFS) was defined as the time from commencement of induction chemotherapy to the date of death from any cause, relapse, or non-achievement of CR, whichever occurred first. Overall survival (OS) was defined as the time from commencement of induction chemotherapy to the date of last follow-up, or death from any cause. Patients undergoing allogeneic HSCT were not censored at the time of transplantation.

Using the ELN classification, subjects were stratified into two categories. The favorable group included those with mutations in *NPN1* but not *FLT3-ITD,* or single or double mutations in *CEBPA* (NK-AML patients). The intermediate-I group included NK-AML patients with mutations in *NPM1* and *FLT3-ITD*; wild-type *NPM1* and *FLT3-ITD*; or wild-type *NPM1* with mutated *FLT3-ITD*. [1]

Statistical analysis

We measured *TET2* mutation frequencies in NK-AML patients. We categorized *TET2* mutations as single, double heterozygous, or homozygous, and compared the outcomes of such patients with those of *TET2* wild-type patients. Clinical characteristics and treatment outcomes were compared by reference to *TET2* mutational status. Descriptive statistics are presented as frequencies with percentages for categorical variables, and as medians with ranges for continuous variables. The chi-squared test was used to compare differences in the distributions of categorical data, and Student's t-test and logistic regression analysis were used to evaluate the significance of differences in continuous variables. Statistical significances were tested by oneway analysis of variables among three groups.

EFS and OS were estimated using Kaplan-Meier survival curves, and differences among groups were compared using the log-rank test. The prognostic impacts of various risk factors on EFS and OS were evaluated via univariate and multivariate analyses using a time-dependent Cox's proportional hazard

model. RI values were calculated using a cumulative incidence method that considered competing risks, and Gray's test was used to perform comparisons.[2] Univariate and multivariate analyses included the following variables: age (<65 years vs. ≥65 years): *FLT3-ITD* mutational status (wild-type vs. mutated); *NPM1* mutational status (wild-type vs. mutated); *CEBPA* mutational status (wild-type vs. mutated); *TET2* mutational status (wild-type vs. mutated); and use of allogeneic HSCT (a time-dependent covariate). All of 6 variables were included in the final multivariate model. We also performed survival analysis by *TET2* mutational subtype (wild-type vs. non-homozygous vs. homozygous). *P*-values of less than 0.05 were considered to reflect statistical significance. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using a predetermined reference risk value of unity.

All statistical analyses were performed using the SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA) and EZR software employing the R-language (available at http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html).[2]

Supplementary references

1. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010 Jan 21;115(3):453-74.
2. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013 Mar;48(3):452-8.

Supplementary Table S1. The sequences of PCR primers used for analysis of *CEBPA* mutational status.

Amplicon	**F-Primer (5'-3')**	**R-Primer (5'-3')**
Fragment 1	CACCTGCAGTTCCAGATCG	AGGCCAGGCTTTCAGGAG
Fragment 2	GCCGGGAGAACTCTAACTCC	GCTTGGCTTCATCCTCCTC
Fragment 3	GCTGGTGATCAAGCAGGAG	GGTCATTGTCACTGGTCAGC

Amplification featured initial denaturation at 95℃ for 5 min; followed by 40 cycles at 94℃ for 30 s, 62℃ for 30 s, and 72℃ for 1 min, and a final extension at 72℃ for 10 min. Three overlapping PCR products covering the entire coding sequence were generated and sequenced using the following PCR primers. The amplification products were sequenced on an ABI 3100 platform using a cycle sequencing kit (BigDye Terminator; Applied Biosystems, Foster City, CA).

Abbreviations: *CEBP*A, CCAAT/enhancer binding protein α.

Supplementary Table S2. Mutation patterns in 61 patients with *TET2* mutations.

Allele	Type of mutation	Reference allele	Mutant allele	Amino acid change
Single	nonsense	C	T	Q1825X
	frameshift	-	AGA	C1271fs
		A	(A)2	K1005fs
		A	(A)2	K1488fs
		G	(G)2	S165fs
		A	-	Q1274fs
		A	-	Q631fs
		A	(A)2	K1004fs
		C	-	S825fs
		C	-	P1012fs
		T	(T)2	C1298fs
		-	AGAAGCC	F1035fs
		A	(A)2	T1047fs
		C	(C)2	A1272fs
		A	(A)2	Y620fs
		CT	-	L1777fs
		C	-	H1764fs
	missense	G	A	G1361D
		T	A	W1847R
		C	T	A1882V
		G	A	A1882T
		T	A	H1868Q
		A	T	N1156I
		A	G	H1380R
		G	C	C1289S
		G	C	C1221S
		G	A	G1282D
	silent	A	G	T1995T
		A	C	P286P
		G	A	K1799K
		G	A	E1874E
		C	T	S1853S
		A	C	P286P
		C	T	N202N
Double	frameshift missense	G A	- G	I264fs N1387S
		T C	- T	F1130fs S1898F
		CT T	- C	S1775fs L1322P
	missense silent	T T	G C	C1211W Y1645Y
	frameshift frameshift	CTCCA AGCACCAGAG	- -	L757fs R1354fs

		A -	- TT	Q1053fs Q1053fs
		TCT G	- -	F1285fs Q1507fs
		C CA	- -	I274fs V1062fs
	frameshift nonsense	CATGGAGCATGTACTACAATGGATGTAAG TTT C	(CATGGAGCATGTACTACAATGGATGTA AGTTT)2 T	S1290fs Q1783X
		A C	(A)2 T	I1116fs R1465X
	missense missense	G T	A C	G1370E C1875R
	missense nonsense	C C	T T	R1359C Q1783X
		A G	G T	H1904R G773X
Homozygous	frameshift	CTGAAGGAAGGCCGTCCATTCTCAGGG	-	G1361fs
	missense	T	C	Y1294H
		G	T	G1936V
		C	G	S1203R
		A	C	N1266H
	nonsense	C	T	Q1664X
		A	T	K1493X
		C	T	Q910X
		T	A	Y1579X
		A	T	K1692X
		C	G	S792X
		C	G	S385X
		C	T	Q740X
		C	T	Q652X

Abbreviation: *TET*, ten-eleven translocation.

A total of 7 cases had synonymous coding changes, leaving 54 patients with somatic mutations

Supplementary Table S3. Clinical characteristics of 407 patients with acute myeloid leukemia of normal karyotype by *TET2* mutational status.

Parameter; no. of patients (%)	Total (n = 407)	*TET2* mutation, (n = 54)	*TET2* wild-type (n = 353)	*p* value*
Age, median (range), years	52 (15–84)	62 (16–83)	50 (15–84)	<0.001†
Age group (years)				
15– <25	25 (6.1)	3 (5.6)	22 (6.2)	
25– <35	45 (11.1)	1 (1.9)	44 (12.5)	
35– <45	70 (17.2)	2 (3.7)	68 (19.3)	
45– <55	86 (21.1)	7 (13.0)	79 (22.4)	
55– <65	115 (28.3)	18 (33.3)	97 (27.5)	
65– <75	58 (14.3)	20 (37.0)	38 (10.8)	
≥75	8 (2.0)	3 (5.6)	5 (1.4)	
Elderly, age ≥ 65 years	66 (16.2)	23 (42.6)	42 (12.1)	<0.001
Gender, male	203 (49.9)	27 (50.0)	176 (49.9)	0.985
WBC, median value ($\times 10^9$/L)	27.3 (0.3–397.21)	46.9 (0.9–282.00)	25.2 (0.3–397.2)	0.013†
Bone marrow blasts (%), median (range)	72 (3–100)	75 (3–100)	70 (10–100)	0.184
Peripheral blast ($\times 10^9$/L), median (range)	8.4 (0–373.4)	19.6 (0–270.7)	6.5 (0–373.4)	0.063
FLT3-ITD mutation	111 (27.3)	15 (27.8)	96 (27.2)	0.929
NPM1 mutation	180 (44.2)	32 (59.3)	148 (41.9)	0.017
NPM1 mutation without *FLT3*-ITD mutation	115 (28.3)	22 (40.7)	93 (26.3)	0.029
CEBPA mutation	80/404 (19.8)	11/54 (20.4)	69/350 (19.7)	0.910
ELN risk group				0.105
Favorable	184/406 (45.5)	30/54 (55.6)	154/352 (43.8)	
Intermediate-I	222/406 (55.0)	24/54 (44.4)	198/352 (56.2)	

***** The *p* values refer to comparisons between the two groups by *TET2* mutational status.

†Upon logistic regression analysis, a *TET2* mutation was associated with increasing age (OR: 1.065, 95% CI 1.038-1.092) and elevated white cell counts (measured on a log scale) (OR: 1.907; 95% CI 1.203-3.023).

Abbreviations: *TET*, ten-eleven translocation; WBC, white blood cells; *FLT3*-ITD, fms-related tyrosine kinase 3-internal tandem duplication; *NPM1*, nucleophosmin1; *CEBPA*, CCAAT/enhancer binding protein α; ELN, European LeukemiaNet.

Supplementary Table S4. Clinical outcomes by *TET2* mutational status in patients with normal-karyotype acute myeloid leukemia, with CR rates; 5-year overall and event-free survival data; and relapse incidence values.

Parameter; no of patients (%)	Total	*TET2* mutation	*TET2* wild-type	*p* value*
All patients	407	54	353	
CR rate (%)	332 (81.6)	41 (75.9)	291(82.4)	0.250
Received allogeneic HCT (%)	131 (32.1)	12 (22.2)	119 (33.7)	0.154
5-year RI rate (95% CI)	43.8% (38.2-49.3)	47.6% (30.3-62.9)	43.3% (37.3-49.2)	0.717
5-year EFS rate (95% CI)	33.5% (28.6-38.4)	28.0% (15.3-40.7)	34.5% (29.2-39.8)	0.391
5-year OS rate (95% CI)	37.2% (32.3-42.1)	35.8% (22.3-49.3)	37.4% (32.1- 42.7)	0.581
ELN favorable-risk group	184	30	154	
CR rate (%)	163 (88.6)	26 (86.7)	137 (89.0)	0.718
5-year RI rate (95% CI)	31.3% (24.0-38.8)	36.9% (17.7-56.3)	30.1% (22.3-38.3)	0.580
5-year EFS rate (95% CI)	49.1% (41.5-56.7)	38.0% (19.8-56.2)	51.5% (43.3-59.7)	0.244
5-year OS rate (95% CI)	53.2% (45.8-60.6)	48.1% (29.5-66.7)	54.3% (46.1-62.5)	0.499
ELN intermediate-I risk group	222	24	198	
CR rate (%)	168 (75.7)	15 (62.5)	153(76.3)	0.111
5-year RI rate (95% CI)	57.2% (48.8-64.7)	67.0% (31.9-86.9)	56.2% (47.4-64.1)	0.333
5-year EFS rate (95% CI)	20.1% (14.0-26.2)	13.5% (0-29.2)	20.4% (14.3-26.5)	0.318
5-year OS rate (95% CI)	23.4% (17.5-29.3)	19.3% (2.4-36.2)	24.0% (17.7-30.3)	0.305

***** The *p* values refer to comparisons between the two groups by *TET2* mutational status.

Abbreviations: *TET*, ten-eleven translocation; CR, complete remission; HCT, hematopoietic cell transplantation; RI, relapse incidence; EFS, event free survival; OS, overall survival; ELN, European LeukemiaNet.

Supplementary Table S5. Multivariate analysis of overall survival (OS), event-free survival (EFS), and relapse rate (RI) in AML patients.

Measure	Variable	Hazard ratio	95% CI	*p* value
Relapse	Age (>65 years)	1.278	0.640-2.551	0.490
	Allogeneic HCT	0.282	0.176-0.452	<0.001
	NPM1 mutation	0.451	0.280-0.726	0.001
	FLT3-ITD mutation	3.119	1.952-4.983	<0.001
	CEBPA mutation	0.353	0.179-0.696	0.003
	TET2 mutation	1.372	0.708-2.665	0.350
EFS	Age (>65 years)	1.129	0.744-1.714	0.569
	Allogeneic HCT	0.368	0.228-0.594	<0.001
	NPM1 mutation	0.472	0.343-0.648	<0.001
	FLT3-ITD mutation	2.148	1.566-2.947	<0.001
	CEBPA mutation	0.579	0.391-0.858	0.007
	TET2 mutation	1.076	0.688-1.682	0.750
OS	Age (>65 years)	1.041	0.675-1.606	0.856
	Allogeneic HCT	0.382	0.233-0.629	<0.001
	NPM1 mutation	0.515	0.372-0.712	<0.001
	FLT3-ITD mutation	2.217	1.607-3.059	<0.001
	CEBPA mutation	0.634	0.424-0.949	0.027
	TET2 mutation	1.058	0.661-1.696	0.814

Abbreviations: HCT, hematopoietic cell transplantation; *NPM1*, nucleophosmin; *FLT3*-ITD, fms-related tyrosine kinase 3-internal tandem duplication; *CEBP*A, CCAAT/enhancer binding protein α; *TET*, ten-eleven translocation.

Supplementary Table S6. Clinical characteristics of normal-karyotype acute myeloid leukemia patients with homozygous *TET2* mutation versus others.

Parameter; no. of patients (%)	Total (n = 407)	Homozygous *TET2* mutation, (n = 14)	Non-Homozygous *TET2* mutation, (n=40)	*TET2* wild-type (n = 353)	*p* value* (between the three subgroups)	*p* value† (homozygous vs non-homozygous)	*p* value‡ (homozygous vs other subgroups)
Age, median (range), years	52 (15–84)	67 (20–74)	61 (16-83)	50 (15–84)	<0.001	0.480	0.002
Age group (years)							
15– <25	25 (6.1)	1 (7.1)	2 (5.0)	22 (6.2)			
25– <35	45 (11.1)	0	1 (2.5)	44 (12.5)			
35– <45	70 (17.2)	1 (7.1)	1 (2.5)	68 (19.3)			
45– <55	86 (21.1)	1 (7.1)	6 (15.0)	79 (22.4)			
55– <65	115 (28.3)	3 (21.4)	15 (37.5)	97 (27.5)			
65– <75	58 (14.3)	8 (57.1)	12 (30.0)	38 (10.8)			
≥75	8 (2.0)	0	3 (7.5)	5 (1.4)			
Elderly, age ≥ 65 years	66 (16.2)	8 (57.1)	15 (37.5)	42 (12.1)	<0.001	0.201	<0.001
Gender, male	203 (49.9)	8 (57.1)	19 (47.5)	176 (49.9)	0.824	0.535	0.580
WBC, median value ($\times 10^9$/L)	27.3 (0.3–397.21)	40.8 (2.6–204.0)	46.9 (0.9–282.0)	25.2 (0.3–397.2)	0.044	0.721	0.394
Bone marrow blasts (%), median (range)	72 (3–100)	75 (5-90)	73 (3-100)	70 (10–100)	0.128	0.185	0.473

Peripheral blast ($\times 10^9$/L), median (range)	8.4 (0–373.4)	18.7 (0.2–155.0)	19.6 (0-270.7)	6.5 (0–373.4)	0.170	0.731	0.567
FLT3-ITD mutation	111 (27.3)	3(27.3)	12(30.0)	96 (27.2)	0.823	0.538	0.617
NPM1 mutation	180 (44.2)	4 (28.6)	28 (70.0)	148 (41.9)	0.002	0.007	0.230
NPM1 mutation without *FLT3*-ITD mutation	115 (28.3)	3 (21.4)	19 (47.5)	93 (26.3)	0.017	0.088	0.564
CEBPA mutation	80/404 (19.8)	2/14 (14.3)	9/40 (22.5)	69/350 (19.7)	0.798	0.511	0.598
ELN risk group					0.062	0.083	0.462
Favorable	184/406 (45.5)	5 (35.7)	25 (62.5)	154/352 (43.8)			
Intermediate-I	222/406 (55.0)	9 (64.3)	15 (37.5)	198/352 (56.2)			

*****The *p* values refer to comparisons among groups with homozygous *TET2* mutations, non-homozygous *TET2* mutations, and wild-type *TET2*.

†The *p* values refer to comparisons among groups with homozygous *TET2* mutations, non-homozygous *TET2* mutations.

‡ The *p* values refer to comparisons between those with homozygous *TET2* mutations and others (with non-homozygous *TET2* mutations or who were *TET2* wild-type).

Abbreviations: *TET*, ten-eleven translocation; WBC, white blood cells; *FLT3*-ITD, fms-related tyrosine kinase 3-internal tandem duplication; *NPM1*, nucleophosmin; *CEBPA*, CCAAT/enhancer binding protein α; ELN, European LeukemiaNet.

Supplementary Table S7. Clinical outcomes of normal-karyotype acute myeloid leukemia patients with homozygous *TET2* mutations versus others.

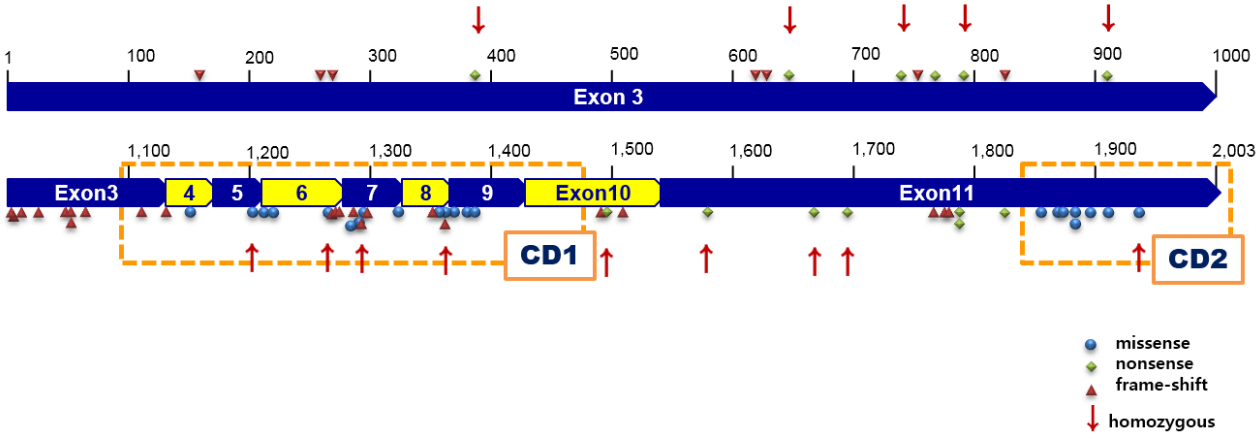
Parameter; no. of patients (%)	Total	Homozygous *TET2* mutation,	Non-Homozygous *TET2* mutation,	*TET2* wild-type	*p* value* (between the three subgroups)	*p* value† (homozygous vs non-homozygous)	*p* value‡ (homozygous vs other subgroups)
All patients	407	14	40	353			
CR rate	332 (81.6)	9 (64.3)	32 (80.0)	323 (82.2)	0.220	0.237	0.090
Received allogeneic HCT	131 (32.2)	2 (14.3)	10 (25.0)	129 (32.8)	0.321	0.599	0.283
5-year RI rate (95% CI)	43.8% (38.2-49.3)	100% (100-100)	36.4% (19.3-53.8)	42.4% (36.7-48.0)	0.029	0.006	0.010
5-year EFS rate (95% CI)	33.5% (28.6-38.4)	0% (0-0)	35.6% (20.1-51.1)	34.6% (29.7- 39.5)	0.136	0.073	0.047
5-year OS rate (95% CI)	37.2% (32.3-42.1)	23.6% (0.3-46.9)	40.1% (24.2-56.0)	37.7% (32.6- 42.8)	0.534	0.422	0.279
ELN-favorable	184	5	25	179			
CR rate	163 (88.6)	4 (80.0)	22 (88.0)	159(88.8)	0.821	0.631	0.540
5-year RI rate (95% CI)	31.3% (24.0-38.8)	100% (100-100)	28.8% (11.0-49.6)	30.0% (22.7-37.5)	0.093	0.021	0.030
5-year EFS rate (95% CI)	49.1% (41.5-56.7)	0% (0-0)	42.7% (22.7-62.7)	50.2% (42.6-57.8)	0.323	0.310	0.196
5-year OS rate, (95% CI)	53.2% (45.8-60.6)	40.0% (0-82.9)	50.1% (29.7-70.5)	53.6% (46.0-61.2)	0.713	0.807	0.587
ELN intermediate-I	222	9	15	213			
CR rate	168 (75.7)	5 (55.6)	10 (66.7)	163(76.5)	0.233	0.586	0.151
5-years RI rate (95% CI)	57.2% (48.8-64.7)	100% (100-100)	52.0% (16.0-79.2)	55.4% (46.9-63.1)	0.251	0.327	0.095
5-year EFS rate (95% CI)	20.1% (14.0-26.2)	0% (0-0)	23.3% (0.8-45.8)	20.5% (14.6-26.4)	0.371	0.452	0.161
5-year OS rate, (95% CI)	23.4% (17.5-29.3)	13.0% (0-37.1)	22.9% (0.6-45.2)	23.8% (17.7-29.9)	0.706	0.787	0.406

*****The *p* values refer to comparisons among groups with homozygous *TET2* mutations, non-homozygous *TET2* mutations, and wild-type *TET2*.

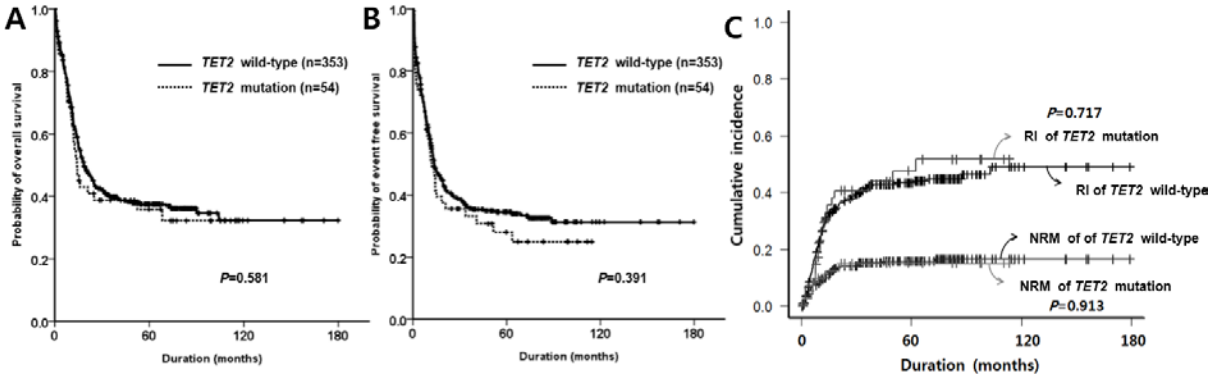
† The *p* values refer to comparisons among groups with homozygous *TET2* mutations, non-homozygous *TET2* mutations.

‡ The *p* values refer to comparisons between those with homozygous *TET2* mutations and others (with non-homozygous *TET2* mutations or who were *TET2* wild-type).

Abbreviations: *TET*, ten-eleven translocation; CR, complete remission; HCT, hematopoietic cell transplantation; RI, relapse incidence; EFS, event-free survival; OS, overall survival; ELN, European LeukemiaNet.



Supplementary Figure S1. The locations of *TET2* mutations. Missense mutations were included in the analysis only when they were located within either of two evolutionarily conserved domains (amino acids 1,104-1,478 and 1,845-2,002). The 65 observed mutations were spread across all exons, and some detected in more than one patient. Identical mutations were detected in two locations (two silent mutations in p289p, two missense mutations in A1882T). The three loci encoding amino acids nos. 1,053, 1,361, and 1,882 had both types of mutation. Single and double mutations in *TET2* were detected in 34 and 27 patients, and included 14 doubly homozygous mutations (red arrows).



Supplementary Figure S2. Clinical outcomes by *TET2* mutational status in normal-karyotype acute myeloid leukemia patients: A. overall survival; B. event-free survival; C. relapse incidence (RI) and non-relapse mortality (NRM).