Clonal leukemic evolution in myelodysplastic syndromes with *TET2* and *IDH1/2* mutations

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ABSTRACT

Somatic mutations of *TET2, IDH1*, and *IDH2* have been described in myelodysplastic syndrome. The impact of these mutations on outcome of myelodysplastic syndrome and their progression to secondary acute myeloid leukemia remains unclear. Mutation status of *TET2, IDH1* and *IDH2* was investigated in a cohort of 46 paired myelodysplastic syndrome/acute myeloid leukemia samples and 122 non-paired cases with *de novo* myelodysplastic syndrome, to clarify their roles in the evolution of myelodysplastic syndrome to acute myeloid leukemia. Among the 168 *de novo* myelodysplastic syndrome patients, the frequency of *TET2, IDH1,* and *IDH2* mutations was 18.5%, 4.2% and 6.0%, respectively. *TET2/IDH* mutations had no impact on survivals, while *TET2* mutations were significantly associated with rapid progression to acute myeloid leukemia. Seventeen of the 46 paired myelodysplastic syndrome/secondary acute myeloid leukemia samples harbored *TET2/IDH* mutations; none acquired these mutations in acute myeloid leukemia phase. Progression to acute myeloid leukemia was accompanied by evolution of a novel clone or expansion of a minor pre-existing subclone of one or more distinct mutations in 12 of the 17 cases with *TET2/IDH* mutations. A minor subclone in 3 cases with biallelic *TET2* inactivation subsequently expanded, indicating biallelic *TET2* mutations play a role in acute myeloid leukemia progression. Twelve patients acquired other genetic lesions, and/or showed increased relative mutant allelic burden of *FLT3*-ITD, *N/K-RAS*, *CEBPA* or *RUNX1* during acute myeloid leukemia progression. Our findings provide a novel insight into the role of *TET2/IDH* mutation in the pathogenesis of myelodysplastic syndrome and subsequent progression to acute myeloid leukemia.

Introduction

Myelodysplastic syndromes (MDS) are highly heterogeneous clonal hematologic malignancies characterized by ineffective hematopoiesis and associated with an increased risk of transformation to secondary acute myeloid leukemia (sAML).[1] During the past ten years, plenty of novel mutational gene targets have been identified through high-throughput genomic studies, promoting our understanding of molecular pathogenesis of MDS, among which *ten eleven translocation 2* (*TET2*) has been shown to represent one of the most frequently mutated genes in MDS.

TET2 was identified within micro-deletions at 4q24 found in MDS cases[2] and has been shown to be mutated also at high frequencies in other myeloid neoplasms, including myeloproliferative neoplasm (MPN), MDS/MPN, and *de novo* AML,[3-8] indicating the critical role of its mutations in myeloid leukemogenesis. TET2 is a member of the TET family of proteins which encode α-ketoglutalate-dependent oxygenases, involved in conversion of 5-methylcytosine to 5-hydroxymethylcytosine,[9,10] and that loss of TET2 function by gene mutations was associated with widespread dysregulated DNA methylation. Interestingly, it has been demonstrated that TET2 function is also compromised by 2-hydroxyglutarate, an oncogenic metabolite produced by gain-of-function mutations of isocitrate dehydrogenase (IDH) 1 and 2, which has been shown to occur mutually exclusive with regard to *TET2* mutations in 3.5-12% of MDS patients.[5,8,11-16]

Until now, several studies carried out have addressed the clinical relevance of *TET2* and *IDH1/2* mutations in MDS.[4,6,8,11-13] The prevalent view is that there is no significant impact of *TET2* mutations on overall survival (OS). However, an initial report[4] suggests the impact of *IDH* mutations is more conflicting. The *IDH1* but not *IDH2* mutations were found to be associated with a higher risk of sAML transformation and poor prognosis in patients with MDS in one study,[11] whereas another study reported the opposite results showing no difference between patients with and without *IDH1/2* mutations in terms of prognostic impact.[13] In the present study, we examined both *TET2* and *IDH* mutations on a large cohort of *de novo* MDS patients from Taiwan and Japan. In particular, we investigated a large number of paired MDS/sAML samples (n=46) for mutational analysis to assess the role of *TET2* and *IDH1/2* mutations in the sAML evolution. The role of *TET2/IDH* and additional gene mutations as well as other genomic alterations in sAML evolution were also investigated in patients carrying *TET2* or *IDH1/2* mutations at initial diagnosis of MDS.

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Methods

Patients and DNA samples

Bone marrow-derived genomic DNA from 168 patients with *de novo* MDS (112 from Chang Gung Memorial Hospital, Taiwan and 56 from Japan) was analyzed for mutations of *TET2* and *IDH1/2*. Patients were diagnosed between 1990 and 2011; their demographic features are summarized in Table 1. The Revised International Prognostic Scoring System (IPSS-R)[17] was available in 122 cases. SNP array-based copy number data were available for 107 samples.[18] The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital and the University of Tokyo.

Analysis of gene mutations

All coding exons of *TET2* and the hot spots on exon 4 of *IDH1/2* were PCR-amplified from genomic DNA and subjected to direct sequencing as previously described.[19] The mutational status of *IDH1/2* in 71 of the 168 patients had been reported before.[13] For 10 cases with *TET2* mutations and 7 cases with *IDH* mutations, mutation status of the additional genes, including *FLT3*-ITD, *FLT3*-TKD, *KIT, C-FMS, N-RAS* and *K-RAS, PTPN11, JAK2*V617F, *CBL, TP53, CEBPA, RUNX1, NPM1, DNMT3A* and *ASXL1*, were investigated as described previously.[18,20-30]

Measurements of allelic burden of mutations

The allelic burden of mutations found in 12 of the 17

Table1. Clinico-hematologic and *TET2* mutation characteristics of patients with myelodysplastic syndromes.

N.	Gender	Age	Subtype	BM blast (%)	Karyotype	IPSS-R	sAML evolution	Time to sAML (months)	Survival (months)	SNP array data	TET2 amino acid change	Ho/He	Exon	Type of mutation
T-002	M	67	RAEB-1	5.0	46,XY[10]	H	+	27.5	35.0	Normal	A1512Nfs*58	He	10	Frameshift
T-007	F	59	RAEB-2	16.3	46,XX[15]	VH	+	1.8	8.7	1p-CNN-LOH	[Q278*(;)Y1255*]	He+He	3+6	Nonsense
T-012	F	73	RAEB-2	15.6	ND	NA	+	1.0	4.0	5q del, 19p gain,+21	[S231Vfs*2 3(;)Q916*]	He+He	3+3	Frameshift+ Nonsense
T-016	M	45	RAEB-1	2.5	47,XY,+8[2]/ 46,XY[3]	I	+	3.6	24.2	+8	M638V	He	3	Missense
T-018	F	67	RAEB-1	7.6	46,XX[20]	I	+	11.4	24.0	Normal	F1104Lfs*2	He	3	Frameshift
T-024	M	88	RAEB-2	14.0	47,XY,+8[12]/ 48,XY,+8,+9[5]	VH	+	3.2	9.9	4q-CNN-LOH, +8	A1512Efs*59	Ho	10	Frameshift
T-048	M	73	RAEB-2	NA	47,XY,+X[18]/ 46,XY[2]	VH	+	1.4	6.3	Normal	[T313Nfs*18(;) Q324*]	He+He	3+3	Frameshift + Nonsense
T-055	F	88	RAEB-1	7.5	46,XX[7]	H	+	10.1	12.0	ND	[L1360Q(;)Q1654*]	He+He	9+11	Missense+ Nonsense
T-070	M	80	RAEB-2	10.4	ND	NA	–	25.8+	25.8	ND	[Q649*(;)A1876V]	He+He	3+11	Nonsense + Missense
T-073	M	74	RAEB-1	6.2	46,XY[20]	I	–	81.2+	81.2+	Normal	S1303R	He	7	Missense
T-075	F	65	RAEB-2	14.1	46,XX,-5,-6,del (7)(p21),-10, -18,add(19)(q13), +4mar[12]/46,XX[2]	VH	+	4.8	5.1	ND	L560Rfs*8	He	3	Frameshift
T-083	M	57	RAEB-1	7.0	46,XY[20]	H	–	8.4+	8.4	Normal	3981n-1G>A	He	5	Splice site mutation
T-095	M	77	RAEB-2	15.2	ND	NA	+	8.7	9.8	ND	A1224Qfs*2	He	6	Frameshift
T-099	M	67	RCMD	3.4	47,XY,+mar[2]/ 46,XY[15]	I	+	11.0	13.0	1p gain, 7q and 14q-CNN-LOH	Q635*	He	3	Nonsense
T-100	F	62	RCMD	0.2	47,XX,+mar1[3]/ 46~47,XX,-9, +mar1,+mar2 [cp4]/46,XX[9]	H	+	5.1	6.54	7q and 19q-CNN-LOH	[R1214W(;) R1359P]	He+He	6+9	Missense
T-108	M	78	RCMD	2.2	46,XY[19]	I	+	11.4	15.0	ND	[R814C(;) P480Lfs*6]	He+He	3+3	Missense + Frameshift
T-123	M	59	RCMD	2.4	47,XY,+8,add(9) (p21)[3]/46,XY[26]	L	–	62.9+	62.9	CNN-LOH in Chr 8, 18, 12q, loss of 2q, 3p, 5q, 9p, 9q, 10p, 11p,13q, 14q, 17p, 18q, 20q, gain of 6q, 7q, 8q, 13q	Q321del	He	3	Deletion
T-126	M	62	RAEB-2	15.2	45,XY,-7[17]/ 46,XY[9]	VH	+	6.2	13.3+	- 7	[R544*(;) G1861Efs*26]	He+He	3+11	Nonsense + Frameshift
T-133	M	77	RAEB-1	8.7	45,X,-Y[25]	L	–	19.9+	19.9+	Normal	C1271W	He	7	Missense

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T-137	M	86	RAEB-1	8.0	46,XY[26]	I	-	27.4+	27.4+	Normal	Q323*	He	3	Nonsense
T-141	F	72	RARS	1.5	46,XX[28]	L	-	5.8+	5.8+	20p-CNN-LOH	Y380Sfs*46	He	3	Frameshift
T-147	M	85	RCMD	3.9	46,XY,+1,der(1;7)(q10;p10)[18]/46,XY[6]	I	-	4.4+	4.4+	+1q, 7q-	L1276Wfs*87	He	7	Frameshift
T-150	M	83	RAEB-2	12.5	46,XY[31]	VH	-	6.1+	6.1+	ND	Q1540*	He	11	Nonsense
J-070	M	NA	MDS–U	NA	ND	NA	NA	NA	NA	Normal	[Q317Rfs*30(;) C1289F]	He+He	3+7	Frameshift + Missense
J-104	M	73	RCMD	3.7	46,Y,t(X;17)(p10;p10)[2]	NA	+	NA	3.0	4p-, del(4)(q21.3-35.2), 5q-, -7, +9p, 9q-, -16, +19p, 19q-CNN-LOH	P1889L	Ho	11	Missense
J-119	M	76	RAEB-2	NA	47,XY,+9[20]	NA	NA	NA	3.8	7q-CNN-LOH, +9	Q591*	He	3	Nonsense
J-129	M	69	RAEB-1	5.2	47,XY,+8[15]	H	+	NA	16.0	4q-CNN-LOH, +8	Q910*	Ho	3	Nonsense
J-146	F	75	RAEB-1	7	46,XX,-7,+mar[9]	H	+	NA	12.0	del(7)(p22.3-13, q31.1-36.3), 22q-CNN-LOH	[S714*(;) Q1852*]	He+He	3+11	Nonsense
J-147	F	74	RCMD	3.2	47,XX,+8[5]	H	-	NA	12+	+8	[R1216*(;) T1726Lfs*20]	He+He	6+11	Nonsense + Frameshift
J-148	M	70	RAEB-1	8.8	46,XY[3]	H	-	NA	9.5+	del(1)(p36.32-35.2), del(5)(q14.3-34), -7	Q1547*	He	11	Nonsense
J-156	M	59	RAEB-2	NA	46,XY,-7,+13[18]	VH	+	NA	10.5	del(5)(q13.2-35.3), del6q21,-7,+8,17p-	R1261H	He	6	Missense

RAEB: refractory anemia with excess blasts; RCMD: refractory cytopenia with multilineage dysplasia; RARS: refractory anemia with ring sideroblasts; MDS-U: myelodysplastic syndrome, unclassifiable; BM: bone marrow; NA: not available; ND: not done; IPSS-R: revised International Prognostic Scoring System; H: high-risk group of IPSS-R; VH: very high-risk group of IPSS-R; I: intermediate-risk group of IPSS-R; L: low-risk group of IPSS-R; sAML: secondary acute myeloid leukemia; SNP: single nucleotide polymorphism; CNN-LOH: copy number neutral-loss of heterozygosity; Ho: homozygous; He: heterozygous.

Table 2. Univariate analysis of time to sAML evolution in patients with myelodysplastic syndromes.

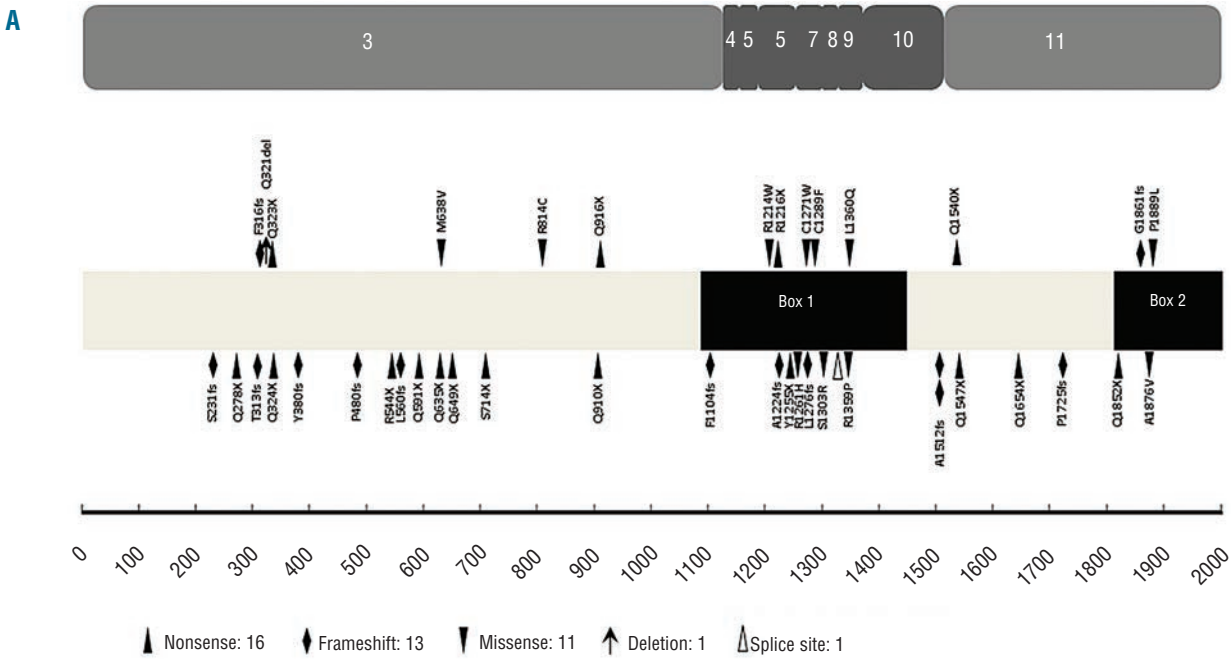
	Whole group Univariate		RAEB-2 Univariate		IPSS-R-3 Univariate	
	P	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)
Gender	0.532	0.85 (0.50 – 1.44)	0.525	0.76 (0.33 – 1.76)	0.160	0.58 (0.27 – 1.25)
Age	0.473	1.24 (0.69 – 2.22)	0.381	1.57 (0.57 – 4.33)	0.376	1.48 (0.62 – 3.53)
Hb	0.194	0.62 (0.30 – 1.28)	0.420	1.57 (0.52 – 4.73)	0.573	0.57 (0.08 – 4.21)
Platelet count	0.110	0.66 (0.39 – 1.10)	0.783	0.89 (0.38 – 2.09)	0.439	0.72 (0.32 – 1.65)
WHO subtype	0.745	0.91 (0.50 – 1.65)	–	–	0.399	1.85 (0.43 – 7.99)
TET2	0.145	1.56 (0.85 – 2.85)	0.001	4.52 (1.66 – 12.30)	<0.001	4.97 (1.89 – 13.09)
IDH1/2	0.030	2.17 (1.06 – 4.45)	0.498	1.45 (0.49 – 4.33)	0.303	1.88 (0.56 – 6.38)
IPSS	0.032	1.84 (1.04 – 3.25)	0.798	0.77 (0.10 – 5.92)	–	–
IPSS-R	0.005		0.357		–	–
IPSS-R-2		1.41 (0.51 – 3.91)		1.00*	–	–
IPSS-R-3		3.30 (1.26 – 8.68)		1.92 (0.44 – 8.40)	–	

**Only IPSS-R-2 and IPSS-R-3 risk group patients were found in RAEB-2 patients. The comparison was performed between these two groups. sAML: secondary acute myeloid leukemia; Hb: hemoglobin; IPSS: International Prognostic Scoring System; IPSS-R: revised International Prognostic Scoring System; IPSS-R-2: IPSS-R intermediate- and high-risk groups; IPSS-R-3: IPSS-R very high-risk group; CI: confidence interval; RAEB-2: refractory anemia with excess blasts-2.*

TET2/IDH mutated cases were measured both at diagnosis of MDS and on progression to sAML by pyrosequencing and/or deep sequencing of the relevant mutant alleles. Pyrosequencing was performed as previously described.[31] The sequences of primer pairs, sequencing primers, and sequences to be analyzed for individual mutations are shown in *Online Supplementary Table S1*. For deep sequencing, in total, 46 genomic fragments harboring each target mutation under interest were PCR-amplified from the corresponding samples using Not1-tagged primers, where each fragment was designed to be ~400bp in length. Touch-down PCRs using high-fidelity DNA polymerase (LA Taq HS, TAKARA Ltd., Tokyo, Japan) were performed for each amplification. An equimolar mixture of all PCR products was prepared for random deep sequencing using MySeq (Illumina), according to the manufacture's protocol with a 150-base-pair end read option. All reads were as aligned to the set of target sequences having primer sequences, using blat (*http://users.soe.ucsc.edu/~kent/src/*). Among the successfully mapped reads, the following reads were removed from further analysis: those mapped to multiple sites, mapped with more than 4 mismatched bases, or had more than 10 clipped bases. All the valid reads with more than 15 base qualities were enumerated with regard to the mutated bases.

SNP microarray analysis

High-density SNP microarray combined with CNAG/AsCNAR



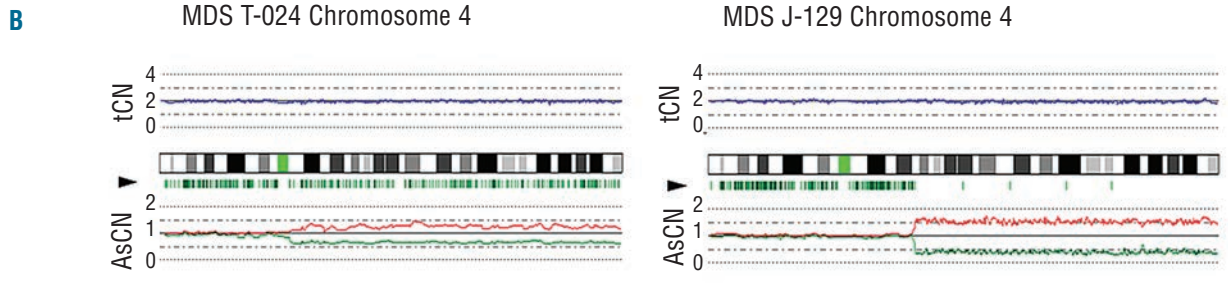


Figure 1. (A) Schematic representation of the locations and patterns of the 42 *TET2* mutations identified in 31 patients with *de novo* MDS. (B) SNP microarray analysis of chromosome 4 showing presence of CNN-LOH in 2 cases. Total copy numbers (tCN) and allele-specific copy numbers (AsCN) with heterozygous SNP calls (arrow head) are shown. Dissociation of AsCN plots indicates the presence of CNN-LOH in 4q; near complete LOH was detected in J-129 and CNN-LOH in a subset of clones in T-024.

Table 3. Univariate and multivariate analysis of overall survival in patients with myelodysplastic syndromes.

	Whole group				RAEB-2				IPSS-R-3	
	Univariate		Multivariate		Univariate		Multivariate		Univariate	
	P	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)
Gender	0.097	1.37 (0.94 – 2.00)	–	–	0.617	1.15 (0.66 – 1.98)	–	–	0.332	1.34 (0.75 – 2.40)
Age	0.008	1.71 (1.15 – 2.54)	0.061	1.62 (0.98, 2.69)	0.010	2.24 (1.19 – 4.20)	0.017	2.16 (1.15, 4.07)	0.050	1.83 (0.99 – 3.37)
Hb	0.031	0.57 (0.34 – 0.96)	0.302	0.67 (0.31, 1.43)	0.528	0.78 (0.36 – 1.68)	–	–	0.485	0.66 (0.20 – 2.14)
Platelet count	0.107	0.73 (0.50 – 1.07)	–	–	0.173	0.65 (0.35 – 1.22)	–	–	0.263	0.71 (0.39 – 1.30)
WHO subtype	0.001	0.49 (0.31 – 0.76)	0.854	1.08 (0.47, 2.50)	–	–	–	–	0.568	1.51 (0.36 – 6.29)
TET2	0.255	1.30 (0.83 – 2.05)	–	–	0.026	2.24 (1.08 – 4.68)	0.050	2.08 (1.00, 4.35)	0.082	1.90 (0.91 – 3.99)
IDH1/2	0.267	1.37 (0.78 – 2.41)	–	–	0.636	1.20 (0.56 – 2.57)	–	–	0.425	0.62 (0.19 – 2.02)
IPSS	<0.001	2.61 (1.77 – 3.85)	–	–	0.693	1.23 (0.44 – 3.42)	–	–	–	–
IPSS-R	<0.001		0.001		0.324					
IPSS-R-2		2.55 (0.87 – 7.47)	0.299	1.90 (0.57, 6.32)		1.00*	–	–	–	–
IPSS-R-3		7.13 (2.51 – 20.27)	0.009	5.49 (1.53, 19.41)		1.82 (0.54 – 6.11)				

**Only IPSS-R-2 and IPSS-R-3 risk groups patients were found in RAEB-2 patients. The comparison was performed between these two groups. sAML: secondary acute myeloid leukemia; Hb: hemoglobin; IPSS: International Prognostic Scoring System; IPSS-R: revised International Prognostic Scoring System; IPSS-R-2: IPSS-R intermediate- and high-risk groups; IPSS-R-3: IPSS-R very high-risk group; CI: confidence interval; RAEB-2: refractory anemia with excess blasts-2.*

software analysis was performed using Affymetrix GeneChip 50K XbaI, HindIII or 250K NspI in 107 patients as reported before.[18]

Statistical analysis

Fisher's exact test, χ^2 analysis, or t-test was used to make comparisons between groups. The Kaplan-Meier method was applied to estimate survival. Comparisons of survival curves were analyzed by the log rank test and hazard ratio by Cox regression. A multivariate Cox regression hazard model was applied to multivariate analysis using parameters with $P<0.05$ under univariate analysis. Statistical analyses were carried out using SPSS 17.0 software for Windows (SPSS Inc., Chicago, USA).

Results

Mutations of TET2, IDH1 and IDH2 in de novo MDS

In total, 50 *TET2* variants were detected. After excluding confirmed SNPs (P29R, V218M, R550Q, F868L, S1039L, L1721W, and I1762V) and another variant located in non-conserved region (Q80E), 42 variants were found in 31 patients, including 16 nonsense, 11 missense, 13 frameshift, 1 splice site change, in addition to a 4-bp deletion (Figure 1A and Table 1). Eleven of the 31 patients (35%) carried two distinct *TET2* mutations. In accordance with previous reports, exons 3 (n=20) and 11 (n=8) were the most common mutation sites. Nine of the 11 missense mutations located in the highly conserved regions, while the remaining 2, M638V and R814C, which were confirmed as somatic (*Online Supplementary Figures S1 and S2*), were located outside the conserved regions. The frequency of *TET2* mutations was 18.5% in the present cohort of MDS patients, there were no significant variations among subtypes with 18.4% (7 of 38) in RCMD, 20.4% (11 of 54) in RAEB-1, 16.9% (11 of 65) in RAEB-2, and 18.2% (2 of 11) in the remaining subtypes. Characteristics of *TET2*-mutated patients are summarized in Table 1. Cytogenetic and SNP array data were available in 27 and 25 out of the 31 *TET2*-mutated patients, respectively. Sixteen patients had abnormal karyotypes and 11 normal karyotypes (Table 1). Although no cytogenetic changes involving the *TET2* locus (4q24) were detected by metaphase karyotyping, 3 cases had loss of heterozygosity (LOH) of 4q in SNP array analysis, for which homozygous *TET2* mutation was suspected; patients T-024 and J-129 had copy number neutral-LOH (CNN-LOH) (Figure 1B) and patient J-104 had a focal deletion involving the *TET2* locus (Table 1).

Mutations of *IDH1* were detected in 7 of the 168 patients (4.2%) (3 R132H, 3 R132C, and 1 R132L), while *IDH2* was mutated in 10 patients (6.0%) (8 R140Q, 1 R140W, and 1 R172K). Types and frequencies of *IDH1/2* mutations were largely comparable to the previous reports, although *IDH2* R172K found in an RAEB-1 case had not been reported in MDS. None of *TET2, IDH1* and *IDH2* mutations coexisted in our patients.

Clinical impact of TET2/IDH mutations in MDS

Treatments consisted of single agent low-dose chemotherapy in 12.6% of patients, combination chemotherapy in 9.6%, and supportive care with transfusion or no therapy in the remaining 77.8% of patients. No patients received erythroid-stimulating agents or demethylation agents. The treatment modality did not dif-

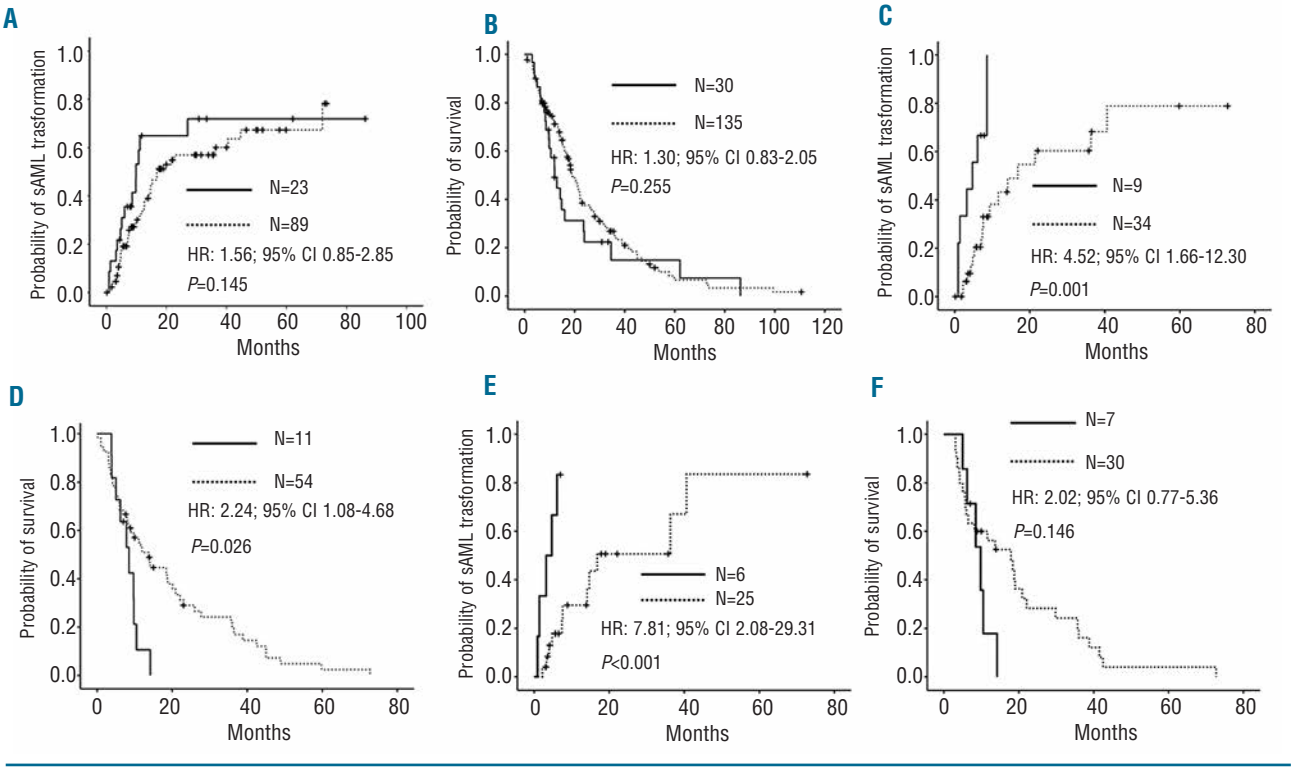


Figure 2. Kaplan-Meier survival curves in patients with MDS according to the *TET2* mutation status (solid line indicating mutation-positive and dotted line indicating mutation-negative). Time to sAML transformation (A) and overall survival (B) in the whole cohort of patients according to *TET2* mutation status; time to sAML transformation (C) and overall survival (D) in patients in RAEB-2 subgroup according to *TET2* mutation status; time to sAML transformation (E) and overall survival (F) in MDS in IPSS-R very high-risk group according to *TET2* mutation status.

fer in any way between *TET2*-mutated and unmutated patients (P=0.674), *IDH1*-mutated and unmutated patients (P=0.149), *IDH2*-mutated and unmutated patients (P=0.981), and *IDH1/2*-mutated and unmutated patients (P=0.563). Among the 165 patients with available follow-up data, with a median follow up of 15.1 months (range 0.2-110.6 months),128 patients died, 37 were alive, and 78 patients developed sAML. *TET2*, but not *IDH1* or *IDH2*, mutations were significantly associated with older age (71.4 *vs.* 62.9 years; P<0.001) and lower platelet counts, which were in accordance with previous reports. Otherwise, there was no statistical difference in WHO subtypes and IPSS-R risk groups between either *TET2*-mutated and unmutated or *IDH1/2*-mutated and unmutated groups (*Online Supplementary Tables S2 and S3*). We also compared the clinico-hematologic features between *TET2*

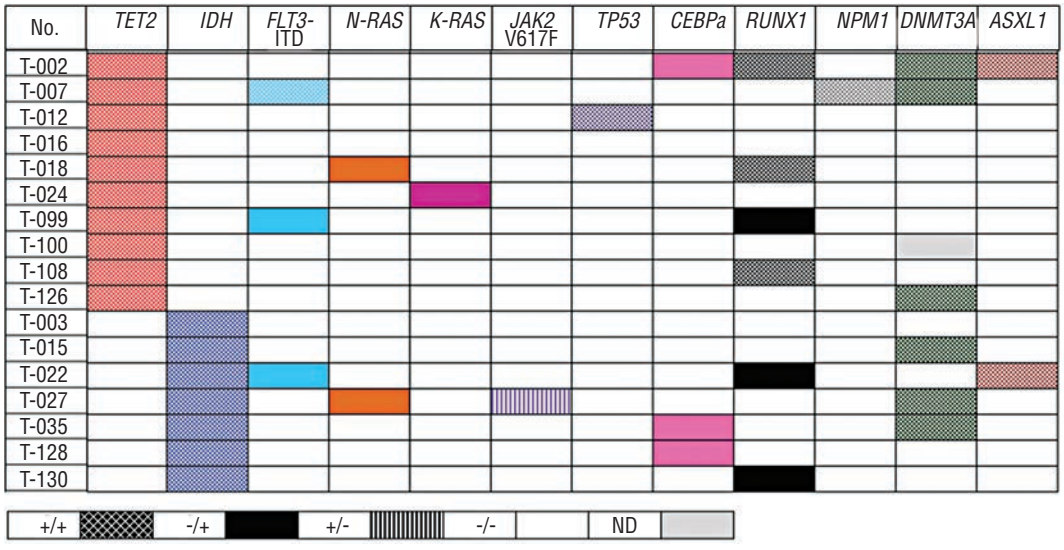


Figure 3. Additional gene mutations in patients with myelodysplastic syndromes harboring *TET2/IDH* mutations during sAML transformation. "+" indicating mutation-positive and "-" mutation-negative. "+/+" indicating gene mutation at both MDS and sAML phases; "-/-" indicating no mutated gene at both MDS and sAML phases; "+/-" indicating loss of gene mutation and "-/+" indicating acquisition of gene mutation during sAML evolution. ND: not done.

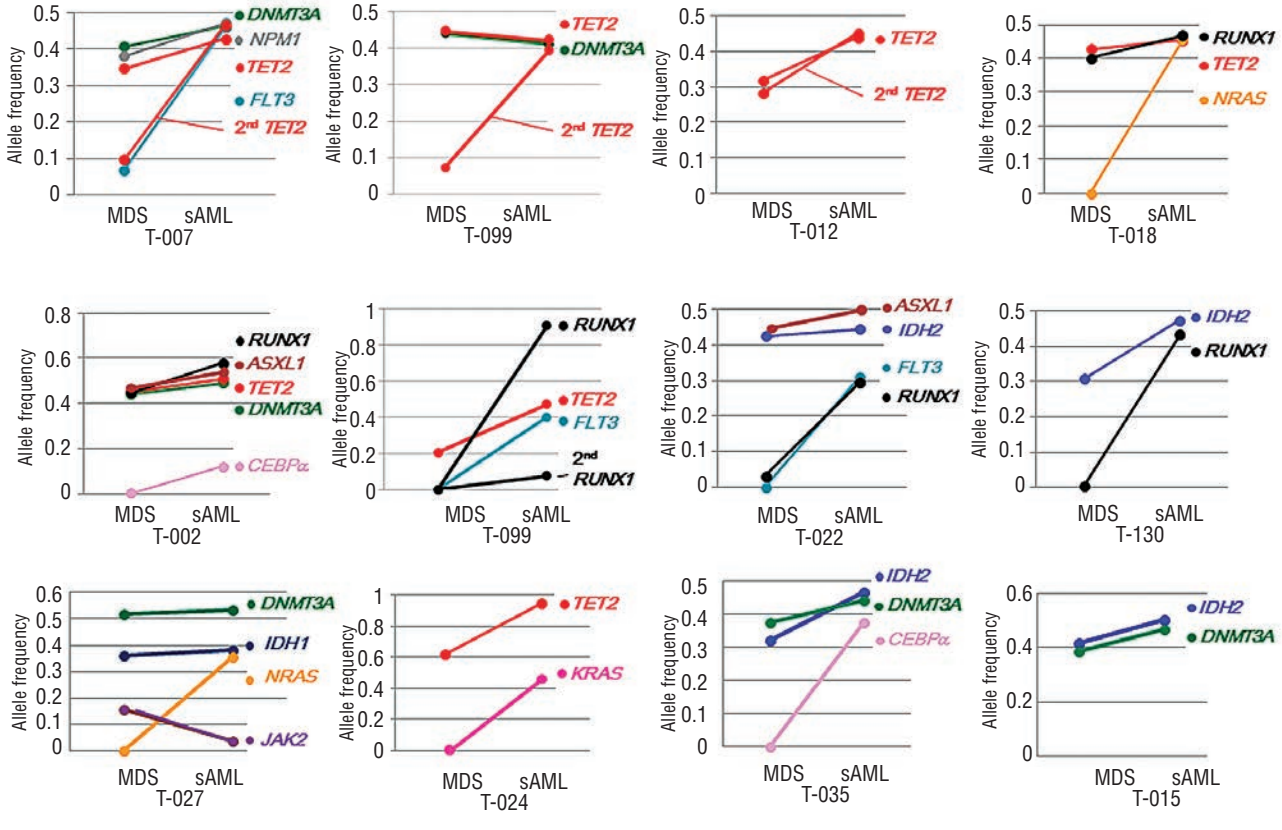


Figure 4. Comparison of mutant allele frequencies in 12 cases between MDS and sAML.

mutation-positive and *IDH1/2* mutation-positive patients. No significant difference was observed (*Online Supplementary Table S4*). In univariate analysis, neither time to progression to sAML nor overall survival (OS) was significantly affected by the mutation status of *TET2* (Figure 2A and 2B and Tables 2 and 3) or *IDH1/2* (Tables 2 and 3), although *TET2*- (but not *IDH1/2*-) mutated cases tended to show a shorter time to progression to sAML (HR=4.52; 95%CI: 1.66-12.30) and OS (HR=2.24; 95%CI: 1.08-4.68) among cases with RAEB-2 (Figure 2C and D) (but not among other WHO subgroups). Similarly, *TET2*-mutated cases also showed a shorter time to progression to sAML (HR=7.81; 95%CI: 2.08-29.31) and tended to have a shorter OS (HR=2.02; 95%CI: 0.77-5.36) among cases with IPSS-R very high-risk group (Figure 2E and F). To see this in more detail, we tested the association of *TET2* mutations with WHO subtypes or IPSS-R risk groups being stratified. After stratification, the *TET2* mutation status was significantly associated with a shorter time to sAML progression (although not with OS) independent of WHO subtype (P=0.041) but not of IPSS-R risk group (P=0.112). There was no difference in time to sAML between different treatment modalities in the RAEB-2 subgroup (P=0.847) and in the IPSS-R very high-risk subgroup (P=0.754). There was also no difference in OS between different treatment modalities (P=0.279 for RAEB-2 and 0.786 for IPSS-R very high-risk subgroup). To exclude the possibility that other mutations co-occurring with *TET2* or *IDH1/2* mutations may influence the prognosis, we also analyzed *DNMT3A, ASXL1* and *TP53* genes recognized for their influence on the prognosis in the entire RAEB-2 or IPSS-R very high-risk subgroups. The *DNMT3A* and *ASXL1* mutations did not have an impact on time to sAML and OS in both RAEB-2 and IPSS-R very high-risk subgroups (*data not shown*), while TP53 mutation conferred an adverse OS (P=0.004) but not the time to sAML (P=0.236) in the RAEB-2 subgroup. Only *TET2* mutation had an adverse impact on OS by multivariate analysis (P=0.044) (*Online Supplementary Table S5*).

TET2, IDH1 and IDH2 mutations in paired samples at MDS/sAML phases

Forty-six cases had genomic DNA from sAML cells available for the comparative analysis of *TET2* and *IDH1/2* mutations both at diagnosis of MDS and at the time of progression to sAML by Sanger sequencing, and followed by pyrosequencing/deep sequencing in 12 cases harboring *TET2/IDH* mutations. Of the 46 patients with paired samples, 23 (50%) of them received supportive care or no therapy, 15 were treated with single agent low-dose chemotherapy, and 8 received combination chemotherapy in MDS phase. At diagnosis of MDS, 15 *TET2* mutations as well as 2 *IDH1* and 5 *IDH2* mutations were identified in 17 cases (10 *TET2*, 2 *IDH1*, and 5 *IDH2* mutated cases), all of which were also detected at the time of sAML progression. No *TET2* or *IDH1/2* mutations were newly iden-

Figure 5. Genomic alterations in patients with myelodysplastic syndromes harboring ***TET2/IDH*** mutations during sAML transformation. (A) Trisomy 21 in patient T-002, (B) 3q gain and 6p deletion in patient T-003, (C) 3q CNN-LOH in patient T-016, (D) retaining 7q CNN-LOH and acquisition of 21q CNN-LOH in patient T-099, (E) 1p CNN-LOH in patient T126.

tified in sAML samples. Five patients carried two distinct *TET2* mutant alleles. Additional gene mutations involving common mutational targets in MDS and other myeloid neoplasms, including *FLT3*-ITD, *N-RAS*, *JAK2*V617F, *CEBPA, RUNX1, NPM1, DNMT3A* and *ASXL1*, were also evaluated at both phases in 17 cases with either *TET2* or *IDH1/2* mutations in a similar manner (Figure 3). In total, 26 additional mutations were identified in 14 of the 17 cases, *RUNX1* and *DNMT3A* were the most commonly mutated (n=6), followed by *FLT3* (n=3), *CEBPA* (n=3), *N-RAS* and *ASXL1* (n=2) and genes mutated in single cases (*NPM1, TP53*, *JAK2*V617F, and *K-RAS*). These mutations were either found at the diagnosis of MDS (15 mutations in 10 cases) or had newly appeared at the time of progression to sAML (11 mutations in 9 cases). According to the analysis of relative allele frequencies of coexisting mutations, progression of *TET2*- or *IDH1/2*-mutated MDS to sAML was frequently accompanied by appearance of a novel clone that had been absent at the diagnosis of MDS or evolution of a pre-existing minor clone, which were characterized by distinct coexisting mutations, including *RUNX1* (T-002, T-022, T-099, and T-130), *TET2* (T-007, T-012, T-024, and T-126), *CEBPA* (T-002 and T-035), *N-RAS* (T-018 and T-027), *FLT3* (T-007, T-022 and T-099), and *K-RAS* (T-024) (Figures 3 and 4). Most of the mutations found at the diagnosis of MDS were also detected in sAML samples, except for a *JAK2*V617F mutation that showed 16% allelic burden at the diagnosis of MDS but disappeared at the sAML phase when the *N-RAS* mutated clone emerged.

Twenty-seven of the 46 paired samples had SNP array-based copy number analysis at both phases of the disease and 11 of them had additional genomic changes at sAML phase. Of the 17 *TET2/IDH1/IDH2* mutation-positive patients, 5 of them had additional genomic alterations including trisomy 21 (T-002), combination of 3q gain and 6p deletion (T-003), 3q CNN-LOH (T-016), loss of 1p gain and 14q CNN-LOH with acquisition of 21q CNN-LOH and retained 7q CNN-LOH (T-099), and 1p CNN-LOH (T-126) (Figure 5). Combining gene mutations and SNP array results in conjunction with clonal expansion, 15 of 17 MDS patients had additional genetic or genomic events during sAML evolution.

Discussion

The frequency of *TET2* mutations in the present cohort of MDS patients was comparable to the results from the previous studies reporting mutation rates of 12-26%.[3-8,32,33] The impact of *TET2* mutations on clinical outcomes in MDS has been previously investigated in several studies.[4,6,8] Kosmider *et al*. reported a favorable outcome in *TET2*-mutated patients,[35] whereas Smith *et al*., Langemeijer *et al*., and Bejar *et al*. indicated no significant impact of *TET2* mutations on OS and/or progression to sAML.[6,8,32] In the current study, *TET2* mutations did not seem to significantly affect OS, supporting the observations in the latter studies. On the other hand, we observed that *TET2* mutations were significantly associated with a shorter time to progression to sAML, especially among the RAEB-2 or IPSS-R very high-risk group, which has not been reported in previous studies. The exact reason for the apparent discrepancy between overall and sAML-free survivals is unclear. To exclude the possibility that other gene mutations co-occurring with *TET2* mutation might influence the outcome, we also analyzed *DMNT3A, ASXL1* and *TP53* in the entire RAEB-2 and very high-risk IPSS-R risk groups, only *TET2* mutation was the independent unfavorable risk factor for outcome. The prognostic impact of *IDH1/2* mutations in MDS has been less intensively investigated in previous studies with conflicting results. Two groups reported an inferior OS and a higher rate of transformation into sAML in *IDH1*-mutated cases,[11,12] while two other studies showed no impact of *IDH1/2* mutations on OS in patients with MDS.[8,16] Patnaik *et al*. observed that *IDH2* mutations had no impact on OS and sAML-free survival in MDS patients.[12] In the present series, we found that *IDH1* or *IDH2* mutation alone did not influence time to sAML transformation or OS, although the small numbers of patients with *IDH1/2* mutation-positive cases precluded any meaningful detection of their impact if there was one. Clearly, further studies are warranted to determine the prognostic impact of *IDH* mutations.

The strength of our study was that the mutation status of *TET2/IDH1/2* was evaluated in a relatively large cohort of paired MDS/sAML samples (n=46), although Kosmider *et al*.[4] and Thol *et al*.[11] also reported the analysis of *TET2* or *IDH* mutations in 12 and 7 paired samples, respectively. Through the analysis, we obtained several new findings of potential interest as for the role of *TET2/IDH* mutations in the development of MDS and/or subsequent progression to sAML. First, none of our patients newly acquired *TET2* or *IDH1/2* mutations at the time of progression to sAML. Conversely, all the *TET2* and *IDH1/2* mutations found in MDS persisted in sAML phase. Second, as expected from the recent genetic study by Walter *et al*.,[34] leukemic transformation of *TET2*- and *IDH1/2*-mutated MDS was frequently accompanied by evolution or expansion of a distinct subclone, which was confirmed in 12 (70.6%) of the 17 paired MDS/sAML cases, where the clonal evolution/expansion was recognized by the appearance of new genetic lesions (T-002, T-018, T-022, T-024, T-027, T-035, T-099, T-128, T-130) that had not been present at diagnosis or by an increased relative allelic burden of pre-existing mutations (T-002, T-007, T-012, T-024, T-099 and T-126). Since the accompanying gene mutations were analyzed only for 15 genes, we could have underestimated the frequency of clonal evolution, which would have been more fully addressed by whole genome/exome analysis. Finally, the clonal evolution/expansion was most commonly associated with mutations in *FLT3* (n=3), *N/K-RAS* (n=3), and *RUNX1* (n=3) genes, but also commonly accompanied by expansion of a pre-existing subclone with biallelic *TET2* inactivation (n=3), which were caused by either two distinct mutations (T-007, T-012 and T-126) or loss of the remaining wild-type allele (T-024). Together, our findings indicated that the first *TET2* mutation is likely to represent founder changes and could be involved in the initiation of MDS by causing haploinsufficiency of TET2, while the mutation/deletion involving the remaining intact allele leads to total loss of TET2 function, which could possibly contribute to the progression of sAML. We demonstrated that *TET2* or *IDH1/2* mutations confirmed by deep sequencing are a recurrent contributor to the founding clone.

In conclusion, *TET2* and *IDH* mutations did not have any significant impact on OS, while *TET2* mutations were significantly associated with progression to sAML. *TET2* mutations are likely to be among the initial changes that

contribute to initiation of MDS, while biallelic *TET2* mutations are more likely to represent a subclone in MDS phase, which later expand in sAML phase, suggesting their role in progression to sAML.

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