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ORIGINAL ARTICLE

Dynamics of *ASXL1* mutation and other associated genetic alterations during disease progression in patients with primary myelodysplastic syndrome

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Recently, mutations of the additional sex comb-like 1 (*ASXL1*) gene were identified in patients with myelodysplastic syndrome (MDS), but the interaction of this mutation with other genetic alterations and its dynamic changes during disease progression remain to be determined. In this study, *ASXL1* mutations were identified in 106 (22.7%) of the 466 patients with primary MDS based on the French-American-British (FAB) classification and 62 (17.1%) of the 362 patients based on the World Health Organization (WHO) classification. *ASXL1* mutation was closely associated with trisomy 8 and mutations of *RUNX1*, *EZH2*, *IDH*, *NRAS*, *JAK2*, *SETBP1* and *SRSF2,* but was negatively associated with *SF3B1* mutation. Most *ASXL1*-mutated patients (85%) had concurrent other gene mutations at diagnosis. *ASXL1* mutation was an independent poor prognostic factor for survival. Sequential studies showed that the original *ASXL1* mutation remained unchanged at disease progression in all 32 *ASXL1*-mutated patients but were frequently accompanied with acquisition of mutations of other genes, including *RUNX1*, *NRAS, KRAS, SF3B1*, *SETBP1* and chromosomal evolution. On the other side, among the 80 *ASXL1*-wild patients, only one acquired *ASXL1* mutation at leukemia transformation. In conclusion, *ASXL1* mutations in association with other genetic alterations may have a role in the development of MDS but contribute little to disease progression.

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INTRODUCTION

The myelodysplastic syndromes (MDSs) are a heterogenous group of diseases, characterized by cytopenia, but usually hypercellular bone marrow with dysplastic hematopoiesis, and a propensity to acute leukemia transformation.[1,2] The pathogenesis that causes these pre-leukemic disorders is not quite clear yet, but immune deregulation, abnormal microenvironment and accumulation of genetic alterations may all have some roles.[3–7] Mutations in *ASXL1* (additional sex combs 1) have been identified in MDS[8] and other myeloid malignancies, like acute myeloid leukemia (AML),[9] chronic myelomonocytic leukemia (CMML)[10] and myelo-proliferative neoplasms.[11] The function of *ASXL1* protein is not fully delineated,[12] but it is suggested that it may be involved in DNA and/or histone modification.[13] *ASXL1* mutations are all disclosed in exon 12 of the gene and are believed to lead to the truncation of the plant homeodomain[10] at the C-terminus of the protein, which is involved in chromatin modification.[14,15] Mutations in *ASXL1* result in global decrease of histone 3 lysine 27 methylation, a histone marker associated with repression of transcription.[16] Recently, the animal models showed that C-terminal-truncating *ASXL1* mutations or deletion/loss of *ASXL1* lead to MDS-like disease in mice.[17–19] Further, the deficiency of the BAP1, a nuclear-localized deubiquitinating enzyme, resulted in a CMML-like phenotype, and the interaction with *ASXL1* is critical for the enzymatic activity of BAP1.[16,20,21] *ASXL1* mutation is found in a substantial proportion (11–18.5%) of WHO-defined MDS patients[8,10,22–24] and is correlated with unfavorable outcome.[23,24] However, the association of *ASXL1* mutation with other genetic alterations in the pathogenesis of MDS and their dynamic changes during disease progression remain unclear. In this large cohort of MDS patients, we found that *ASXL1* mutation was statistically closely associated with trisomy 8 and mutations of *RUNX1*, *EZH2*, *IDH*, *NRAS*, *JAK2*, *SETBP1* and *SRSF2*, suggesting cooperation of these gene alterations with *ASXL1* mutation may contribute to the development of MDS. Moreover, sequential analyses showed all *ASXL1*-mutated patients retained the original *ASXL1* mutation during disease progression, but frequently acquired other novel genetic alterations, including *RUNX1*, *NRAS*, *KRAS*, *SF3B1* and *SETBP1* mutations and chromosomal evolution, at the same time.

MATERIALS AND METHODS

Patients

Four hundred and sixty-six adult patients who were diagnosed as having *de novo* MDS according to the FAB classification at the National Taiwan

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University Hospital (NTUH) and had cryopreserved bone marrow cells for study were recruited for gene mutation analyses. Among them, the disease of 362 patients fulfilled the criteria of MDS according to the 2008 WHO classification. All patients signed informed consents for sample collection in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board of the NTUH.

Mutation analysis

The *ASXL1* exon 12 until the stop codon was amplified by three pairs of primers and sequenced by another six internal primers, as described by Gelsi-Boyer *et al.*,[10] with mild modification.[9] The PCR reaction included 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min. The mutations were confirmed at least twice. When the mutations were not obvious because of location near the sequencing primers, sequencing from the other direction was done to solve this issue. Mutation analyses of other 15 relevant molecular genes, including class I mutations, such as *FLT3*/ITD,[25] *NRAS*,[26] *KRAS*[26] and *JAK2*[26] mutations, and class II mutations, such as *MLL*/PTD,[27] *RUNX1*[28] and *WT1* mutations,[29] as well as mutations of genes involving in epigenetic modifications, such as *IDH1*,[30] *IDH2*,[31] including R140 and R172 mutations, *DNMT3A*[32] and *EZH2*[33] mutations, splicing machinery mutations, such as *U2AF1*,[34] *SRSF2*[35] and *SF3B1*[34] mutations, and *SETBP1* mutation,[36] were performed as previously described. Sequential studies of all these genes were also performed in 305 samples from 112 patients during clinical follow-ups.

Cytogenetics

Bone marrow cells were harvested directly or after 1–3 days of unstimulated culture, and the metaphase chromosomes were banded by the G-banding method as described earlier.[37]

TA-cloning analysis

For the patients with discrepancy of the mutation status of the *ASXL1* in paired samples, Taq polymerase-amplified (TA) cloning was performed in the samples without detectable mutant by direct sequencing. The DNA spanning the mutation spots of *ASXL1* detected at either diagnosis or during subsequent follow-ups was amplified and the PCR products were then cloned into the TA-cloning vector pGEM-T Easy (Promega, Madison, WI, USA). More than 10 clones were selected for sequencing as previously described.[38]

Statistics

The χ^2-test was performed to calculate the significance of association between *ASXL1* mutation and other parameters, including sex, the FAB subtypes, the 2008 WHO classification, karyotypes, international prognostic scoring system (IPSS) score,[1] revised IPSS (IPSS-R) score[39] and mutations of other genes. Fisher's exact test was used if any expected value of the contingency table was less than 5. The Mann–Whitney test method was used to compare continuous variables and medians of distributions. Overall survival (OS) was measured from the date of first diagnosis to the

Table 1. Comparison of clinical manifestation and laboratory features between MDS patients with and without *ASXL1* mutation

Variables	*Total* (n = *466*)	*ASXL1 mutated* (n = *106, 22.7%*)	*ASXL1 wild* (n = *360, 77.3%*)	P-*value*
Sex				0.01
Male	308	81 (26.3)	227 (73.7)	
Female	158	25 (15.8)	133 (84.2)	
Age (year)[a]	66 (18–98)	71 (26–89)	64 (18–98)	0.001
Lab data[a]				
WBC (per μl)	3870 (440–355 300)	5340 (1090–355 300)	3610 (440–227 200)	<0.001
Hb (g/dL)	8.2 (3.0–15.0)	8.6 (3.0–14.0)	8.1 (3.0–15.0)	0.084
Platelet (× 1000 per μl)	74 (2–931)	80.5 (3–931)	74 (2–721)	0.253
LDH ($U\,l^{-1}$)	485 (145–6807)	531 (225–3756)	469 (145–6807)	0.275
FAB subtype[b]				<0.001
RA	171	18 (10.5)	153 (89.5)	<0.001
RARS	34	4 (11.8)	30 (88.2)	0.138
CMML	52	28 (53.8)	24 (46.2)	<0.001
RAEB	157	40 (25.5)	117 (74.5)	0.350
RAEBT	52	16 (30.8)	36 (69.2)	0.16
WHO classification 2008[b]	*N* = 362	62 (17.1)	300 (82.9)	0.004
RCUD	73	10 (13.7)	63 (86.3)	0.384
RARS	20	4 (20.0)	16 (80.0)	0.726
RCMD	109	8 (7.3)	101 (92.7)	0.001
RAEB1	78	18 (23.1)	60 (76.9)	0.115
RAEB2	79	22 (27.8)	57 (72.2)	0.004
MDS-U	3	0 (0)	3 (100.0)	0.429
IPSS[b,c]				0.022
Low/INT-1	254	50 (19.7)	204 (80.3)	
INT-2/High	181	53 (29.3)	128 (70.7)	
IPSS-R[b,d]				0.002
Very low/low/INT	229	40 (17.5)	189 (82.5)	
High/very high	206	63 (30.6)	143 (69.4)	

Abbreviations: CMML, chronic myelomonocytic leukemia; FAB, French-American-British classification; IPSS, international prognostic scoring system; IPSS-R, revised IPSS; MDS-U, unclassified; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEBT, refractory anemia with excess blasts in transformation; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCUD, refractory cytopenia with unilineage dysplasia; WBC, white blood cell. There were no patients with MDS with isolated del(5q) in this study. [a]Median (range). [b]Number of patients (% of patients with or without *ASXL1* mutation in the subgroup). [c]International prognosis scoring system: low, 0; intermediate (INT)-1, 0.5–1; INT-2, 1.5–2; and high, ⩾2.5. [d]Revised international prognosis scoring system: very low: ⩽1.5; low: >1.5–3; intermediate (INT): >3–4.5; high: >4.5–6; and very high: >6.

date of last follow-up or death from any cause. Multivariate Cox proportional hazard regression analysis was used to investigate independent prognostic factors for OS. The Kaplan–Meier estimation was used to plot survival curves, and log-rank tests were used to calculate the difference of OS between groups. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS 17 software (SPSS Inc., Chicago, IL, USA) and Statsdirect (Cheshire, UK).

RESULTS

Mutation status of *ASXL1* in patients with MDS

Among the 466 MDS patients according to the FAB classification, 106 (22.7%) patients had *ASXL1* mutations, including 96 patients with frameshift mutations and 10 with nonsense mutations. The most common mutation was c.1934dupG that occurred in 66 patients. All 106 patients showed single heterozygous mutation. (Supplementary Table 1) All these mutations resulted in truncation of the plant homeodomain of ASXL1. Patients with CMML had a high incidence (53.8%) of *ASXL1* mutations (Table 1). Patients with refractory anemia (RA) and RA with ring sideroblasts had a lower incidence (10.5% and 11.8%, respectively) of *ASXL1* mutation than patients with RA with excess blasts (RAEB, 25.5%) or RAEB in transformation (30.8%; $P<0.001$).

Among the 362 patients with MDS according to the 2008 WHO classification, 17.1% patients had *ASXL1* mutation. MDS patients with RAEB1/RAEB2 had a significantly higher incidence of *ASXL1* mutation than those with other subtypes (25% vs 10.7%, $P<0.001$, Table 1).

Clinical features and biological characteristics of *ASXL1*-mutated patients

Most of the patients received conservative and supportive care and only 91 (19.5%) patients received AML-directed chemotherapy, including 56 patients (12%) who underwent allogeneic hematopoietic stem cell transplantation. We could not find the treatment difference between the patients with *ASXL1* mutations and those without *ASXL1* mutations (data not shown). The comparison of clinical and hematologic characteristics of patients with and without mutation of *ASXL1* is shown in Table 1. The patients with *ASXL1* mutation were predominantly male, older (median, 71 years vs 64 years, $P=0.001$) and had higher white blood cell counts ($P<0.001$) and higher IPSS-R score ($P=0.002$) at diagnosis. There was no difference in hemoglobin levels and platelet counts between the patients with and without *ASXL1* mutation.

Among the 435 patients with cytogenetic data for analysis, clonal chromosomal abnormalities were detected in 44.4% of the MDS patients based on the FAB classification and in 44.2% of those based on the 2008 WHO classification. The mutation rate was especially high in the patients with trisomy 8 (45.5%, $P=0.02$; Supplementary Table 2).

Table 2. Comparison of other genetic alterations between MDS patients with and without the *ASXL1* mutation

Mutation	*Percentage of patients with the other gene mutation*				
	No. examined	*Total pts*	*ASXL1-mutated pts*	*ASXL1-wild pts*	P-*value*
FLT3/ITD	464	1.1	0.9	1.1	>0.999
NRAS	464	4.7	10.4	3.1	0.007
KRAS	462	1.3	2.9	0.8	0.134
JAK2	462	1.1	3.8	0.3	0.011
MLL/PTD	445	1.1	1.0	1.2	>0.999
RUNX1	459	12.6	32.4	6.8	<0.001
WT1	252	0.4	0	0.5	>0.999
IDH	463	4.5	11.4	2.5	<0.001
DNMT3A	464	9.9	5.7	11.2	0.137
EZH2	464	6.0	22.6	1.1	<0.001
U2AF1	462	7.4	9.5	6.7	0.334
SRSF2	462	13.0	34.3	6.7	<0.001
SF3B1	462	10.6	2.9	12.9	0.003
SETBP1	461	2.8	10.5	0.6	<0.001

Abbreviation: pts, patients.

Association of *ASXL1* mutation with other genetic alterations

Ninety (85%) of the *ASXL1*-mutated patients had concurrently other gene mutations (Supplementary Table 1 and Table 2). The patients with *ASXL1* mutation had significantly higher incidences of concurrent *RUNX1* mutation (32.4% vs 6.8%, $P<0.001$), *EZH2* mutation (22.6% vs 1.1%, $P<0.001$), *IDH* mutation (11.4% vs 2.5%, $P<0.001$), *NRAS* mutation (10.4% vs 3.1%, $P=0.007$), *JAK2* mutation (3.8% vs 0.3%, $P=0.011$), *SETBP1* mutation (10.5% vs 0.6%, $P<0.001$) and *SRSF2* mutation (34.3% vs 6.7%, $P<0.001$), but had a lower incidence of concurrent *SF3B1* mutation (2.9% vs 12.9%, $P=0.003$) than those with wild-type *ASXL1*. There was no correlation of *ASXL1* mutation with other gene mutations studied (Table 2).

Analysis of *ASXL1* mutation in sequential samples

To investigate the role of *ASXL1* mutation in disease progression, sequential analyses of the gene mutation were performed in 305 samples from 112 patients, including 32 patients with *ASXL1* mutation at diagnosis and 80 patients without the mutation. Among the 32 *ASXL1*-mutated patients, 27 had disease progression including 19 with AML transformation. Two patients (patients 60 and 92) lost the original *ASXL1* mutation at remission status following transplantation. Among the remaining 30 patients, the same mutations were retained in 29 patients during follow-ups (Table 3) but could not be detected by direct sequencing in one patient at the time of disease progression (patient 1). As direct sequencing might not be sensitive enough to detect low level of *ASXL1* mutant, we therefore did TA cloning of the sample obtained at RAEB from this patient. The original *ASXL1* mutation was detected in 2 of the 10 clones analyzed.

On the other hand, among the 80 *ASXL1*-wild patients who were sequentially studied, 36 patients had disease progression, including 20 patients with AML transformation. Two of them (patients 107 and 108) acquired *ASXL1* mutations when the disease progressed to AML and RAEB, respectively. Actually, we could not find any *ASXL1* mutation in the 44 clones analyzed at diagnosis using cloning technique in patient 107. Patient 108 who was diagnosed as having RA had *EZH2* mutation initially. He acquired *RUNX1, ASXL1* and *SETBP1* mutations when the disease progressed to RAEB 90 months later. Interestingly, using a more sensitive cloning technique, we could identify *ASXL1* mutation in one of the 11 clones, but no *RUNX1* and *SETBP1* mutations in the 41 clones and 40 clones analyzed at diagnosis, respectively. Therefore, a total of 31 patients had *ASXL1* mutations at both MDS diagnosis and subsequent follow-ups (Table 3 and Supplementary Table 3). Among them, eight patients acquired mutations of other genes, including *RUNX1* in four (patients 22, 51, 83 and 108; Table 3), *NRAS* in three (patients 26, 56 and 80), *KRAS* in one (patient 35), *SF3B1* in one (patient 22) and *SETBP1* in one (patient 108) during disease progression, whereas other six patients had chromosomal evolution (patients 5, 24, 38, 71, 98 and 100).

Influence of *ASXL1* mutation on clinical outcome

With a median follow-up duration of 58.2 months (range, 0.1–250.7 months), there was a close correlation between *ASXL1* mutations and acute leukemia transformation (39.0% vs 17.7%; $P<0.001$). If the analysis was restricted to the 362 MDS patients

Table 3. Sequential studies in the 34 MDS patients who had *ASXL1* mutations at diagnosis and/or at follow-ups[a]

UPN	*Date*	*Status*	*Karyotype*	*ASXL1 mutation*	*Other mutations*
1	26/01/2006	RA	47,XY, + 8	G646WfsX12	*RUNX1, IDH2, SRSF2*
	19/07/2006	RAEB2	N	G646WfsX12[b]	*RUNX1, IDH2, SRSF2*
3	27/12/2005	CMML	N	G646WfsX12	*EZH2*
	22/09/2006	RAEBT	ND	G646WfsX12	*EZH2*
5	18/11/1997	RA	N	G646WfsX12	*RUNX1*
	11/09/1998	RAEBT	47,XY, + 21	G646WfsX12	*RUNX1*
	15/05/1999	AML	47,XY, + 21/46,XY,add(6)(p22)	G646WfsX12	*RUNX1*
11	26/07/2007	RAEBT	46,XY,i(17)(q10)	G646WfsX12	*NRAS, SETBP1, SRSF2*
	06/12/2007	AML	46,XY,i(17)(q10)	G646WfsX12	*NRAS, SETBP1, SRSF2*
14	22/08/2008	RAEB	N	S665fsX1	*SRSF2*
	09/12/2008	AML	N	S665fsX1	*SRSF2*
	06/10/2009	AML	ND	S665fsX1	*SRSF2*
21	26/08/2008	CMML	N	G646WfsX12	*NRAS, RUNX1, SRSF2*
	04/11/2008	CMML	ND	G646WfsX12	*NRAS, RUNX1, SRSF2*
	03/03/2009	AML	ND	G646WfsX12	*NRAS, RUNX1, SRSF2*
22	19/06/2008	RARS	N	E635RfsX15	*EZH2*
	15/09/2011	CMML	N	E635RfsX15	*RUNX1, EZH2, SF3B1*
24	01/12/2005	RA	46,XX,i(17)(q10)	G646WfsX12	*SETBP1, SRSF2*
	28/11/2006	AML	46,XY,i(17)(q10)/45,idem, − 7	G646WfsX12	*SETBP1, SRSF2*
26	18/02/1997	RA	45,XY, − 7	G646WfsX12	*RUNX1*
	13/06/1997	RA	45,XY, − 7	G646WfsX12	*RUNX1*
	23/12/1998	RAEB	45,XY, − 7	G646WfsX12	*RUNX1*
	27/04/1999	RAEB	ND	G646WfsX12	*NRAS, RUNX1*
	04/01/2000	AML	45,XY, − 7	G646WfsX12	*RUNX1*
32	24/04/1995	RAEB	N	E635RfsX15	*RUNX1, SRSF2*
	03/06/1997	RAEBT	N	E635RfsX15	*RUNX1, SRSF2*
	03/11/1997	AML	ND	E635RfsX15	*RUNX1, SRSF2*
35	11/06/1999	RAEB2	N	G646WfsX12	*SRSF2*
	07/10/1999	AML	N	G646WfsX12	*KRAS, SRSF2*
38	10/06/1995	RAEBT	N	G646WfsX12	*JAK2, U2AF1*
	01/03/1996	RAEBT	46,XY,del(12)(q13q21)	G646WfsX12	*JAK2, U2AF1*
42	27/12/1999	RAEBT	N	W960X	*IDH1, DNMT3A*
	11/12/2000	AML	ND	W960X	*IDH1, DNMT3A*
44	08/05/1997	CMML	N	S846QfsX5	—
	04/07/1997	CMML	N	S846QfsX5	—
48	13/11/1997	RAEBT	47,XY, + 8	G646WfsX12	*RUNX1*
	04/02/1998	RA (s/p C/T)	ND	G646WfsX12	—
49	18/06/2002	RARS	N	R860SfsX3	*IDH2, SRSF2*
	19/11/2004	RARS	N	R860SfsX3	*IDH2, SRSF2*
51	02/09/1998	RAEBT	N	T1139K	*SF3B1*
	26/02/1999	RAEBT	ND	T1139K	*SF3B1*
	03/12/1999	RAEBT	N	T1139K	*SF3B1*
	13/07/2001	AML	N	T1139K	*RUNX1, SF3B1*
52	04/12/1998	RA	N	G646WfsX12	*IDH2, DNMT3A*
	23/03/1999	CMML	N	G646WfsX12	*IDH2, DNMT3A*
54	05/12/2002	RA	N	A619FfsX17	*MLL*-PTD, *U2AF1*
	25/02/2003	AML	N	A619FfsX17	*MLL*-PTD, *U2AF1*
56	11/01/2001	CMML	N	Y591X	*EZH2, SETBP1*
	29/01/2002	AML	ND	Y591X	*NRAS, EZH2, SETBP1*
60	04/01/2005	RAEB	N	G646WfsX12	—
	06/04/2005	s/p HSCT	N	—	—
71	03/07/2003	RA	N	G646WfsX12	*EZH2*
	29/06/2006	AML	46,XY,inv(7)/47,XY, + 8	G646WfsX12	*EZH2*
74	19/02/2004	CMML1	45,XY, − 7	E635RfsX15	*EZH2*
	19/08/2004	CMML2	45,XY, − 7	E635RfsX15	*EZH2*
80	07/03/2006	RAEB1	N	*Q803X*	*RUNX1, EZH2*
	28/12/2006	RAEB2	N	*Q803X*	*RUNX1, EZH2, NRAS*
83	22/05/2008	CMML1	N	G646WfsX12	*SETBP1*
	03/03/2009	CMML2	N	G646WfsX12	*RUNX1, SETBP1*
88	27/03/2008	CMML	N	G646WfsX12	*RUNX1, EZH2, SETBP1, SF3B1*
	05/02/2009	AML	ND	G646WfsX12	*RUNX1, EZH2, SETBP1, SF3B1*
92	18/11/2008	RAEB1	47,XY, + 8	G646WfsX12	*U2AF1*
	19/02/2009	RAEB2	47,XY, + 8	G646WfsX12	*U2AF1*
	29/07/2009	AML	46,XY,del(11)(q23q25)	G646WfsX12	*U2AF1*
	09/03/2010	s/p HSCT	ND	—	—
	19/08/2010	in CR	ND	—	—
	12/04/2011	in CR	ND	—	—
93	25/12/2008	CMML1	47,XY, + 21	G646WfsX12	*NRAS, RUNX1, EZH2*
	01/09/2009	CMML2	47,XY, + 21	*G646WfsX12*	*NRAS, RUNX1, EZH2*
	21/01/2010	AML	47,XY, + 21	*G646WfsX12*	*NRAS, RUNX1, EZH2*

Table 3. (Continued)

UPN	*Date*	*Status*	*Karyotype*	*ASXL1 mutation*	*Other mutations*
98	15/12/2009	RAEBT	47,XX,+8	R693X	*NRAS, RUNX1*
	17/06/2010	AML	47,XX,+8/47,idem,del(7)(q11q32)	R693X	*NRAS, RUNX1*
100	21/01/2010	RAEB	N	D954GfsX16	*NRAS, EZH2, SETBP1*
	19/07/2010	AML	47,XY,+8	D954GfsX16	*EZH2, SETBP1*
105	28/08/2008	RAEB	N	G646WfsX12	*RUNX1, EZH2*
	30/12/2008	RAEB	N	G646WfsX12	*RUNX1, EZH2*
	16/02/2009	RAEBT	N	G646WfsX12	*RUNX1, EZH2*
	09/06/2009	RAEBT	N	G646WfsX12	*RUNX1, EZH2*
106	18/04/2008	RAEB1	N	W1065X	*SETBP1, U2AF1*
	10/03/2009	RAEB2	N	W1065X	*SETBP1, U2AF1*
	10/06/2009	AML	N	W1065X	*SETBP1, U2AF1*
107	14/06/2002	CMML	46,XY,t(3;3;12)	—	*KRAS*
	06/11/2003	AML	ND	T600Pfs103	*KRAS*
108	23/06/1995	RA	N	E635RfsX15[b]	*EZH2,*
	12/12/2002	RAEB	ND	E635RfsX15	*EZH2, RUNX1, SETBP1*

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; CR, complete remission; C/T, chemotherapy; FAB, French-American-British classification; HSCT, hematopoietic stem cell transplantation; ND, not done; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEBT, refractory anemia with excess blasts in transformation. [a]The 78 patients without *ASXL1* mutation at both diagnosis and during sequential follow-ups are not shown in this table. MDS entity with bone marrow blasts 20–29% was subclassified as RAEBT according to FAB classification and that with bone marrow blasts more than 30% was subclassified as AML. [b]The *ASXL1* mutation could be detected by TA cloning, but not by direct sequencing, in patient 1 at disease progression and in patient 108 at diagnosis.

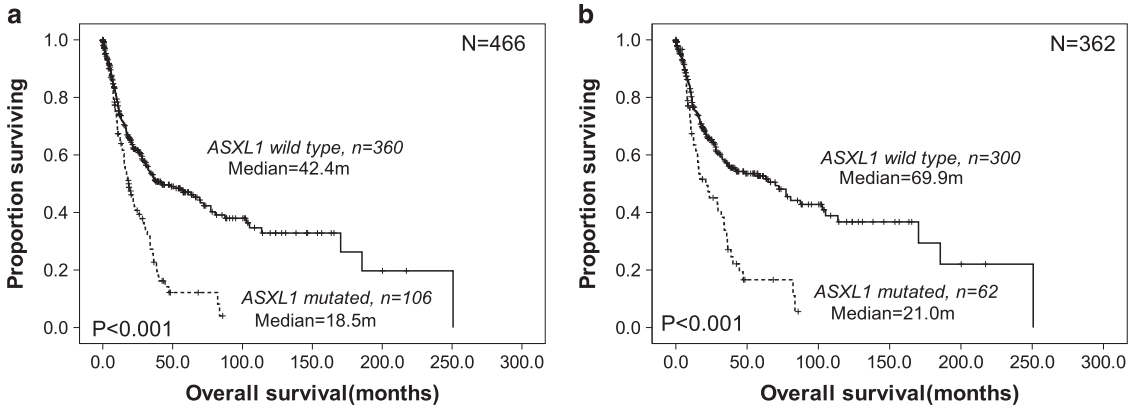

Figure 1. Kaplan–Meier survival curves for OS in MDS patients stratified by *ASXL1* mutation status: (**a**) in all MDS patients based on the FAB classification; (**b**) in all MDS patients based on the 2008 WHO classification.

based on the 2008 WHO classification, those with *ASXL1* mutation also had a significant higher incidence of acute leukemia transformation (32.5% vs 13.0%; $P<0.001$) than those without the mutation. MDS patients, based either on the FAB or 2008 WHO classification, had a significantly shorter OS if they harbored *ASXL1* mutation than those who did not (median, 18.5 vs 42.4 months, $P<0.001$; and 21 vs 69.9 months, $P<0.001$, respectively; Figure 1). The difference remained significant when the analysis was performed in the subgroup of patients with lower-risk MDS defined by the FAB classification (including RA and RA with ring sideroblasts), the WHO classification (other than RAEB, subtypes with blasts $<5\%$), IPSS-R (including very low, low and intermediate groups) and those with favorable-risk cytogenetics (median, 36.1 vs 170.2 months, $P<0.001$, 36.1 vs 170.2 months, $P<0.001$, 33.8 vs 113.7 months, $P<0.001$ and 18.5 vs 69.3 months, $P<0.001$, respectively; Figure 2). However, there was no prognostic impact of *ASXL1* mutation on the MDS patients with higher-risk MDS based on the FAB/WHO classification, IPSS-R or poor-risk cytogenetics.

In univariate analysis, older age, unfavorable cytogenetics, higher IPSS-R score, *IDH, ASXL1*, *RUNX1, NRAS, EZH2* and *SRSF2* mutations were poor prognostic factors for the OS (Supplementary Table 4). The patients with *DNMT3A* mutations had a trend of poorer OS than those without ($P=0.060$). In multivariate analysis using covariables including age $\geqslant 50$ years, IPSS-R and mutations of *ASXL1, RUNX1, NRAS*, *IDH*, *SRSF2* and *DNMT3A* (Table 4), *ASXL1* mutation was an independent poor prognostic factor for OS. Interestingly, in the 242 MDS patients with normal karyotype, *ASXL1* mutation *w*as also an independent poor prognostic factor (hazard ratio $=2.307$, 95% confidence interval $=1.410$–3.775, $P=0.001$) in addition to older age and *NRAS* mutation (Table 5).

DISCUSSION

In the present study, *ASXL1* mutations were detected in 22.7% and 17.1% of MDS patients defined either by the FAB or the 2008 WHO classification, respectively. A majority of *ASXL1*-mutated patients had concurrently other gene mutations, most commonly *RUNX1* and *EZH2* mutations. All *ASXL1* mutations detected at diagnosis remained unchanged during disease progression but were frequently accompanied with acquisition of other novel genetic alterations. Moreover, *ASXL1* mutation was associated with distinct clinical and biological features and a poorer outcome.

We only counted frameshift mutations and nonsense mutations of *ASXL1* gene as true mutations in this study as

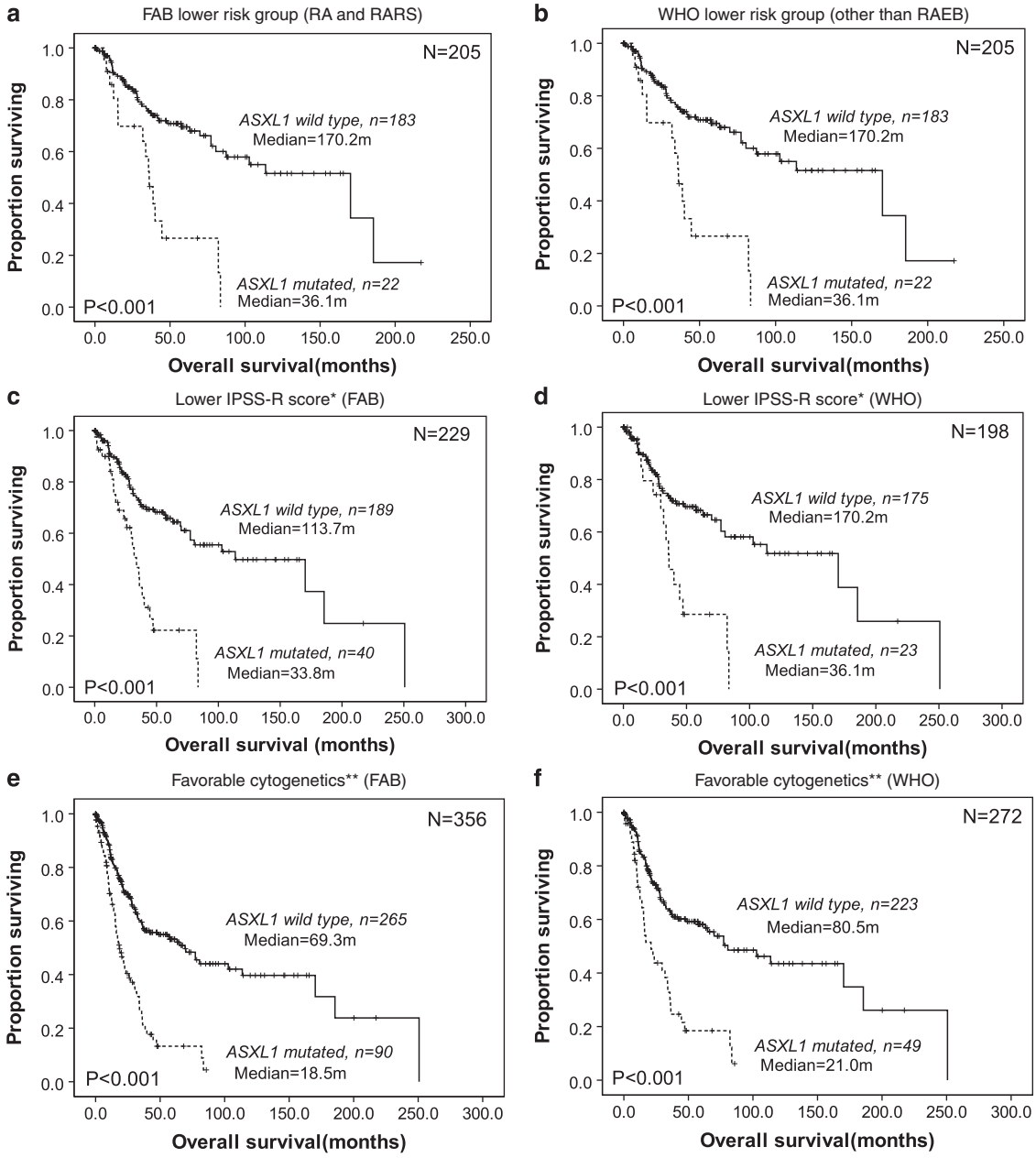

Figure 2. Kaplan–Meier survival curves in the subgroup of patients with lower-risk MDS. (**a**) Lower-risk group (RA and RA with ring sideroblasts (RARS)) defined by the FAB classification; (**b**) lower-risk group (other than RAEB, subtypes with bone marrow (BM) blasts $<5\%$) defined by the 2008 WHO classification; (**c**, **d**) patients with lower IPSS-R score; (**e**, **f**) patients with favorable/intermediate-risk cytogenetics. *Lower IPSS-R groups include very low, low and intermediate subgroups. **Favorable cytogenetics include very good, good and intermediate-risk cytogenetic changes.

previously reported.[9,10] Missense mutations were excluded because their significance could not be verified owing to lack of normal tissue for comparison, not being reported previously or not at the sites well conserved among different species (data not shown). Thol *et al.*[23] analyzed the prognostic effect of *ASXL1* point and frameshift mutations separately. They found that frameshift mutations, but not point mutations, had an independent prognostic effect in MDS patients. The frequency of *ASXL1* mutation in this study (22.7% in MDS patients defined by the FAB classification and 17.1% in those by the 2008 WHO classification) was comparable to that in the West (19.3%[22] and 11–18.5%).[8,10,22–24] The mutation rate in CMML was also similar between this study and others. (45.5% vs 43–49%).[8,40] So were the incidences of *ASXL1* mutation among patients with higher-risk MDS (RAEB by the 2008 WHO classification) and those with lower-risk MDS (other subtypes with bone marrow blasts less than 5; 25.5 vs 18.9–31% and 10.5% vs 8.7–14.2%).[22,23] If the comparison was made separately for these two groups, the mutation occurred more frequently in higher- than in lower-risk MDS.

The report concerning interaction of *ASXL1* mutation with other genetic alterations in the pathogenesis of MDS and its progression is very limited. In the report of Rocquain *et al.*,[8] three of 65 MDS patients had both *ASXL1* and *RUNX1* mutations.[8,41] Another study of 24 patients with CMML showed the co-occurrence of *ASXL1* and *TET2* mutations in seven cases and *ASXL1* and *EZH2* mutations in two cases.[41] However, because of small patient number in these two studies, no statistical analyses were done to evaluate whether

Table 4. Multivariate analysis (Cox regression) for the overall survival in all patients ($N = 466$)

Variable[a]	*HR*	*Overall survival*		P-*value*
		Lower 95% CI	*Upper 95% CI*	
Age ⩾ 50 years	3.672	2.423	5.565	<0.001
Higher IPSS-R[b]	3.983	2.729	5.812	<0.001
ASXL1	1.425	1.024	1.983	0.035
RUNX1	1.199	0.800	1.797	0.380
NRAS	1.227	0.677	2.226	0.500
DNMT3A	1.482	0.964	2.280	0.073
IDH	0.913	0.493	1.691	0.771
SRSF2	1.101	0.731	1.659	0.646

Abbreviations: CI, confidence interval; HR, hazard ratio; IPSS, international prognostic scoring system; IPSS-R, revised IPSS. [a]The model was generated from a stepwise Cox regression model that included age, IPSS-R and gene mutations of *ASXL1, RUNX1, NRAS, DNMT3A, IDH* and *SRSF2*. [b]Higher IPSS-R (high, very high and intermediate) vs lower IPSS-R (very low and low).

Table 5. Multivariate analysis (Cox regression) for the overall survival in MDS patients with normal karyotype ($N = 242$)

*Variable**	*HR*	*Overall survival*		P-*value*
		Lower 95% CI	*Upper 95% CI*	
Age ⩾ 50 years	3.642	1.789	7.413	<0.001
ASXL1	2.307	1.410	3.775	0.001
RUNX1	1.552	0.892	2.699	0.120
NRAS	2.418	1.140	5.130	0.021
EZH2	1.370	0.724	2.594	0.333
IDH	1.747	0.833	3.661	0.140
SRSF2	1.176	0.684	2.021	0.558

Abbreviations: CI, confidence interval; HR, hazard ratio; MDS, myelodysplastic syndrome. *The model was generated from a stepwise Cox regression model that included age and gene mutations of *ASXL1, RUNX1, NRAS, EZH2, IDH1/2* and *SRSF2*.

there was a significant association of *ASXL1* mutation with these mutations. Our study showed 85% (90 of 106) of *ASXL1*-mutated patients had concurrent other gene alterations. Furthermore, *ASXL1* mutation was closely associated with mutations of *RUNX1, EZH2, IDH, NRAS, JAK2, SETBP1* and *SRSF2* in MDS patients. In other words, the *ASXL1* mutation coincided with mutations of genes involved in the signal transduction pathway (*JAK2* and *NRAS*), transcription factor (*RUNX1*), epigenetic modification (*IDH* and *EZH2*) or splicing machinery (*SRSF2*) in MDS patients. The manner in which the *ASXL1* mutation was associated with these genetic aberrations in the MDS pathogenesis needs further investigation.

The reports regarding sequential studies of *ASXL1* mutation in MDS are even less. In one report,[8] the *ASXL1* mutation detected in one patient with RAEB2 at diagnosis was retained at the time of AML transformation. To the best of our knowledge, this is the largest study to evaluate the dynamic change of *ASXL1* mutation during disease progression in MDS. We found that with the exception of the two patients who received transplantation, the remaining 30 *ASXL1*-mutated patients analyzed retained the same mutation during serial follow-ups, including the one (patient 1) whose original mutation could be detected only by a sensitive gene-cloning technique, but not by direct sequencing, at the time of disease progression. These findings suggest that *ASXL1* mutation may constitute an early hit in the pathogenesis of MDS. Similar to our findings, most *ASXL1* mutations detected at leukemic transformation of myeloproliferative neoplasm patients were already present at chronic phase.[14] Interestingly, during disease progression, *ASXL1*-mutated patients frequently acquired other novel genetic alterations, most commonly *RUNX1* and *NRAS* mutations, (Table 3) or had chromosomal evolution indicating the additional genetic aberrations contributed to the progression of MDS in these patients. On the other hand, only one (patient 107) of the 80 patients without *ASXL1* mutation at diagnosis acquired novel *ASXL1* mutation at the time of AML transformation. Although no *ASXL1* mutation could be detected at diagnosis even using a more sensitive cloning technique, we could not exclude the possibility that minor subpopulations of cells with the *ASXL1* mutant existed initially. Another one patient (patient 108) with no detectable *ASXL1* mutation at diagnosis by direct sequencing had in fact low level of *ASXL1* mutant as shown by a more sensitive TA-cloning technique and the mutant expanded at disease progression. Altogether, these findings imply that *ASXL1* mutations may have little, if any, role in the progression of MDS in *ASXL1*-wild patients.

ASXL1 mutation was shown to predict poor outcome in WHO-defined MDS and CMML patients,[10,42] and was associated with a reduced time to AML transformation.[40] Thol *et al.*[23] and Bejar *et al.*[24] further demonstrated *ASXL1* mutation as an independent poor prognostic factor in MDS patients. In our study, *ASXL1*-mutated MDS patients had poorer OS and higher rates of AML progression, especially in lower-risk patients, but not in the higher-risk ones. *ASXL1* mutation was an independent poor prognostic factor irrespective of age, IPSS-R and mutations of *RUNX1*, *NRAS, DNMT3A, IDH* and *SRSF2*. Intriguingly, in MDS patients with normal karyotype, *ASXL1* mutation was also an independent poor prognostic factor. *ASXL1* mutation is thus helpful for risk stratification of MDS patients with normal karyotype, which is categorized as an intermediate-risk cytogenetic group.

In summary, *ASXL1* mutations were detected in a substantial portion of MDS patients and were closely associated with trisomy 8 and mutations of *RUNX1, EZH2, IDH, NRAS, JAK2, SETBP1* and *SRSF2*. The presence of *ASXL1* mutations predicted shorter survival, especially in the patients with lower-risk MDS. For patients with normal karyotype, the mutation was also an independent poor prognostic factor for OS. Sequential study during the clinical course showed *ASXL1*-mutated patients retained the original *ASXL1* mutation, but frequently acquired other novel genetic alterations during disease evolution.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

T-C Chen was responsible for literature collection, data management and interpretation, statistical analysis and manuscript writing; H-AH was responsible for study design, literature collection, data management and interpretation, statistical analysis and manuscript writing; C-YL was responsible for statistical analysis and interpretation of the statistical findings; Y-YK and M-C L were responsible for mutation analysis and interpretation; C-YC, W-CC, M-Y, S-YH, J-LT, B-SK, S-CH, S-JW, WT and Y-CC contributed patient samples and clinical data; M-CL, M-HT, C-FH, Y-CC, C-YL, F-YL and M-CL performed the gene mutation and chromosomal studies and H-FT planned, designed and coordinated the study over the entire period and wrote the manuscript.

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