INTRODUCTION

The myelodysplastic syndromes (MDSs) are a heterogeneous group of diseases, characterized by cytopenia, but usually hypercellular bone marrow with dysplastic hematopoiesis, and a propensity to acute leukemia transformation. The pathogenesis that causes bone marrow with dysplastic hematopoiesis, and a propensity of diseases, characterized by cytopenia, but usually hypercellular

The myelodysplastic syndromes (MDSs) are a heterogenous group of diseases in mice. Further, the deficiency of the BAP1, a nuclear-localized deubiquitinating enzyme, resulted in a CMLML-like phenotype, and the interaction with ASXL1 is critical for the enzymatic activity of BAP1. ASXL1 mutation is found in a substantial proportion (11–18.5%) of WHO-defined MDS patients and is correlated with unfavorable outcome. 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 and mutations of RUNX1, EZH2, IDH, NRAS, JAK2, SETBP1 and SF3B1, 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 University Hospital, Department of Internal Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan; 2Department of Laboratory Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan; 3Graduate Institute of Oncology, College of Medicine, National Taiwan University, Taipei, Taiwan; 4Department of Pathology, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan; 5Biostatistics Consulting Laboratory, Department of Nursing, National Taiwan University College of Nursing and Health Science, Taipei, Taiwan. Correspondence: Dr H-F Tien, Department of Internal Medicine, National Taiwan University Hospital, No 7, Chung-Shan South Road, Taipei 100, Taiwan.

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ORIGINAL ARTICLE

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

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 SF3B1, 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.
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., with mild modification. 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, NRAS, KRAS, and JAK2 mutations, and class II mutations, such as NLR/PTD, RUNX1 and WT1 mutations, as well as mutations of genes involving in epigenetic modifications, such as IDH1, IDH2, including R140 and R172 mutations, DNMT3A and EZH2 mutations, splicing machinery mutations, such as U2AF1, SRSF2 and SF3B1 mutations, and SETBP1 mutation, were performed as previously described. Sequential studies of all these genes were also performed in 305 samples from 112 patients during clinical follow-ups.

Cyto-genetics
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. 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.

Statistics
The $\chi^2$-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, revised IPSS (IPSS-R) score 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 date of death or the last follow-up.

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**                        |                 |                                |                            |         |
| Male                           | 308             | 81 (26.3)                      | 227 (73.7)                 | 0.01    |
| Female                         | 158             | 25 (15.8)                      | 133 (84.2)                 |         |
| **Age (year)**                 | 66 (18–98)      | 71 (26–89)                     | 64 (18–98)                 | 0.001   |
| **Lab data**                   |                 |                                |                            |         |
| 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 1−1)                    | 485 (145–6807)  | 531 (225–3756)                 | 469 (145–6807)             | 0.275   |
| **FAB subtype**                |                 |                                |                            |         |
| 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**    |                 |                                |                            |         |
| 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**                       |                 |                                |                            |         |
| Low/INT-1                      | 254             | 50 (19.7)                      | 204 (80.3)                 | 0.022   |
| INT-2/High                     | 181             | 53 (29.3)                      | 128 (70.7)                 |         |
| **IPSS-R**                     |                 |                                |                            | 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. *Number of patients (% of patients with or without ASXL1 mutation in the subgroup). #International prognosis scoring system: low, 0; intermediate (INT)-1, 0.5–1; INT-2, 1.5–2; and high, ≥2.5. 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 StatisticF (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. 106 patients showed single heterozygous mutation. (Supplementary Table 1) All these mutations resulted in truncation of the plant homeodomain of ASXL1. Patients with CMMML 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).

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).

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

| UPN | Date       | Status | Karyotype                  | ASXL1 mutation | Other mutations            |
|-----|------------|--------|----------------------------|----------------|---------------------------|
| 1   | 26/01/2006 | RA     | 47,XY, + 8                 | G646WfsX12     | RUNX1, IDH2, SRSF2        |
| 3   | 27/12/2005 | CMMML  | N                          | G646WfsX12     | EZH2                     |
| 5   | 18/11/1997 | RA     | G646WfsX12                 |                |                           |
| 11  | 15/09/1997 | RAEBT  | 47,XY, + 21                | G646WfsX12     | RUNX1                    |
| 11  | 26/07/2007 | RAEBT  | 46,XY; (+1)(q10)           | G646WfsX12     | NRAS, SETBP1, SRSF2      |
| 14  | 06/12/2007 | AML    | 46,XY; (+1)(q10)           | G646WfsX12     | NRAS, SETBP1, SRSF2      |
| 21  | 26/08/2008 | CMML   | N                          | G646WfsX12     | NRAS, RUNX1, SRSF2       |
| 35  | 11/09/2008 | AML    | 47,XY, + 21                | G646WfsX12     | SRSF2                    |
| 38  | 10/06/1995 | RAEBT  | 46,XY; (+1)(q10)           | G646WfsX12     | JAK2, U2AF1              |
| 42  | 27/12/1999 | RAEBT  | 46,XY, + 7                 | G646WfsX12     | JAK2, U2AF1              |
| 44  | 08/05/1997 | CMMML  | N                          | G646WfsX12     |                           |
| 48  | 13/11/1997 | RAEBT  | 46,XY, + 8                 | G646WfsX12     | RUNX1                    |
| 49  | 18/06/2002 | RARS   | N                          | G646WfsX12     |                           |
| 52  | 04/12/1998 | RA     | (s/p C/T)                  | ND             |                           |
| 54  | 05/12/2002 | RA     | N                          | G646WfsX12     |                           |
| 56  | 11/01/2001 | CMMML  | N                          | G646WfsX12     |                           |
| 60  | 04/01/2005 | RAEB   | N                          | G646WfsX12     |                           |
| 71  | 03/07/2003 | RA     | N                          | G646WfsX12     | EzH2                     |
| 74  | 19/02/2004 | CMMML1 | 45,XY, + 7                 | G646WfsX12     | EzH2                     |
| 80  | 07/03/2006 | RAEB1  | N                          | Q803X          | EzH2                     |
| 83  | 22/05/2008 | CMMML1 | N                          | G646WfsX12     | RUNX1, SETBP1            |
| 88  | 27/03/2008 | CMMML2 | N                          | G646WfsX12     | RUNX1, SETBP1, SFR3B1    |
| 92  | 18/11/2008 | RAEB1  | 47,XY, + 8                 | G646WfsX12     | U2AF1                    |
| 93  | 25/12/2008 | CMMML1 | 47,XY, + 21                | G646WfsX12     | NRAS, EzH2, SETBP1       |
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. 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.

Table 3. (Continued)

| UPN | Date       | Status       | Karyotype            | ASXL1 mutation       | Other mutations |
|-----|------------|--------------|----------------------|----------------------|----------------|
| 98  | 15/12/2009 | RAEBT        | 47XX, +8             | R693X                | NRAS, RUNX1    |
| 100 | 21/01/2010 | RAEB         | N                    | D954GfsX16            | NRAS, RUNX1    |
| 105 | 28/08/2008 | RAEB         | N                    | G646WfsX12            | RUNX1, EZH2    |
| 107 | 14/06/2002 | CMML         | 46XY(13;12)           | —                    | KRA5           |
| 108 | 23/06/1995 | RA           | N                    | E635RfsX15b           | EZH2           |
| 12/12/2002 | RAEB         | N              | 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. aThe 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. bThe 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 69.9 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 ≥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 was 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...
previously reported. 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. 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 2008 WHO classification; 17.1% in those by the 2008 WHO classification) was comparable to that in the West (19.3% and 11–18.5%). The mutation rate in CMML was also similar between this study and others (45.5% vs 43–49%). 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% vs 25.5 vs 18.9–31% and 10.5% vs 8.7–14.2%). 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., three of 65 MDS patients had both ASXL1 and RUNX1 mutations. 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. However, because of small patient number in these two studies, no statistical analyses were done to evaluate whether
were already present at chronic phase. 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 not detectable ASXL1 mutation at diagnosis by direct sequencing had in fact low level of ASXL1 mutation 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 and was associated with a reduced time to AML transformation. Moreover, Bejar et al. 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 mutation of RUNX1, NRAS, DNMT3A, IDH1 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.
REFERENCES

1 Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997; 89: 2079–2088.

2 Hofmann WK, Koeffler HP. Myelodysplastic syndrome. Annu Rev Med 2005; 56: 1–16.

3 Acquaviva C, Geli-Boyer V, Bimbbaum D. Myelodysplastic syndromes: lost between two states? Leukemia 2010; 24: 1–5.

4 Kulasekararaj AG, Mohamedali AM, Mufti GJ. Recent advances in understanding the molecular pathogenesis of myelodysplastic syndromes. Br J Haematol 2013; 162: 587–605.

5 Bejar R, Levine R, Ebert BL. Unraveling the molecular pathophysiology of myelodysplastic syndromes. J Clin Oncol 2011; 29: 504–515.

6 Stooland EM, Rezvani K. The role of the immune system in myelodysplasia: implications for therapy. Semin Hematol 2008; 45: 39–48.

7 Raaijmakers MH. Myelodysplastic syndromes: revisiting the role of the bone marrow microenvironment in disease pathogenesis. Int J Hematol 2012; 95: 17–25.

8 Rocquain J, Carbuccia N, Trouplin V, Raynaud S, Murati A, Nezri M et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC Cancer 2010; 10: 401.

9 Chou WC, Huang HH, Hou HA, Chen CY, Tang JL, Yao M et al. Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (ASXL1) mutations. Blood 2010; 116: 4086–4094.

10 Geli-Boyer V, Trouplin V, Adelaide I, Bonansea J, Cervera J, Cervera N, Carbuccia N et al. Mutations of polycomb-associated gene AXSL1 in myelodysplastic syndromes and chronic myelomonocytic leukemia. Br J Haematol 2009; 145: 788–800.

11 Martinez-Aviles L, Besses C, Alvarez-Larran A, Torres E, Serrano S, Bellosillo B. Characterization of Asxl1, a murine homolog of additional sex combs, and analysis of the Asx-like gene family. Gene 2006; 369: 109–118.

12 Braubert T, Walter MJ. Genetics of myelodysplastic syndromes: new insights. Hematology Am Soc Hematol Educ Program 2011; 2011: 543–549.

13 Fisher CL, Randazzo F, Humphries RK, Brock HW. Characterization of ASXL1, a murine homolog of additional sex combs, and analysis of the Asxl-like gene family. Gene 2006; 369: 109–118.

14 Abdel-Wahab O, Manshouri T, Patel P, Harris K, Yao J, Hedvat C et al. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. Cancer Res 2010; 70: 447–452.

15 Sood AV, D’Andrea AJ, Hoang B, Fieno JS, Bais BC et al. Loss of heterozygosity at chromosome 5q in myelodysplastic syndrome with del(5q). Leukemia 2009; 23: 616–620.

16 Dey A, Seihasaeye D, Noubade R, French DM, Liu J, Chaurasiah S et al. Loss of the tumor suppressor BAP1 causes myeloid transformation. Science 2012; 337: 1541–1546.

17 Scheuermann JC, de Ayala Alonso AG, Oktaba K, Ly-Hartig N, McGinty RK, Fraterman S et al. Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. Nature 2010; 465: 243–247.

18 Boulthwood J, Penny J, Pellegatti A, Fernandez-Mercado M, Fernandez-Santamaria C, Calasanz MJ et al. Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. Leukemia 2010; 24: 1062–1065.

19 Thol F, Friesen I, Damm F, Yun HY, Weissinger EM, Krauer J et al. Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. J Clin Oncol 2011; 29: 2499–2506.