



Gene mutation spectrum of patients with myelodysplastic syndrome and progression to acute myeloid leukemia

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Aim: This study aimed to investigate the regularity of gene mutations in patients with myelodysplastic syndrome (MDS) and in those that progressed to acute myeloid leukemia (MDS/AML). **Patients & methods:** High-throughput sequencing technology was used to detect gene mutations in 99 newly diagnosed patients with MDS or MDS/AML. **Results:** Gene mutations were detected in 88 patients. The mutation incidence in the MDS/AML group was significantly higher than that in the MDS group. Statistically significant differences were observed between the MDS with refractory anemia (MDS-RA) and MDS-RA with excess blasts groups and between the MDS/AML and MDS-RA groups. **Conclusion:** Our data demonstrate that there is a cumulative accumulation of gene mutations, especially in transcription factor genes, during disease progression in MDS and MDS/AML.

Lay abstract: This study investigated the regularity of gene mutations in patients with myelodysplastic syndrome (MDS) and in those that have progressed to acute myeloid leukemia (MDS/AML). High-throughput sequencing was used to detect mutations in 58 genes with known clinical significance in 99 patients who were newly diagnosed with MDS or MDS/AML. A total of 28 mutated genes and 214 mutations were detected in 88 (88.9%) patients. The most frequently mutated gene was *U2AF1* (13.55%; 29/214), followed by *ASXL1* (10.28%; 22/214), *TP53* (7.09%; 15/214), and *RUNX1* (7.09%; 15/214). The mutation rate in the MDS/AML group was significantly higher than in the MDS group (100 vs 84.51%; $p = 0.031$). The average number of mutations per patient was 1.40, 2.20 and 2.64 in the MDS-refractory anemia (RA), MDS-RA with excess blast (RAEB) and MDS/AML groups, respectively. Statistically significant differences were observed between the MDS-RA and MDS-RAEB groups ($p = 0.031$) and between the MDS/AML and MDS-RA groups ($p = 0.003$). Signal transduction gene mutations were more frequent in the MDS/AML than in the MDS group (50% vs 22.54%; $p = 0.014$), especially in the *FLT3* (14.29% vs 0; $p = 0.005$) and *PTPN11* (17.86 vs 2.82%; $p = 0.018$) genes. Statistically significant ($p < 0.05$) correlations were found in 12 mutated gene combinations. *TP53* mutations were mutually exclusive with RNA splicing factor gene mutations ($p = 0.001$). *U2AF1* S34 mutations were associated with trisomy 8 (22.22 vs 5.97%; $p = 0.03$), and *TP53* mutations were associated with complex karyotypes. Our data demonstrate that there is cumulative accumulation of gene mutations, especially in transcription factor genes, during disease progression in MDS and MDS/AML. The data also indicate there are synergistic pathogenicity and mutually exclusive effects among gene mutations and chromosomal abnormalities.

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Myelodysplastic syndrome (MDS) is a group of hematological stem cell clonal diseases with high heterogeneity. The clinical features of pathological hematopoiesis and ineffective hematopoiesis lead to peripheral blood cell

Table 1. Clinical characteristics of 99 MDS and MDS/AML patients.

Clinical characteristics	Total cohort	MDS	MDS/AML
Number of patients	99	71	28
Gender (%)			
– Male	67	50 (74.6)	17 (25.4)
– Female	32	21 (65.6)	11 (34.4)
Median age years (range)	40 (2–76)	43 (2–76)	29.5 (2–63)
Median WBC ($\times 10^9/l$)	2.6	2.6	2.94
– Range	0.4–105	0.64–11.72	0.4–105
Median HB (g/l)	74	72	74.5
– Range	28–145	36–145	28–135
Median PLT ($\times 10^9/l$)	39	47	35.5
– Range	4–525	4–524	5–190
Median BM blasts, %	12.00	8.0	30.5
– Range	0.34–86	0.34–18.5	20–86
Cytogenetics			
– No. with date available	94	68	26
– Normal	30	23	7
– Trisomy 8	10	8	2
– Complex	24	16	8
– Others	30	21	9
Disease subtype			
– RA/RARS	6	4/2	–
– RCMD	15	15	–
– RAEB-I	22	22	–
– RAEB-II	28	28	–

AML: Acute myeloid leukemia; BM: Bone marrow; HB: Hemoglobin; MDS: Myelodysplastic syndrome; MDS/AML: MDS progressed to acute myeloid leukemia; PLT: Platelets; RA: Refractory anemia; RAEB-I: Refractory anemia with excess blasts-I; RAEB-II: Refractory anemia with excess blasts-II; RARS: Refractory anemia with ring sideroblasts; RCMD: Refractory cytopenia with multilineage dysplasia; WBC: White blood cells.

reduction and the risk of transformation into acute myeloid leukemia [1]. With the progress in molecular biology and high-throughput sequencing technology, increasing numbers of mutant genes have been identified in MDS [2,3].

More than 90% of patients with MDS have been found to harbor at least one known pathogenic gene mutation, and some of these mutations have been associated with clinical features [4]. Analysis of these mutant genes is of great significance for exploring the molecular biology of the initiation and progression of MDS. Herein, we aimed to investigate the rate and regularity of gene mutations in patients with MDS and in those that progressed to acute myeloid leukemia (MDS/AML). This study provides more evidence to further explain the similarities and differences between MDS and MDS/AML and can help deepen the understanding of the evolution trajectory of these two myeloid malignancies.

Methods

Patients

We retrospectively analyzed 71 patients with newly diagnosed MDS and 28 patients with MDS that progressed to acute myeloid leukemia (MDS/AML) who were enrolled in the Hebei Yanda Lu Daopei Hospital from May 2015 to December 2017. There were 67 males and 32 females, with a median age of 40 years (2–76 years). Both hospitals are centers mainly focused on patients who need allogeneic hematopoietic stem cell transplantation (allo-HSCT), so the patients referred do not represent the overall MDS population. This study was approved by the ethics committees of the Lu Daopei Hospital, and all patients (or legal guardians) enrolled in this study signed informed consent according to approved protocols. The diagnosis of MDS and MDS/AML was based on the WHO 2008 classification criteria [5]. Patients with MDS included four cases of refractory anemia (RA), two cases of refractory anemia with ring sideroblasts (RARS) and 15 cases of refractory cytopenia with multiple dysplasia (RCMD). There were 50 cases of refractory anemia with excess blasts (RAEB) and 28 cases of MDS/AML (Table 1).

Genomic DNA extraction

DNA was extracted from 3–5 ml of bone marrow (92 cases) or bone marrow smears (seven cases) retained at the patients' first diagnosis. Fresh bone marrow samples were subjected to cell counting after lysis of red blood cells, and 1.0×10^7 nucleated cells were used to extract genomic DNA. The bone marrow smear cells were repeatedly purged with a pipette and pure water, and the obtained cell suspension was used to extract genomic DNA. Genomic DNA was extracted using a silica-column-based DNA extraction kit (Tiangen Biotech Co., Ltd, Beijing, China; cat. no. DP318-03) according to the manufacturer's protocol, as previously described [6].

Gene mutation detection and analysis

High-throughput targeted amplicon sequencing (Ion Torrent PGM platform) was used to detect mutation hotspots or full-length coding regions of 58 commonly mutated genes associated with hematological tumors [6]. For the mutation analysis of 58 genes implemented in this study, only hotspot mutations and mutations with clear pathological significance in hematological tumors were reported and analyzed. Average sequencing depth was greater than 1000 \times , single nucleotide variation (SNVs) and short fragment insertion/deletion mutations were identified by TVC 5.0-13 software. The SNVs recognition threshold was set to the minimum coverage depth of 100 \times and the variant allele frequency (VAF) was >5%. The dbSNP, 1000 Genomes, ExAC, ClinVar, and COSMIC databases and the bioinformatics software programs PolyPhen-2 and SIFT were used for mutation analysis. Mutations of *FLT3-ITD*, *NPM1* and *CALR* genes were detected by fragment analysis (AB 3500XL sequencer), the results of which were analyzed using GeneMapper ID V3.2 software to calculate the length and VAF of the inserted/deleted fragments.

Statistical analysis

SPSS 23.0 statistical software was used to analyze data of patients in different disease groups. The number of genetic mutations carried by patients in different disease groups was compared using a nonparametric approach Kruskal–Wallis analysis of variance, while categorical data was analyzed using χ^2 or Fisher exact tests. P-values are unadjusted and two-sided, and <0.05 was considered statistically significant.

Results

Overall gene mutation frequency

A total of 214 gene mutations were detected in 28 genes, among which the most frequently mutated gene was *U2AF1* (13.55%, 29/214), followed by *ASXL1* (10.28%, 22/214), *TP53* (7.09%, 15/214) and *RUNX1* (7.09%, 15/214; **Figure 1**). Mutations were detected in 88.89% (88/99) of the patients, among which the positive mutation rate was 84.51% (60/71) in the MDS group and 100% (28/28) in the MDS/AML group. The median number of mutated genes carried in the 99 patients was 2 (0–6); 27.27% (27/99) of patients carried a single gene mutation, and 61.62% (61/99) carried two or more gene mutations.

We classified the mutated genes into six classes according to their gene function: epigenetic regulatory genes *ASXL1*, *ASXL2*, *DNMT3A*, *EZH2*, *IDH1*, *IDH2* and *TET2*; transcription factor genes *CEBPA*, *ETV6*, *GATA2*, *NPM1* and *RUNX1*; splicing factor genes *SF3B1*, *SRSF2* and *U2AF1*; signal transduction genes *CBL*, *CSF3R*, *FLT3*, *JAK2*, *JAK3*, *NRAS*, *PTPN11* and *KRAS*; tumor suppressor genes *TP53*, *WT1* and *PHF6*; and other genes, *CALR* and *SETBP1* [3,7,8]. The mutation rates of the six types of genes were as follows: epigenetic regulatory genes 23.83% (51/214), transcription factor genes 21.50% (46/214), splicing factor genes 18.69% (40/214), signal transduction genes 18.22% (39/214), tumor suppressor genes 11.21% (24/214) and the other genes 6.54% (14/214; **Figure 1**).

Gene mutation spectrum analysis

Twelve mutated gene combinations showed statistical significance ($p < 0.05$). Of the epigenetic regulation genes, 95.45% (21/22) of *ASXL1* mutations co-occurred with other gene mutations and 11 co-occurred with *U2AF1* mutations, which was the most frequent concomitant mutation (50%, 11/22). *ASXL1* mutations were more likely to co-occur with other gene mutations such as *ETV6*, *RUNX1*, *SRSF2*, *PHF6* and *SETBP1* (**Table 2** & **Figure 2**).

Of the RNA splicing factor genes, 82.76% (24/29) of *U2AF1* mutations co-occurred with other genes. The combination of *U2AF1* mutations with *CBL* was higher than with other genes (13.79 vs 0%; $p = 0.001$). This type of gene mutation in the MDS patient group was mutually exclusive, while only one patient in the MDS/AML group carried a combination of mutations of *SF3B1*, *U2AF1*, *TET2* or *SETBP1* (**Figures 2** & **3**).

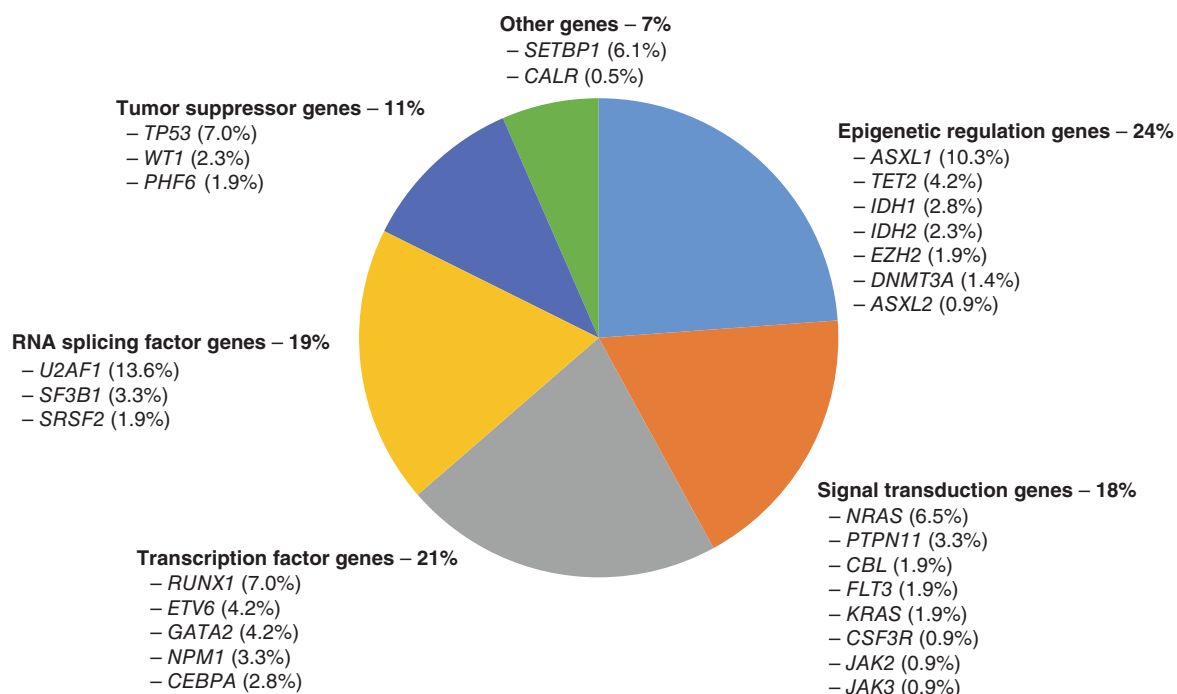


Figure 1. Composition of mutant genes.

Table 2. ASXL1 accompanying mutations.

	ASXL1 M (n = 22)	ASXL1 W (n = 66)	p-value
ASXL1	–	–	–
EZH2	2	2	0.259
IDH1	3	3	0.162
IDH2	3	2	0.097
TET2	1	8	0.440
CBL	2	2	0.259
FLT3	0	4	0.568
NRAS	4	10	0.743
PTPN11	2	5	1
KRAS	1	3	1
CEBPA	2	4	0.637
ETV6	6	3	0.007
GATA2	2	7	1
NPM1	0	7	0.185
RUNX1	9	6	0.002
SF3B1	0	7	0.185
SRSF2	3	1	0.047
U2AF1	11	18	0.067
TP53	2	13	0.338
WT1	0	5	0.325
PHF6	3	1	0.047
SETBP1	7	6	0.016

M: Mutated; W: Wild-type. Genes mutated in fewer than four patients are not listed.

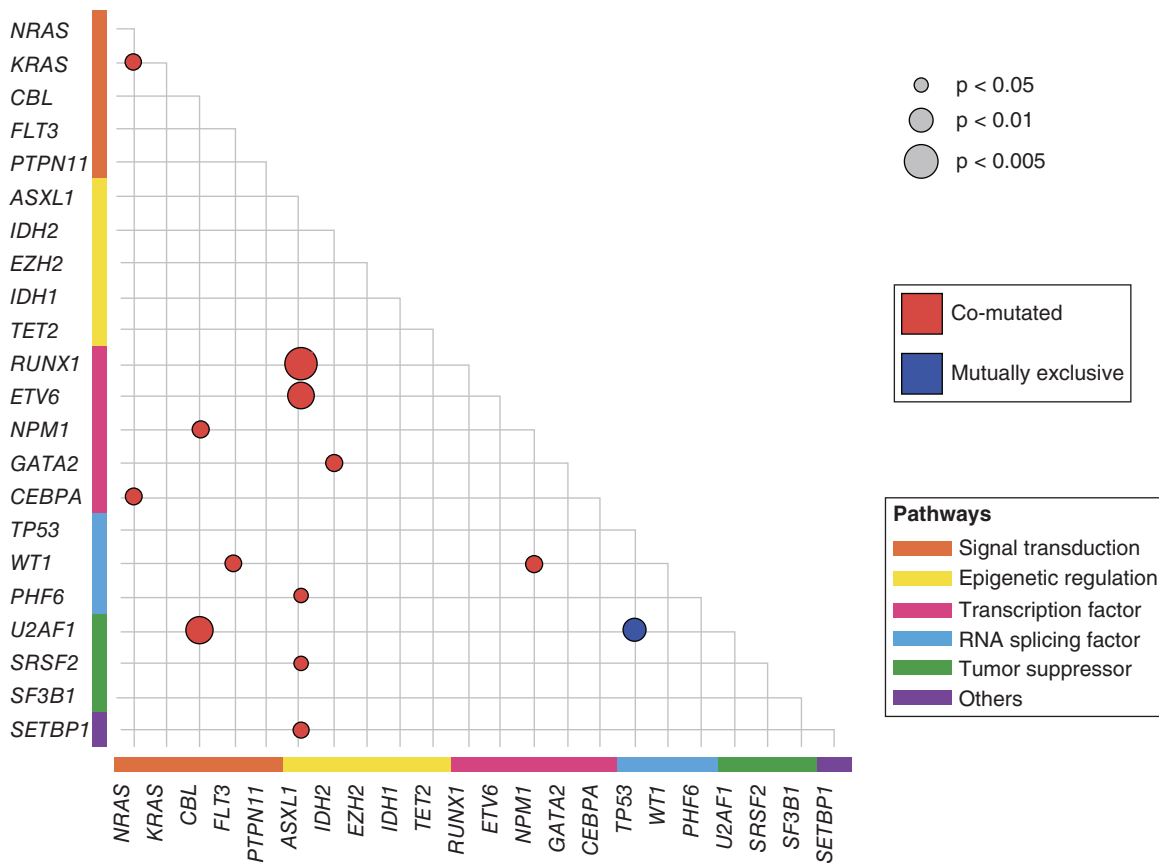


Figure 2. Ninety-nine cases of myelodysplastic syndrome and myelodysplastic syndrome/acute myeloid leukemia patients with genetic mutations. Correlation between mutations was found in 22 genes associated with myelodysplastic syndrome and myelodysplastic syndrome/acute myeloid leukemia. The correlation and p-values are represented by the size of the circles and the color gradient.

Regarding NRAS mutations, 92.86% (13/14) co-occurred with other gene mutations, with *KRAS* and *CEBPA* being common companion mutations (21.43 vs 1.35%; $p = 0.012$; and 21.43 vs 4.05%; $p = 0.049$, respectively) (Figure 2). In addition, *WT1* mutations often co-occurred with *FLT3* mutations (40 vs 2.41%; $p = 0.015$; Figure 2).

Correlation between gene mutations and chromosomal abnormalities

In this study, we observed that *U2AF1* mutations were more likely to be associated with trisomy 8, and *TP53* mutations were associated with complex karyotypes. Of the 29 patients with *U2AF1* mutations in this study, 27 were S34 mutations (S34Y, $n = 11$; S34F, $n = 16$), and the other two were Q157R and R156H mutations. *U2AF1* S34 mutations were associated with trisomy 8 (6/27, 22.22% vs 4/67, 5.97%; $p = 0.030$). *TP53* gene mutations were often associated with complex karyotypes (93.33 vs 12.66%; $p < 0.001$) and were mutually exclusive with RNA splicing factor gene mutations (6.67 vs 52.05%, $p = 0.001$), especially with *U2AF1* gene mutations (3.45 vs 23.73%; $p = 0.017$; Figure 3).

Gene mutations in different disease groups

There were certain differences in gene mutation rates and mutant gene types between MDS and MDS/AML in different disease groups. The overall gene mutation rate in the MDS/AML group was significantly higher than that in the MDS group (100 vs 84.51%; $p = 0.031$). The most commonly mutated gene types in the MDS group were epigenetic regulation genes and RNA splicing factor genes; however, there was no statistically significant difference when compared with the MDS/AML group. The most common mutations in the MDS/AML group were in signal transduction genes, and this was statistically different compared with the MDS group (50 vs 22.54%; $p = 0.014$).

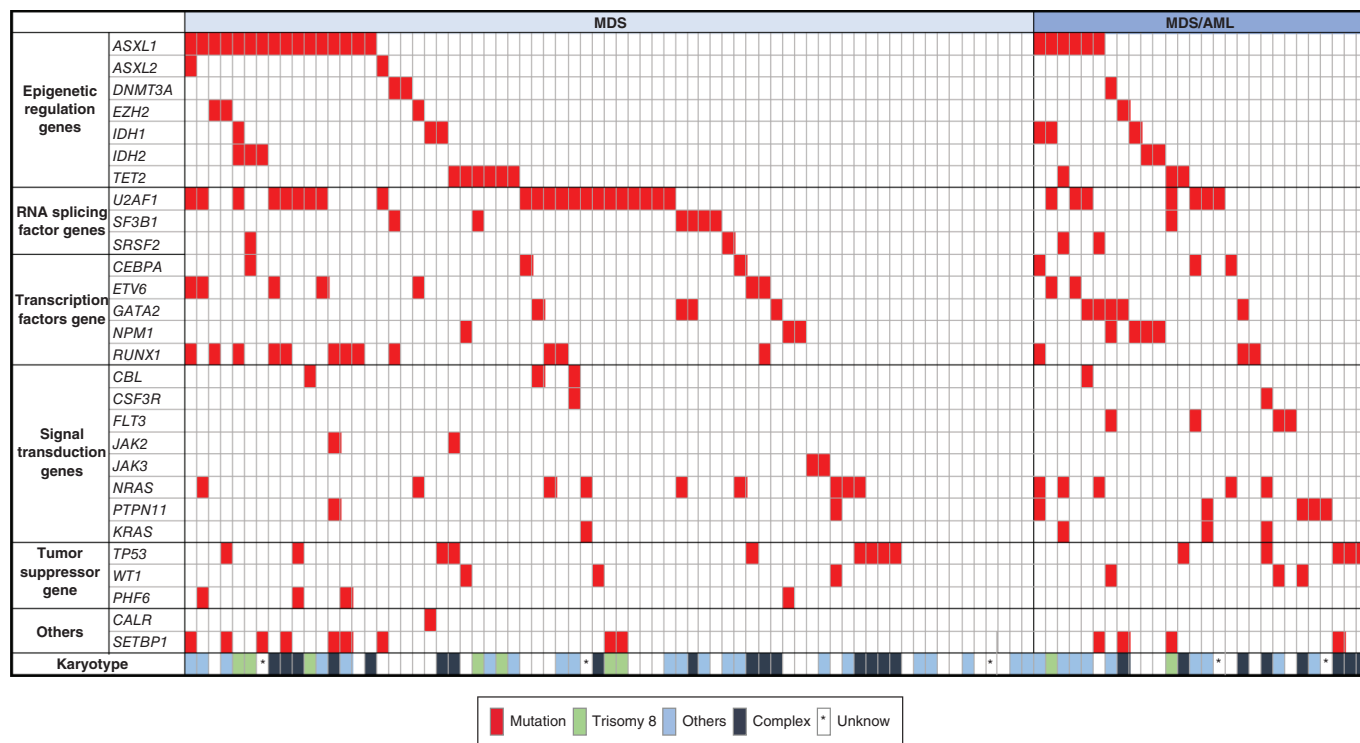


Figure 3. Gene mutation spectrum and karyotype in 99 patients with Myelodysplastic syndrome and MDS/AML. The rows represent individual gene mutations or karyotype, and the columns represent individual patients. AML: Acute myeloid leukemia; MDS: Myelodysplastic syndrome.

Four *FLT3* gene mutations were detected in 99 patients, all of which were found in the MDS/AML group (*FLT3-ITD*, $n = 3$; *FLT3-TKD*, $n = 1$), and there was a statistically significant difference compared with the MDS group (14.29% vs 0; $p = 0.005$). Seven *PTPN11* gene mutations were detected, five in the MDS/AML group and two in the MDS group (17.86 vs 2.82%; $p = 0.018$; Table 3 & Figure 3). Seven patients had *SF3B1* gene mutations, and two of them were RARS patients.

We observed that 50% (11/22) of *ASXL1* mutations were associated with *U2AF1* mutations in patients, seven of whom were diagnosed as RAEB-I, three were diagnosed as MDS/AML and one was diagnosed as RCMD. However, among the seven traceable cases, three patients underwent hematopoietic stem cell transplantation, and the remaining four patients eventually progressed to AML.

Also, we compared RA, RARS and RCMD as one disease group (refractory anemia disease group) with the RAEB group (refractory anemia with excess blasts group) and the MDS/AML group (secondary AML group). There were differences in the number of genetic mutations these patients carried ($p = 0.013$), the number of gene mutations between the refractory anemia group and the RAER group (1.4 vs 2.2; $p = 0.027$), and between the refractory anemia group and the MDS/AML group (1.4 vs 2.64; $p = 0.004$). However, there was no statistical difference between the RAEB and MDS/AML groups (2.2 vs 2.64; $p = 0.261$; Figure 4).

Discussion

We used high-throughput gene sequencing to analyze 58 gene mutation profiles in 99 patients with MDS and MDS/AML. The overall positive mutation rate was 88.89% (88/99), which is consistent with results reported in previous studies and was significantly higher than the positive rate (65.98%) of 15 genes previously detected by the Sanger sequencing platform [4,9]. High-throughput sequencing of these 58 gene targets could aid in the identification of genetic lesions in approximately 90% of patients in an economical and efficient manner. This is important for guiding patient diagnosis and prognosis stratification.

There were differences in the types of gene mutations commonly seen in the MDS/AML and MDS groups in this study. The *FLT3* and *PTPN11* genes exhibited significant differences in mutation rates between the two groups. Makishima *et al.* [2]. found that mutated genes common in MDS can be divided into two categories: the

Table 3. The frequency of mutant genes in the MDS and MDS/AML groups.

Gene mutation	MDS (n = 71)	MDS/AML (n = 28)	p-value
<i>U2AF1</i>	30.99% (22/71)	25% (7/28)	0.630
<i>ASXL1</i>	22.54% (16/71)	21.43% (6/28)	1
<i>RUNX1</i>	16.9% (12/71)	10.71% (3/28)	0.546
<i>TP53</i>	12.68% (9/71)	21.43% (6/28)	0.351
<i>NRAS</i>	12.68% (9/71)	17.86% (5/28)	0.530
<i>SETBP1</i>	12.68% (9/71)	14.29% (4/28)	1
<i>ETV6</i>	9.86% (7/71)	7.14% (2/28)	1
<i>GATA2</i>	7.04% (5/71)	14.29% (4/28)	0.266
<i>TET2</i>	8.45% (6/71)	10.71% (3/28)	0.710
<i>NPM1</i>	4.23% (3/71)	14.29% (4/28)	0.097
<i>PTPN11</i>	2.82% (2/71)	17.86% (5/28)	0.018
<i>SF3B1</i>	8.45% (6/71)	3.57% (1/28)	0.669
<i>CEBPA</i>	4.23% (3/71)	10.71% (3/28)	0.347
<i>IDH1</i>	4.23% (3/71)	10.71% (3/28)	0.347
<i>IDH2</i>	4.23% (3/71)	7.14% (2/28)	0.620
<i>WT1</i>	4.23% (3/71)	7.14% (2/28)	0.620
<i>CBL</i>	4.23% (3/71)	3.57% (1/28)	1
<i>EZH2</i>	4.23% (3/71)	3.57% (1/28)	1
<i>FLT3</i>	0	14.29% (4/28)	0.005
<i>KRAS</i>	1.41% (1/71)	10.71% (3/28)	0.067
<i>PHF6</i>	5.63% (4/71)	0	0.575

Genes mutated in fewer than four patients are not listed.
AML: Acute myeloid leukemia; MDS: Myelodysplastic syndrome.

first is associated with MDS progression to AML and consists of *FLT3*, *PTPN11*, *WT1*, *IDH1/2*, *NPM1* and *NRAS*, and the second is associated with high-risk MDS and consists of *GATA2*, *KRAS*, *TP53*, *RUNX1*, *STAG2*, *ASXL1*, *ZRSR2* and *TET2*. Our study compared gene mutations in the MDS group and the MDS/AML group and found that *FLT3* and *PTPN11* mutations in the MDS/AML group were significantly more prevalent than in the MDS group. *FLT3* mutations were only detected in MDS/AML patients, suggesting that patients with MDS who have mutations in the *FLT3* gene have a higher risk of progression to AML [10]. In addition, we found that as the disease progressed, the number of mutated genes carried in the refractory anemia, RAEB and MDS/AML disease groups increased. This suggests that the accumulation of gene mutations occurs in the development of MDS disease. At the same time, we also analyzed the composition of gene mutations in the noted disease groups. Although the frequency of gene mutation in different disease groups was different, no statistical significance was found. This may be due to the relatively small number of cases.

The splicing factor pathway is usually the most affected in MDS, but some cases in this study developed into MDS/AML, which is the main influencing factor leading to the change in mutation rate ranking. Mutations in RNA splicing factor genes were mutually exclusive, meaning they did not occur in the same individual. However, the concomitant occurrence of RNA splicing factor gene mutations has been found in some studies [2,11,12]. In this study, among 39 patients with mutations in the RNA splicing factor genes, one patient with MDS/AML had concomitant mutations in *SF3B1*, *U2AF1*, *SETBP1*, and *TET2*. Related studies have reported that *SF3B1* gene mutations can be used as a favourable prognostic indicator in MDS and are highly correlated with RARS. In this study, *SF3B1* mutations were found in all patients with RARS, further confirming that *SF3B1* is highly correlated with ring-shaped iron granulocytes [13,14].

In this study, epigenetic regulatory genes are the most common mutant genes in MDS, and their mutation rate is 41.41% (41/99) of the total patients. Among the epigenetic regulatory genes, the *ASXL1* mutation rate was the highest and often accompanied by other gene mutations. Related studies have reported that the incidence of *ASXL1* mutation in MDS is approximately 20%, suggesting a poor prognosis and easy progression to AML [15]. The mutation rate of *ASXL1* in this study was 22.22% (22/99) and of the patients with this mutation, 27.3% (6/22) progressed to AML, which is consistent with previous reports [2–4,15]. The most common concomitant

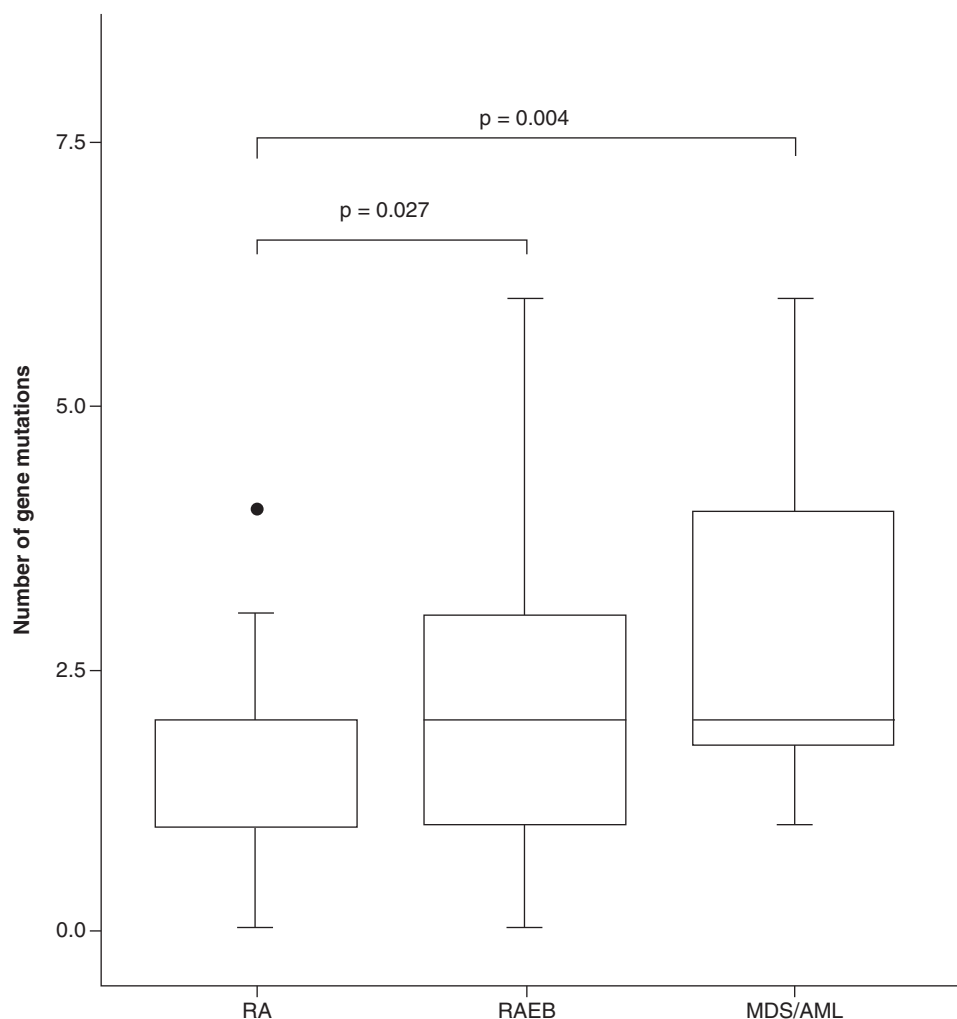


Figure 4. Number of mutations in different disease groups. The RA group includes RA, RARS and refractory cytopenia with multiple dysplasia disease subgroups. The RAEB group includes the RAEB-I and RAEB-II subgroups. AML: Acute myeloid leukemia; MDS: Myelodysplastic syndrome; RA: Refractory anemia; RAEB: Refractory anemia with excess blasts; RARS: Refractory anemia with ring sideroblasts.

association with *ASXL1* was *U2AF1* mutation [2,4,12]. We found that more than half of patients with *ASXL1* and *U2AF1* mutations progressed to AML, suggesting a poor prognosis for *ASXL1* and *U2AF1* comutations. In molecular biology, combinations of different mutations might have different prognostic effects; however, this does not include the undetected gene mutations in this study. Therefore, from the perspective of gene mutation groups, the significance and accuracy of a prognosis based on a single gene mutation are limited.

U2AF1 was the most commonly mutated gene in this study cohort; it was mutated mainly at S34 and often accompanied by trisomy 8. In some reports, the *U2AF1* gene is mutated in Asian populations more frequently than in Caucasians [11,16,17]. Whether there is synergy between the *U2AF1* mutation and the trisomy 8 chromosomal abnormality remains to be further studied. In addition to our studies, previous studies have also shown that *TP53* gene mutations are often associated with complex karyotypes [18,19]. The present study found that there is a mutual exclusion between *TP53* gene mutations and RNA splicing factor gene mutations, especially with the *U2AF1* gene mutation [20,21]. Whether the *TP53* gene mutation has synergistic lethal effects with the RNA splicing factor genes remains to be further studied.

This study found that there are certain patterns in the combinations of gene mutations in patients with MDS and MDS/AML which may be closely related to gene function and may have implications in the pathogenesis of MDS and progression to AML. However, due to the limited number of patients, we did not obtain genetic test

results of MDS/AML patients within the MDS period. In our next work, we will study the dynamic changes of gene mutations in MDS patients with AML to provide patients with more precise treatment.

Conclusion

High-throughput sequencing of these 58 genes can aid in the detection of molecular lesions with known clinical significance in approximately 90% of MDS and MDS/AML patients. In this study, the average number of mutations and signal transduction gene mutations increased with the stage of disease progression, and they may act as significant disease-promoting factors. Data also suggest that *ASXL1* and *U2AF1* may act synergistically. Interestingly, the *U2AF1* S34 mutation was associated with trisomy 8, and the *TP53* mutation and RNA splicing factor gene mutations were mutually exclusive. The mechanism is worthy of further study.

Future perspective

In future work, we will study the dynamic changes of gene mutations in patients with MDS who progress to AML. There are many types of mutated genes in MDS, and there are also numerous different gene mutation combinations. The gene mutations, gene mutation combinations and other clinical factors affect each other. The clinical significance of each gene mutation and combination thus requires more research. However, with the accumulation of relevant medical data and research, and the development and application of various targeted drugs, genetic mutation detection will help facilitate MDS precision medicine.

Summary points

- Gene mutation spectrum of myelodysplastic syndrome (MDS) patients.
- Gene mutation combinations in MDS patients.
- The gene mutation spectrum of MDS patients progressing to acute myeloid leukemia (AML).
- The difference in the number of gene mutations in different MDS subgroups.
- MDS patients with comutation of *ASXL1* and *U2AF1* have poor prognosis.
- The mutation spectrum of MDS patients may be differ among populations.
- In MDS patients, there may be a correlation between gene mutations and chromosomal abnormalities.
- MDS patients with *FLT3* gene mutations are more likely to progress to AML.

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Ethical conduct of research

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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