Mutations of *ASXL1* and *TET2* in aplastic anemia

Acquired aplastic anemia (AA), characterized by pancytopenia in peripheral blood (PB) and bone marrow (BM) hypoplasia, is a bone marrow failure syndrome. The late evolution to myelodysplastic syndromes (MDS)/acute myeloid leukemia (AML) is the most common clonal complication in refractory patients and in those who do not achieve a robust response.[1,2] The reported rates of clonal evolution varied in some studies from 1.7%-57% during an observation period of 5-11 years.[1-3] The evolution of chromosomal abnormalities including monosomy 7 has been associated with a poor prognosis, but some abnormal cytogenetics, for example, +8 and del13q, have been associated with a good response to immunosuppressive therapy (IST).[4] Single nucleotide polymorphism array karyotype abnormalities could identify those AA patients who were at risk of clonal evolution.[5] Mutations of *DNMT3A* and *BCOR* may be associated with a risk of transformation to MDS.[6] However, no reliable biomarkers that predict prognosis and MDS evolution are currently known in AA. AA has genetic instability, and acquired somatic mutations of *ASXL1, TET2, RUNX1, TP53, K-RAS* and *N-RAS* typically occurred in MDS/AML.[7-11] We postulated that these mutations might be an early event in AA evolution to MDS/AML, and could predict MDS/AML evolution and prognosis. In this study, we analyzed mutations in *ASXL1, TET2, RUNX1, TP53, K-RAS* and *N-RAS* in Chinese AA patients and showed that somatic mutations that were common in myeloid malignancies also existed in AA. Moreover, patients with different mutations showed distinct clinical and biological features.

Bone marrow aspirates were collected from 440 patients with pancytopenia between February 2012 and September 2014 at a single institution (Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Science & Peking Union Medical College). A total of 138 patients with AA diagnosed according to standard criteria[12] had complete clinical data for this study; 16 iron deficiency anemia or megaloblastic anemia patients (age range 32-77 years) were analyzed as controls. Tables 1 and 2 list the clinical and biological characteristics of these AA patients.

Table 1. Comparison of clinical and biological characteristics of 138 aplastic anemia patients according to *ASXL1* mutation.

	Total	*ASXL1* mutated	*ASXL1* wild-type	*P*
Sex, n (%)				
Male	81	9(11.1)	72(88.9)	0.654
Female	57	5(8.8)	52(91.2)	
Age, years				
Median	30.7	23.9	31.4	0.137
Range	5-76	6-44	5-76	
Age, n (%)				
5-9 years	19	3(15.8)	16(84.2)	0.184
10-29 years	55	6(10.9)	49(89.1)	
30-49 years	38	5(13.2)	33(86.8)	
>50 years	26	0(0)	26(100)	
Severity of AA				
VSAA	10	2 (20.0)	8 (80.0)	0.364
SAA	71	8 (11.3)	63 (88.7)	
NSAA	57	4 (7.0)	53 (93.0)	
Response to treatment,* n. (%)				
CR	36	4 (11.1)	32 (88.9)	1.000
PR	31	3 (9.7)	28 (90.3)	
NR	33	4 (12.1)	29 (87.9)	
Disease duration, n. (%)				
De novo	63	7 (11.1)	56 (88.9)	0.924
1-12m	19	2 (10.5)	17 (89.5)	
>12m	56	5 (8.9)	51 (91.1)	
PNH clone, n. (%)				
Positive	20	1 (5.0)	19 (95.0)	0.672
Negative	118	13 (11.0)	105 (89.0)	
Cytogenetic, n. (%)				
Normal	130	12 (9.2)	118 (90.8)	0.188
Abnormal	8	2 (25.0)	6 (75.0)	
Interventions,§ n. (%)				
CsA	42	4 (9.5)	38 (90.5)	0.412
CsA+ATG	19	3 (15.8)	16 (84.2)	
Others	14	0 (0)	14 (100)	
MDS/AML evolution,* n. (%)				
Yes	9	3 (33.3)	6 (66.7)	0.044
No	91	7 (7.7)	84 (92.3)	

*SAA: severe aplastic anemia; NSAA: non-severe aplastic anemia; VSAA: very severe aplastic anemia; CR: complete response; PR: partial response; NR: no response; CsA: cyclosporine; ATG: antithymocyte globulin; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; *38 AA patients lost to follow up and were excluded in this analysis. §63* de novo *AA patients who did not receive any treatment at sampling time were excluded this analysis.*

All patients were screened for PNH clone using the combination of FLAER with multicolor flow cytometry to detect expression of GPI-anchored proteins on peripheral blood red cells and granulocytes. Chromosome analyses were performed on unstimulated bone marrow cells after 24 h cultures using the G- and/or R-banding techniques. Somatic mutations of *TET2, ASXL1, RUNX1, TP53, N-RAS* and *K-RAS* genes were searched by direct sequencing exons and consensus splicing sites after PCR amplification of genomic DNA. Exons studied were: 1) *TET2* (reference sequence: NM_001127208.2), exons 3 and 11; 2) *ASXL1* (reference sequence: NM_015338.5), exon 12; 3) *RUNX1* (reference sequence: NM_001754), exons 3-8; 4) *TP53* (reference sequence: NM_000546.5), exons 5-8; 5) *N-RAS* (reference sequence: NM_002524.4), codon 12 and 13; 6) *K-RAS* (reference sequence: NM_004985.3), codon 12 and 13. The primers used for sequencing are listed in *Online Supplementary Table S1*. Previously annotated single nucleotide polymorphisms in the database (*http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes*) were discarded.

A total of 24 of 138 (17.4%) patients with AA were found to harbor mutations, including *ASXL1* mutations in 14 patients, *TET2* in 10 patients; no mutations were detected in *RUNX1*, *TP53*, *K-RAS* and *N-RAS*. All mutations were heterozygous, including missense (n=13), nonsense (n=8), frameshift (n=4), non-frameshift deletion (n=1), and splice site (n=1) changes (Figure 1 and *Online Supplementary Tables S2 and S3*). Comparisons of clinical and biological variables between patients with and without *ASXL1/TET2* mutations are shown in Tables 1 and 2. All *ASXL1* and *TET2* mutations were isolated. *ASXL1* mutations had different relationship with clinical features and biological characteristics compared with those of *TET2* mutations. Somatic mutations of *ASXL1* were the most frequent abnormality and were seen in 14 of 138 (10.1%) patients. Median age of patients with *ASXL1* mutations was lower than those without *ASXL1* mutations (23.9 years *vs.* 31.4 years). Patients under nine years of age had the highest incidence (3 of 19, 15.8%), but the difference was not significant (P=0.184). Also there were no significant differences between patients with or without *ASXL1* mutations in terms of sex (P=0.654), severity of AA (P=0.364), duration of disease (P=0.924), interventions (P=0.412), or response

Table 2. Comparison of clinical and biological characteristics of 138 aplastic anemia patients according to *TET2* mutation.

	Total	*TET2* mutated	*TET2* wild-type	*P*
Sex, n. (%)				
Male	81	8 (9.9)	73 (90.1)	0.155
Female	57	2 (3.5)	55 (96.5)	
Age, years				
Median	30.7	28.9	30.8	0.747
Range	5-76	7-76	5-76	
Age, n. (%)				
5-9 years	19	2 (10.5)	17 (89.5)	0.877
10-29 years	55	4 (7.3)	51 (92.7)	
30-49 years	38	3 (7.9)	35 (92.1)	
>50 years	26	1 (3.8)	25 (96.2)	
Severity of AA				
VSAA	10	2 (20.0)	8 (80.0)	0.234
SAA	71	5 (7.0)	66 (93.0)	
NSAA	57	3 (5.3)	54 (94.7)	
Response to treatment*, n. (%)				
CR	36	6 (16.7)	30 (83.3)	0.013
PR	31	1 (3.2)	30 (96.8)	
NR	33	0 (0)	33 (100.0)	
Disease duration, n. (%)				
De novo	63	4 (6.3)	59 (93.7)	0.813
1-12m	19	2 (10.5)	17 (89.5)	
>12m	56	4 (7.1)	52 (92.9)	
PNH clone, n. (%)				
Positive	20	3 (15.0)	17 (85.0)	0.327
Negative	118	7 (5.9)	111 (94.1)	
Cytogenetic, n. (%)				
Normal	130	10 (7.7)	120 (92.3)	1.000
Abnormal	8	0 (0)	8 (100.0)	
Interventions,§ n (%)				
CsA	42	1 (2.4)	41 (97.6)	0.008
CsA+ATG	19	5 (26.3)	14 (73.7)	
Others	14	0 (0)	14 (100.0)	
MDS/AML evolution,* n. (%)				
Yes	9	1 (11.1)	8 (88.9)	0.543
No	91	7 (7.7)	84 (92.3)	

*SAA: severe aplastic anemia; NSAA: non-severe aplastic anemia,: VSAA: very severe aplastic anemia, CR:complete response, PR: partial response, NR: no response, CsA: cyclosporine,ATG: antithymocyte globulin, MDS: myelodysplastic syndrome, AML: acute myeloid leukemia, *-38 AA patients lost to follow up and were excluded in this analysis. §-63 de novo AA patients who did not receive any treatment at sampling time were excluded in this analysis.*

to treatment (*P*=1.000) (Table 1). *TET2* mutations were seen in 10 of 138 (7.3%) patients, and were closely associated with prior interventions. Mutations were detected in 5 of 19 (26.3%) patients who had received antithymocyte globulin (ATG)-based IST, compared with patients receiving cyclosporine (CsA)-based IST (1 of 42, 2.4%) and others (0 of 14, 0%); these differences were significant (*P*=0.008). *TET2* mutations also were closely related with a good response. The frequency of *TET2* mutations in patients with CR (6 of 36, 16.7%) was higher than those with PR (1 of 31, 3.2%) or NR (0%, 0 of 33); this difference was also significant (*P*=0.013). There was no significant difference between patients with or without *TET2* mutations in terms of age (*P*=0.877), sex (*P*=0.155), severity of AA (*P*=0.234), or duration of disease (*P*=0.813) (Table 2).

Of 138 patients, the PNH clone was negative in 118 patients, positive in 20 (14.5%); 10 were detected at diagnosis, and after diagnosis in the other 10. The metaphase cytogenetic karyotype was normal in 130 patients, trisomy 8 (n=3), del (13) (q12-q21) (n=2), monosomy 7 (n=1), 16qh+ (n=1) and complex karyotype (n=1) were detected in the remaining 8 patients (*Online Supplementary Table S4*). *ASXL1* and *TET2* mutations had no relationship with the PNH clone (*P*=0.672 and *P*=0.327, respectively) (Tables 1 and 2). *ASXL1* mutation were detected in 2 of 8 (25%) patients with abnormal cytogenetics, compared with patients with normal cytogenetics (12 of 130, 9.2%), but the difference was not significant (*P*=0.188) (Table 1), possibly because of the limited number of cases. Surprisingly, *TET2* mutation had no relationship with abnormal cytogenetics (*P*=1.000), and was not detected in any patients with an abnormal cytogenetic profile (Table 2).

Of the 100 AA patients who had complete clinical data available for analysis in evolution to MDS, progression to MDS was seen in 9 patients (*Online Supplementary Table S5*). The median time of progression to MDS was 36.3 months (range 7-86 months), and in 6 of 9 patients occurred less than three years from diagnosis. Seven of 9 patients received CsA-based IST, the others ATG-based IST; 2 patients who evolved to MDS subsequently progressed to AML. The cytogenetic karyotype at the time of evolution was normal (n=5), monosomy 7 (n=1), trisomy 8 (n=1), del(13)(q12-q22) (n=1), and complex karyotype (n=1), respectively. The frequency of *ASXL1* mutations was higher in patients who progressed to MDS than those who did not (3 of 9, 33.3% *vs.* 7 of 91, 7.7%; *P*=0.044) (Table 1). AA patients with *ASXL1* mutations had a greater risk of transformation to MDS in univariate analysis (*P*=0.014) (*Online Supplementary Figure S1A*). Surprisingly, *TET2* mutations had no relationship with evolution to MDS in univariate analysis (*P*=0.464) (*Online Supplementary Figure S1B*); 1 of 9 (11.1%) patients with *TET2* mutation evolved to MDS, compared to 7 of 91 (7.7%) patients without *TET2* mutation, although this difference was not significant (*P*=0.543) (Table 2).

ASXL1 and *TET2* mutations have been reported in various myeloid malignancies,[7-11] especially MDS/AML. Evolution of AA to MDS/AML is a serious and common long-term complication.[1-2,13] Little has been known about somatic mutations in AA until now. In this study, we found mutations in epigenetic regulator genes including *TET2* and *ASXL1* in 17.4% patients with AA. Mutations were detected at any stage of disease. The rate of mutation was similar to those reported in AA patients in the UK.[6] *ASXL1* mutations were the most common mutation in AA, and were associated with a transformation to MDS. Meanwhile, *ASXL1* mutations were relatively more frequently found in patients with abnormal cytogenetics compared with patients with normal cytogenetics. Kulasekararaj *et al.*[6] also found that 7 of 12 AA patients with *ASXL1* mutations showed progression to MDS and were associated with 40% risk of transformation to MDS. In their report, *ASXL1* mutation occurred more frequently in older patients, but we found *ASXL1* mutation was more common in younger Chinese patients and those under nine years of age had the highest incidence. For the first time, *TET2* mutations were found in AA. Surprisingly we found that AA patients with *TET2* mutations had a better response to IST than those without mutations. The advantage resulted at least in part from the fact that *TET2* mutations occurred more frequently in patients who had received ATG-based IST, and that *TET2* mutations were not associated with abnormal cytogenetics and had no adverse effect on transformation to MDS. In addition, *TET2* mutations were also associated with longer survival, lower risk of transformation to AML, and a molecular marker for good prognosis in patients with MDS.[14,15] *TET2* mutations may have a good prognostic implication in AA, but this result needs to be further confirmed with larger series of patients.

In summary, we identified *TET2* and *ASXL1* mutations in Chinese patients with AA. These important data may predict disease outcomes in AA patients of diverse genetic backgrounds.

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