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

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doi:10.3324/haematol.2014.120931

SUPPLEMENTARY INFORMATION

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DNA sequencing

Genomic DNA was extracted from frozen bone marrow samples using the PureLink^{tm} Genomic DNA Kits (Invitrogen Life Technologies). PCR amplifications was performed using an Applied Biosystems® GeneAmp® PCR System 9700 in a total volume of 25μL PCR mix containing 20ng template DNA, 200nM each deoxynucleoside triphosphate, 1.6mM magnesium sulfate, 200nM each forward and reverse primers, buffer and 1U of Platinum® Taq (Invitrogen Life Technologies). PCR amplification conditions were as of follows, initial denaturation was at 94℃ for 10 minutes followed by 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 56℃ for 30 seconds, and extension at 72℃ for 30 seconds. All PCR products were confirmed by 1.5% agarose gel, purified using Exonuclease I and Shrimp Alkaline Phosphatase (Thermofisher). Two microliter of the purified PCR products was used for sequencing using the Big Dye terminator v 3.1 kit (Applied Biosystems) including the forward or reverse primer. Sequence was loaded on an ABI 3730XL automat (Applied Biosystems).

Statistics

Kolmogorov-Smirnow test and homogeneity of variance test were used to analyse if the continuous data were normally distributed and homoscedasticity while comparing the patients with or without mutation. Numerical variables were summarized by median, 95% confidence interval and range. The chi-square test and t-test were used to calculate the significance of association between ASXL1/TET2 mutations and

other parameters, including sex, phage, PNH clone, Response to IST, cytogenetics, MDS/AML evolution and age; Fisher exact test was used if any expected value of the contingency table was <5. Survival curves were generated using the Kaplan-Meier method and differences were assessed by Log-Rank analysis. A *P* value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS 17 software.

Supplementary Table S1: Oligonucleotide primers used for sequencing the coding regions of the ASXL1, TET2, RUNX1, TP53, K-RAS and N-RAS genes.

amplicon	sequence oligonucleotide Forward 5'-3'	sequence oligonucleotide Reverse 5'-3'	**PCR (bp)**
N-RAS code 12/13	AGAACCAAATGGAAGGTCACAC	TTCAGCAGCTAATAAAAATGAACTGT	420
K-RAS code 12/13	CTTAAGCGTCGATGGAGGAG	CCCTGACATACTCCCAAGGA	544
TP53 exon 5	AAGCTCCTGAGGTGTAGACGC	AGGCCCTTAGCCTCTGTAAGC	722
TP53 exon 6	AAAAAGGCCTCCCCTGCTTGC	AGAAATCGGTAAGAGGTGGGC	276
TP53 exon 7/8	GTTGGGAGTAGATGGAGCCTG	TTGTCTTTGAGGCATCACTGC	526
ASXL1 exon 12 PCR1	CCGGCTTGAAGATCGTCAGT	AGGCTGCTCCACTAATCTCT	1232
ASXL1 exon 12 PCR2	AGAGGACCTGCCTTCTCTGA	GGCTCAACAGATGGTATGTG	618
ASXL1 exon 12 PCR3	GGAACTGGCCAAGCTCTTGA	TCCTTGTCCACCGAAGATCC	620
ASXL1 exon 12 PCR4	ACTGAGTCCTCACGGTGAGT	CAGCTATCTGGCAGAAGAGG	573
ASXL1 exon 12 PCR5	CCACGATGACAGCATGTCAG	CATTCTTGACGCTGCCAACA	734
ASXL1 exon 12 PCR6	ATGCCTCTTCCTGCTGAGAT	GTGCTCCTGCCTAAAGAGTA	772
RUNX1 exon 3	AACCACGTGCATAAGGAACA	TTGGAATCAGCAGAAACAGC	372
RUNX1 exon 4	GAGCTGCTTGCTGAAGATCC	CATCCCAAGCTAGGAAGACC	496
RUNX1 exon 5	CATTGCTATTCCTCTGCAACC	CCGAGTTTCTAGGGATTCCA	393
RUNX1 exon 6	AAATTCCGGGAGTGTTGTCA	GCAACTTTTTGGCTTTACGG	382
RUNX1 exon 7	GGGAGAGAGAGGGGAAAGAC	AGTTGGTCTGGGAAGGTGTG	407
RUNX1 exon 8	ACCCTGGTACATAGGCCACA	AGCCACTTCTGCCTTCACAT	494
TET2 exon 3 PCR1	AGCTGTCAGTTGTCACTATG	CCTCCTGCTCATTCAGAATC	773
TET2 exon 3 PCR2	AGTCGTGTGAGTCCTGACTT	TCTGTGCGGAATTGATCTGC	622
TET2 exon 3 PCR3	GCCATTAACAGTCAGGCTAC	GGTAGTGGTGGTGTTCTTCT	562
TET2 exon 3 PCR4	GCACTCTGAATGGTGGAGTT	GGAGATGTTGGTCCACTGTA	684
TET2 exon 3 PCR5	CAACACAGCTGGAGCACAAG	GTGACTTCTGCTCCTGTTCT	801
TET2 exon 3 PCR6	CAAGCGAGTTCGAGACTCAT	CTGACTATGGCAAGACTCAG	548
TET2 exon 3 PCR7	GCTGCTCTAAGGTGGCATCT	ACAAATTGCTGCCAGACTCA	675
TET2 exon 11 PCR1	TATCTTTGCTTAATGGGTGTC	GAAGTGGCCATCCATCTCAT	795
TET2 exon 11 PCR2	GCCGATGGATCTGTATAGGT	AAGCTCTGCTCGCTGTCTGA	600
TET2 exon 11 PCR3	AGCAGCCATTGGCACTAGTC	ATGCCATCTGTGACCACTTG	735

Supplementary Table S2: The mutation patterns, clinical and biological features in 14 AA patients with ASXL1 mutations

UPN	Sex/age (years)	Diagnosis	PNH clone	karotype	ASXL1			Prognosis
					n.t. change	a.a. change	variant class	
43#	M/6	SAA	_	normal	c.2574G>T	p.Gln858His	missense	NR(12m)
64#	M/44	NSAA	_	normal	c.3757A>G	p.Ser1253Gly	missense	missing
93#	F/42	NSAA	_	normal	c.4353-4356 insC	p.Thr1452His fs*4	frame-shift	NR(12m)
94#	M/42	SAA	_	normal	c.2980C>T	p.Pro994Ser	missense	missing
96	F/8	SAA	_	normal	c.3693C>U	p.Ser1231=	nonsense	PR
139#	M/24	NSAA	_	normal	c.3759T>C	p.Ser1276=	nonsense	missing
217	F/16	SAA	_	normal	c.4344G>T	p.Gln1448=	nonsense	CR
246#	F/9	VSAA	_	normal	c.2629G>T	p.Glu877*	stopgain	NR(3m)/HSCT
282	F/22	SAA	_	normal	c.2640A>G	p.Thr880Ala	missense	PR
286#	M/31	NSAA	-	normal	c.3702G>A	p.Gln1234=	nonsense	PR
308	M/19	SAA	-	normal	c.2423C> A	p.Pro808His	missense	NR/Evolution to MDS
					c.2465-2466 insA	p.Leu823Ile fs*10	frame-shift	
					c.2495A> G	p.Asp832Gly	missense	
367	M/14	VSAA	-	-7	c.3098A>T	p.Glu1033Val	missense	NR/Evolution to MDS
400	M/37	SAA	-	normal	c.3827C>U	p.Ser1276Phe	missense	NR/Evolution to MDS
421	M/21	SAA	+	del（13）（q12-q22）	c.4521G>A	p.Ala1507=	nonsense	CR

UPN-unique patient number, SAA-severe aplastic anemia, NSAA-non-severe aplastic anemia, VSAA-very severe aplastic anemia, MDS- myelodysplastic syndrome, HSCT- haematopoietic stem cell transplantation, n.t. change-CCDS sequence change, a.a. change-protein sequence change. CR-complete response, PR-partial response, NR-no response. #-ASXL1 mutation was detected at diagnosis and followed for 12-18m.

Supplementary Table S3: The mutation patterns, clinical and biological features in 10 AA patients with TET2 mutations

UPN	Sex/Age (years)	Diagnosis	PNH clone	karotype	TET2			Prognosis
					n.t. change	a.a. change	Variant class	
27#	M/76	SAA	-	normal	c.828-829insA	p.Ala277Ser fs*7	frame-shift	NR(12m)/Evolution to MDS
					c.1560-1561 insGGT	p.Gly520_Ser 521 insGly	frame-shift	
					c.4630C>A	p.Pro1544Thr	missense	
160#	M/10	NSAA	-	normal	c.651C>U	p.Ser217=	nonsense	missing
207#	F/48	NSAA	-	normal	c.936U>C	p.Asn312=	nonsense	CR
238#	M/24	NSAA	-	normal	c.1058G>A	p.Cys353Tyr	missense	CR
301	M/43	SAA	+	normal	c.4627_4644 del	p.Arg1543_Gln 1548 del	nonframe-shift deletion	CR
331	M/18	SAA	+	normal	c.651C>U	p.Ser217=	nonsense	CR
345	M/19	VSAA	-	normal	c.1972C>T	p.His658Tyr	missense	CR
403	M/37	VSAA	+	normal	c.597A>G	p.Leu199=	nonsense	CR
427	F/7	SAA	-	normal	c.2429A>G	p.Gln810Arg	missense	PR
429	M/7	SAA	-	normal	c.2440C>T	p.Arg814Cys	missense	CR

UPN-unique patient number, SAA-severe aplastic anemia, NSAA-non-severe aplastic anemia, VSAA-very severe aplastic anemia, MDS- myelodysplastic syndrome, n.t. change-CCDS sequence change, a.a. change-protein sequence change. CR-complete response, PR-partial response, NR-no response. #-ASXL1 mutation was detected at diagnosis and followed for 12-18m.

Supplementary Table S4: Characteristics of AA patients with abnormal cytogenetic or PNH clone.

UPN	Sex/age (years)	Diagnosis (at presentation)	karotype	PNH clone	ASXL1	TET2	Prognosis
1	F/22	SAA	del(13)(q12-q22)	-	wildtype	wildtype	CR
41	M/17	NSAA	8	-	wildtype	wildtype	evolution to H-MDS
46	M/17	SAA	8	-	wildtype	wildtype	NR
81	F/7	SAA	+X，+X，-7，+10	-	wildtype	wildtype	evolution to H-MDS
367	M/14	VSAA	-7	-	mutant	wildtype	evolution to H-MDS
393	M/15	SAA	16qh+	-	wildtype	wildtype	missing
398	F/53	SAA	8	-	wildtype	wildtype	PR
421	M/21	SAA	del(13)(q12-q22)	+	mutant	wildtype	evolution to H-MDS
8	M/66	SAA	normal	+	wildtype	wildtype	PR
22	M/76	NSAA	normal	+	wildtype	wildtype	missing
28	M/67	NSAA	normal	+	wildtype	wildtype	missing
55	F/42	NSAA	normal	+	wildtype	wildtype	missing
215	F/18	NSAA	normal	+	wildtype	wildtype	PR
223	F/25	SAA	normal	+	wildtype	wildtype	CR
225	M/21	SAA	normal	+	wildtype	wildtype	CR
227	M/40	SAA	normal	+	wildtype	wildtype	missing
228	F/50	NSAA	normal	+	wildtype	wildtype	CR
235	M/51	NSAA	normal	+	wildtype	wildtype	CR
284	F/22	SAA	normal	+	wildtype	wildtype	CR
315	F/28	NSAA	normal	+	wildtype	wildtype	PR
333	F/21	SAA	normal	+	wildtype	wildtype	NR
391	M/44	SAA	normal	+	wildtype	wildtype	missing
415	F/26	SAA	normal	+	wildtype	wildtype	missing
437	M/62	SAA	normal	+	wildtype	wildtype	PR
301	M/43	SAA	normal	+	wildtype	mutant	CR
331	M/18	SAA	normal	+	wildtype	mutant	CR
403	M/37	VSAA	normal	+	wildtype	mutant	CR

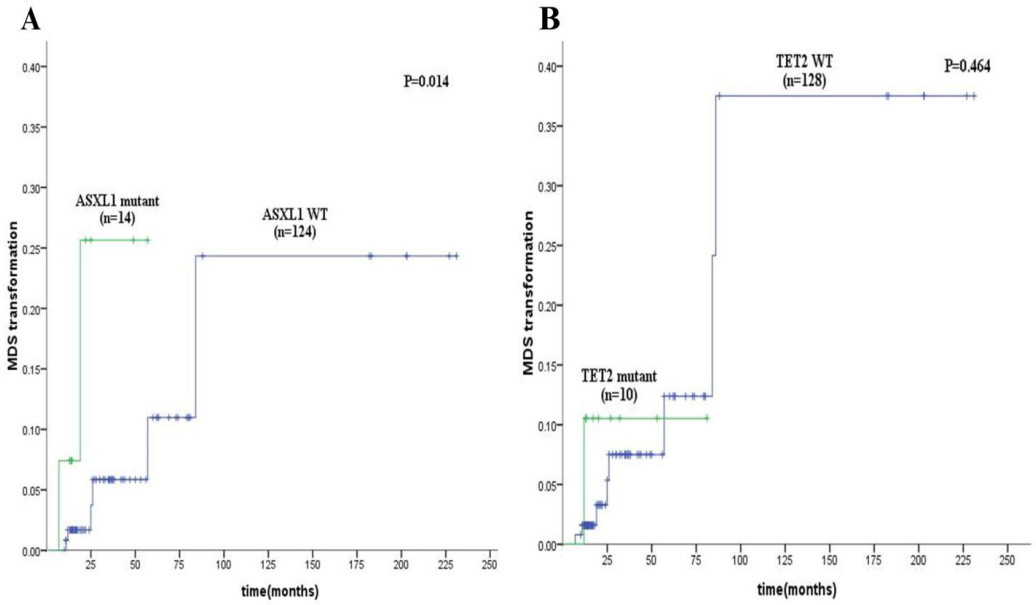
UPN-unique patient number, SAA-severe aplastic anemia, NSAA-non-severe aplastic anemia,VSAA-very severe aplastic anemia, H-MDS-hypoplastic myelodysplastic syndrome, CR-complete response, PR-partial response, NR-no response.

Supplementary Table S5: Characteristics of 9 AA patients who evolved to MDS

UPN	Sex/age (years)	Diagnosis	PNH clone	Karotype (at evolution)	ASXL1	TET2	treatment	Transformation /duration (months)
3#	M/40	SAA	-	normal	wildtype	wildtype	CsA+androgen+G-CSF	MDS-RAEB-Ⅱ/25
27	M/76	SAA	-	normal	wildtype	mutant	CsA+androgen	H-MDS/12
41	M/17	NSAA	-	+8	wildtype	wildtype	CsA+androgen	H-MDS/84
81#	F/7	SAA	-	+X,+X,-7,+10	wildtype	wildtype	CsA+androgen+ATG+G-CSF	H-MDS/11
258	M/22	SAA	-	normal	wildtype	wildtype	CsA+androgen	MDS-RCMD/57
333	F/21	SAA	+	normal	wildtype	wildtype	CsA+androgen+G-CSF	MDS-RCMD/26
367	M/14	VSAA	-	-7	mutant	wildtype	CsA+androgen+ATG+G-CSF	H-MDS/7
400	M/37	SAA	-	normal	mutant	wildtype	CsA+androgen+G-CSF	H-MDS/19
421	M/21	SAA	+	del（13）（q12-q22）	mutant	wildtype	CsA+androgen+G-CSF	H-MDS/86

UPN-unique patient number, SAA-severe aplastic anemia, NSAA-non-severe aplastic anemia,VSAA-very severe aplastic anemia, CsA-ciclosporin, ATG-antithymocyte globulin, G-CSF-granulocyte colony-stimulating factor, H-MDS-hypoplastic myelodysplastic syndrome, MDS-RCMD-myelodysplastic syndrome-refractory cytopenia with multilineage dysplasia, MDS-RAEB- myelodysplastic syndrome-refractory anemia with excess of blasts.

Figure S1



Supplementary Figure S1： Kaplan-Meier curves for time to MDS transformation. (A) Risk to MDS transformation in AA patients with ASXL1 mutated (n=14) and wildtype ASXL1 (n=124), AA patients with ASXL1 mutations had a greater risk of transformation to MDS (log-rank test, *P*=0.014). (B) Risk to MDS transformation in AA patients with TET2 mutated (n=10) and wildtype ASXL1 (n=128), TET2 mutations had no relationship with transformation to MDS (log-rank test, *P*=0.464).