의학박사학위논문

Telomere Length and Somatic Mutations in Correlation with Response to Immunosuppressive Treatment in Aplastic Anemia

재생불량빈혈에서 텔로미어 길이와 체세포 돌연변이에 따른 면역억제제치료 반응과의 상관관계에 관한 연구

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박 희 수

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Telomere Length and Somatic Mutations in Correlation with Response to Immunosuppressive Treatment in Aplastic Anemia

by

Hee Sue Park

A thesis submitted to the Department of Laboratory Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine at Seoul National University College of Medicine, Seoul, Korea

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ABSTRACT

Telomere Length and Somatic Mutations in Correlation with Response to Immunosuppressive Treatment in Aplastic Anemia

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We investigated the frequencies of cytogenetic aberrations and somatic mutations of prognostic relevance in 393 patients with aplastic anemia (AA). Clonality was determined by G-banding/fluorescence in situ hybridization (FISH) (n = 245), and targeted capture sequencing was performed for 88 hematopoiesis-related genes (n = 70). The telomere length (TL) of bone marrow nucleated cells was measured at the single cell level by FISH (n = 135). Eighteen (4.6%) patients showed disease progression, and monosomy 7 (50.0%) was the most predominant cytogenetic evolution at disease transformation. One

third of patients (32.9%) presented at least 1 mutation; the most frequently mutated genes were *NOTCH1*, *NF1*, *SCRIB*, *BCOR* and *DNMT3A*. The patient group with clonal changes (30.7%) showed an adverse response to immunosuppressive treatment (IST), compared to the non-clonal group, but this finding did not show statistical significance. The TL of AA patients was significantly shorter than normal control and patients with clonal changes showed significantly shorter TLs. Patients with TL>5.9 showed a higher response rate to IST (P = 0.048). In conclusion, the patients with clonal changes or TL attrition showed a poor response to IST. Shorter TL can be used not only as a biomarker, but also as a predictive marker for treatment response to IST.

Keywords: aplastic anemia, telomere length, somatic mutations, immunosuppressive treatment

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LIST OF ABBREVIATIONS

AA	Aplastic anemia
Alt.	Alternative sequence
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
BM	Bone marrow
BMT	Bone marrow transplantation
CA/SM	Cytogenetic aberrations and/or somatic mutations
CBC	Complete blood count
CEP	Centrometric eunumeration probes
Chr	Chromosome
FISH	Fluorescent *in situ* hybridization
Hb	Hemoglobin
IST	Immunosuppressive therapy
MDS	Myelodysplastic syndrome
PNH	Paroxysmal nocturnal hemoglobinuria
Ref.	Reference sequence
SNV	Single nucleotide variant
TL	Telomere length
VAF	Variant allele frequency
WBC	White blood cell
WHO	World Health Organization

1. INTRODUCTION

Aplastic Anaemia (AA) is a heterogeneous disease group that exhibits the combination of pancytopenia of the peripheral blood and hypocellular marrow (Guinan, 2011). The underlying causes of AA include damage to hematopoietic stem cells (HPC) due to exposure to toxic drugs or chemicals, and the autoimmune system attacking HPC (Young, 2002). Notably, AA is a well-known pre-malignant disease that can evolve into paroxysmal nocturnal hemoglobinuria (PNH), myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). Damaged HPC in patients with AA is more susceptible to mutations, which can lead to clonal proliferation of hematopoietic cells (Ogawa, 2016).

Epidemiologically, AA is a rare disease. From the point of ethnic difference, the incidences of AA show geographic variation. The estimated incidence is approximately two cases per million in Europe and the incidence rate is 4 - 5 times higher in Asians than Caucasians (Lee et al, 2001b; Kaufman et al, 2006; Young & Kaufman, 2008). In Korea and Japan, AA has an age-adjusted incidence of 11.0 per million populations per year, while it is 2.2 per million in Caucasians (Storb, 1997). However, the occurrence of AA in Korea has decreased significantly over the past few years. Given that official epidemiological data are not extractable

in Korea, because of the absence of a national registry, we assessed the proportion of AA patients among patients who visited Seoul National University Hospital (SNUH), and the proportion of AA has decreased 4.5-fold, from 0.0045% (39/858,744 patients) in 1997 to 0.0001% (21/2,163,861 patients) in 2015 (Annual Report, published by Seoul National University Hospital).

Paradoxically, the rate of malignant evolution is lower in Asians, although the incidence rate of AA in Asians is higher than that in Caucasians (Young et al, 2006): 8% in Germany, about 11% in US and Europe, and about 6% in Japan (Young et al, 2006). It is well known that AA with cytogenetic clonal changes, such as monosomy 7, frequently transform into AML (Maciejewski et al, 2002). Somatic mutations in AA patients, such as *DNMT3A*, *ASX1* and *BCOR*, are similar to those found in myeloid malignancies; these genes are also related to clonal hematopoiesis of aged people, and are considered as clonal hematopoiesis of intermediate significance (Kulasekararaj et al, 2014; Yoshizato et al, 2015). Consequently, the detection of clonality in AA would be a determining point for the exclusion of immunosuppressive therapy (IST).

It is well known that telomere lengths (TLs) in patients with AA are significantly shorter than those in normal controls (Brümmendorf et al, 2001; Lee et al, 2001a). There has been controversy regarding whether shorter TL is as a predictive

marker for treatment response to IST or clonal evolution. Brümmendorf et al. (2001) and Lee et al (2001a) reported that TL in AA patients who had recovered after IST did not differ from controls, whereas non-responders after IST showed significant telomere shortening (Brümmendorf et al, 2001, Lee et al, 2001a.) In a National Institutes of Health (NIH) study, TL was unrelated to response, but was associated with a risk of relapse, clonal evolution and overall survival in severe AA (Cooper et al, 2008; Scheinberg et al, 2010). Meanwhile Sakaguchi et al (2014) reported that shorter TL is associated with adverse response to IST in childhood AA. Another report described accelerated telomere attrition to be characteristic of early myeloid oncogenesis, specifically chromosome 7 loss (Dumitriu et al, 2015).

We aimed to investigate the frequencies of chromosomal clonality and somatic mutations of hematopoietic genes in AA, and their correlation with prognostic relevance and response to immunosuppressive treatment. Due to limitations of G-banding, which cannot detect the cytogenetic changes in cells with lo w mitotic activity, we performed molecular cytogenetic study using interphase fluorescence *in situ* hybridization (iFISH) in combination with G-banding. To detect somatic mutations in hematopoietic-related genes, we performed targeted capture sequencing for 88 hematopoiesis- related genes. Also we assessed the correlation between TL and clonal changes (cytogenetic aberrations or somatic mutations) and tried to find

the threshold of TL that can predict treatment response to IST.

2. Materials and Methods

2.1. Patients

Three hundred and ninety-three patients who were diagnosed with AA and treated at Seoul National University Hospital between January 1997 and December 2013 were included in this study. The inclusion criteria for this study were availability of bone marrow (BM) samples collected at the time of diagnosis or at revisit. At least two of the following must be present for a diagnosis of AA: white blood cell count $\leq 3.5 \times 10^9$/l, platelet count $\leq 50 \times 10^9$/l, and either a hemoglobin concentration ≤ 100 g/l or hematocrit $\leq 30\%$ with a reticulocyte count of $\leq 30 \times 10^9$/l (Kramer et al, 1987). The severity of the disease was graded according to the blood count parameters and bone marrow findings (Camitta et al, 1975; Cooper et al, 2008). To define severe AA, BM cellularity must be <25%, or 25 - 50% with <30% residual hematopoietic cells and at least two of the following: neutrophil count $<0.5 \times 10^9$/l, platelet count $<20 \times 10^9$/l, or reticulocyte count $<20 \times 10^9$/l (Camitta et al, 1975).

To evaluate clinical course according to cytogenetic aberration by G-banding/fluorescence in situ hybridization (FISH) or somatic mutations, we selected 210 patients who were followed-up for more than 8 months (Table 1). They consisted of 105 males and

105 females (median age: 42 years, range 20 - 83). Thirty of these patients had severe AA, 153 had non-severe AA, and 27 patients had very severe AA. The following laboratory and clinical information was obtained for each patient: date of diagnosis and start of therapy, age, sex, ethnicity, hemoglobin level, absolute neutrophil count (ANC), white blood cell count, platelet count and the conventional cytogenetic analyses of BM cells by G-banding. Patients were followed up to December 2013 for disease progression, survival and response to treatment. All BM samples were collected with informed consent, and the study was

reviewed and approved by the Institutional Review Board of Seoul National University College of Medicine (IRB 1504-081-665).

Table 1. Clinical characteristics of 210 patients with AA followed up at least 8 months

Characteristics	Total (n=210)		AA with normal karyotype (n=180)		AA with cytogenetic aberrations and/or somatic mutation (n=30)		*p*-value
Age at diagnosis, years*							
	42	(30-53)	42	(30-54)	42	(33-53)	0.96
Sex, n (%)							
Male	105	(50.0)	91	(50.6)	14	(46.7)	0.42
Female	105	(50.0)	89	(49.4)	16	(53.3)	
Initial CBC at diagnosis*							
Hb (g/l)	83	(67 -97)	83	(65-97)	85	(68-107)	0.53
WBC ($\times 10^9$/l)	2.3	(1.53-3.0)	2.3	(1.48-2.99)	2.44	(1.81-3.27)	0.26
Platelets ($\times 10^9$/l)	30	(18-56)	30	(18-55)	29	(19-65)	0.54
ANC ($\times 10^9$/l)†	0.86	(0.45-1.32)	0.85	(0.46-1.23)	0.99	(0.41-1.5)	0.42
BM cellularity (%)							0.81
<10%	149	(71.0)	128	(71.1)	21	(70.0)	
10-20%	28	(13.3)	23	(12.8)	5	(16.7)	
>20%	33	(15.7)	29	(16.1)	4	(13.3)	
Severity (%)							0.91
Very severe	27	(12.9)	23	(12.8)	4	(13.3)	
Severe	30	(14.3)	25	(13.9)	5	(16.7)	
Non-severe	153	(72.9)	132	(73.3)	21	(70.0)	
Treatment (%)							0.39
BMT	30	(14.3)	27	(15.0)	3	(10.0)	
IST	65	(31.0)	57	(31.7)	8	(26.7)	
BMT after IST	25	(11.9)	23	(12.8)	2	(6.7)	

Supportive care	90	(42.9)	73	(40.6)	17	(56.7)	
PNH clone††							0.48
Positive	15	(14.2)	13	(14.0)	2	(16.7)	
Negative	90	(85.8)	80	(86.0)	10	(83.30	

* The values presented as the median and interquartile range in parentheses.
† Results of ANC were missing in 3 patients.
†† Results of PNH flow cytometry was available in 105 patients.
Abbreviations: Hb, hemoglobin; WBC, white blood cell; ANC, absolute neutrophil count, BM; bone marrow; BMT, bone marrow transplantation; IST, immunosuppressive therapy; PNH, paroxysmal nocturnal hemoglobinuria

2.2. BM histological examination

Two hematopathologists reviewed Wright-stained BM smears and hematoxylin and eosin-stained sections of BM trephine biopsies. The percentages of blasts, presence of ring sideroblasts and morphological dysplasia in each hematopoietic lineage were determined in BM smears. In BM sections, the infiltration of blasts and number of megakaryocytes were determined. Immunohistochemical staining CD34, CD117 and CD61 (all from Dako, Glostrup, Denmark) was performed for differential diagnosis of hypoplastic MDSs.

2.3. Cytogenetic analysis by G-banding

In 232 of 393 patients, cytogenetic studies using standard G-banding techniques on heparinized BM samples were performed as part of the diagnostic work-up. At least 20 metaphases were analysed whenever possible. Clonal abnormalities were defined as two or more cells with the same chromosomal gain or structural rearrangement, or at least three cells with the same chromosome deletion. Karyotypes were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN) 2013 (Shaffer et al, 2013).

2.4. FISH for -5/5q-, -7/7q-, +8, -20/20q-, and +1/1q+

In 199 of 393 patients, iFISH analyses (-5/5q-, -7/7q-, +8, -20/20q-, and +1/1q+) were performed on mononuclear cells of bone marrow aspirates, using Vysis LSI EGR1 (5q31) probe, Vysis LSI D7S522 (7q31) probe, Vysis CEP 8 probe, Vysis LSI Trisomy 1q (1q25) probe and Vysis LSI D20S108 (20q12) probe (Abbott Downers Grove, IL, USA), according to the manufacturer's instructions. Of the 232 patients with pre-existing G-banding results, FISH studies for -5/5q-, -7/7q-, +8, -20/20q-, and +1/1q+ were performed in 186 patients. Additionally, 43 MDS patients and 70 AML patients who were newly diagnosed at SNUH between 2010 and 2012 were enrolled for comparison.

2.5. Quantitative measurement of telomere length using interphase FISH (Q-FISH)

Q-FISH was performed using the Cy3-labelled Telomere peptide nucleic acid (PNA) FISH kit (DakoCytomation Denmark A/S, Glostrup, Denmark) and a fluorescein isothiocyanate (FITC)-labelled PNA probe for the centromere of chromosome 2 (kindly provided by Dako). An aliquot (1 μl) of the chromosome 2 centromere probe was added to 9 μl of the telomere probe. The telomere and centromere Q-FISH hybridizations were performed according to the manufacturer's instructions. Interphase Q-FISH images were captured using a Zeiss Axioplan 2 imaging microscope (Zeiss, Jena, Germany) equipped with the ISIS software (MetaSystems, Altlussheim, Germany). To measure TL, the ISIS-Telomere module (MetaSystems) was used, as previously described (Kim et al, 2015). The software calculates a telomere/centromere (T/C) fluorescence intensity ratio, which is a measure of the TL, for each individual chromosome arm within each metaphase and interphase nucleus (Narath et al, 2005). The T/C ratio was multiplied by 100 and used as the TL. At least 100 interphase nuclei were scanned for each patient. We used normal white blood cells from 147 healthy individuals for comparison.

2.6. Targeted capture sequencing

We selected 88 genes related to hematologic malignancies for target sequencing (Table S1). The shearing of genomic DNA to generate the standard library and the hybridization step, targeting only exonic regions, were performed by Celemics Inc. (Seoul, Korea). The final quality was assessed by analysis on the Agilent 2200 TapeStation system (Agilent, Santa Clara, CA, USA). We sequenced a total target length of 259-kb regions with the paired-end 150-bp rapid-run sequencing mode on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). We achieved over 109 coverage for greater than 97% of targeted regions for each sample. The mean sequencing depth for the targeted regions (259 kb) was 231-fold. Taking into consideration that a matched control sample was not included in this study, we applied a stringent variant selection pipeline to prioritize the high-confidence set of somatic mutations.

2.7. Variant calling

Fastq files from targeted exon sequencing results were aligned to the human reference genome (hg19, GRCh37) by Burrows - Wheeler Aligner (BWA, v062, http://bio-bwa.sour ceforge.net/). Polymerase chain reaction (PCR) duplicate read was removed using Picard 198 (https://broadinstitute. github.io/picard/) and variant was called using 'UnifiedGenotyper' in GATK 27-2 https://software.broadinstitute.org/gatk/). For filtering the low quality variants, variants with a low total depth (<20) and with a low altered allele count (<10) were discarded. Detected single nucleotide variants (SNVs) and insertions/deletions (Indels) were analysed based on the algorithm. Synonymous SNVs and non-coding variants were filtered out. Subsequently, variants were excluded if they were more than 0.01 allele frequencies based on dbSNP137 (www.ncbi.nlm.nih.gov/projects/SNP/). Additionally, an in-house Korean SNP database was applied for filtering out common variants in a normal Korean population: SNVs found in more than 2 out of 273 Korean people were filtered out.

2.8. Statistical analysis

Fisher's exact χ^2 test and the 2 test were used to compare categorical variables, and a paired t-test and the Mann - Whitney U test were used for continuous variables. Pearson correlation and Spearman's rank correlation method were used for an analysis of association between two continuous variables. Linear regression statistics was used to evaluate of the correlation between TL and age. Estimates of the overall survival (OS) were made using the Kaplan - Meier method, and differences among survival curves were analysed using the log-rank test. Cox proportional hazards regression analysis was used to develop a multi-variate model of prognostic factors by considering the factors that were associated with survival. Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). P values <0.05 were considered statistically significant.

3. Results

3.1. Abnormal cytogenetics detected by conventional G-banding and/or FISH

Cytogenetic aberrations by G-banding and/or FISH were observed in 19/245 (7.8%) patients at initial diagnosis; 17 patients by G-banding, and six patients showed aberrations by FISH panel for AA including -5/5q-, -7/7q-, +8, -20/20q-, and +1/1q+. The most common abnormalities by G-banding were numerical abnormality (9/17 patients; 52.9%), followed by structural abnormality (5/17 patients; 29.4%), and combined abnormality (3/17 patients; 17.6%). Aberrant cytogenetics revealed by FISH were trisomy 8 in 66.7% and ≥ 2 abnormalities in 33.3%. Among 20 patients, two patients with trisomy 8 suffered disease progression to MDS (Table 2, Fig 1).

Table 2. Cytogenetic aberration detected by Conventional G-banding and/or FISH in patients with aplastic anemia.

N	Age (yrs)	Sex	Initial G-banding	Initial FISH	Treatment	Disease progression	F/U G-banding	F / U FISH
1	36	M	46,XY,t(12:15)(q24:q22)[24]	NT	IST	No	NT	NT
2	56	F	45,XX,-17[3]/46,XX[17]	Negative for all 5 FISH probes* CEP17 monosomy 2.5%	IST	No	NT	NT
3	45	M	43~45,XY,-22[3]/46,XY[11]	NT	BMT	No	NT	NT
4	52	F	46,XX,del(20)(p13)[2]/46,XX[17]	NT	IST	No	NT	NT
5	56	F	45,X[5]/46,XX[11]	Negative for all 5 FISH probes* X monosomy 2.0%	Supportive	No	NT	NT
6	33	F	46,XX,del(5)(q13q22)[11]/46,XX[13]	NT	IST	No	46,XX,del(5)(q13q22)[1]/46,XX[11]	NT
7	54	F	46,XX,del(12)(p11.2)[4]/46,XX[16]	Negative for all 5 FISH probes* TEL monosomy 2.5%	IST	No	NT	NT
8	58	M	47,XY,+8[17]/46,XY[3]	Trisomy 8 50%	IST	Yes	47,XY,+8[21]	NT
9	38	M	47,XY,+8[6]/46,XY[14]	Trisomy 8 9%	Supportive	No	NT	NT
10	55	F	47,XY,+6[3]/46,XX[18]	Negative for all 5 FISH probes* CEP6 trisomy 6.5%	Supportive	No	NT	NT
11	61	F	43~46,XX,-X,-12,-13,-14,-15,-20,-22,t(1:2)(q44q23:q37),	NT	Supportive	No	NT	NT

			add(7)(p22){cp8}/46,XX{9}					
12	38	F	47,XX,+8[19]/[2]	NT	Supportive	Yes	47,XX,+8[23]	NT
13	62	M	45,XY,-12[3]/46,XY[4]	NT	IST	No	NT	NT
14	49	F	44~47,XY,-9[3],+11[5],+mar [2]/46,XY[13]	NT	Supportive	No	NT	NT
15	45	F	47,XX,+mar[2]/46,XX[12]	NT	BMT	No	//46,XY[20]	NT
16	24	M	47,XX,+8[2]/46,XX[21]	Trisomy 8 15%	BMT	No	//46,XY[20]	NT
17	58	1	47,XY,+8[17]/46,XY[3]	Trisomy 8 50%	IST	F/U Loss	NT	NT
18	39	F	46,XX[10]	Monosomy 7 3.3%, Monosomy 8 1.0%, Trisomy 8 0.5%	Supportive	No	NT	NT
19	20	F	46,XX[20]	Trisomy 8 3.0%, 7q deletion 1.5%	Supportive	No	46,XY[20]	Negative for all 5 FISH probes*

* A FISH panel for aplastic anemia includes -5/5q-, -7/7q-, +8, -20/20q-, and +1/1q+.

Abbreviations: FISH, fluorescent *in situ* hybridization; F/U, followed up; NT, not tested; BMT, bone marrow transplantation; IST, immunosuppressive therapy; CEP, centromeric enumeration probes;

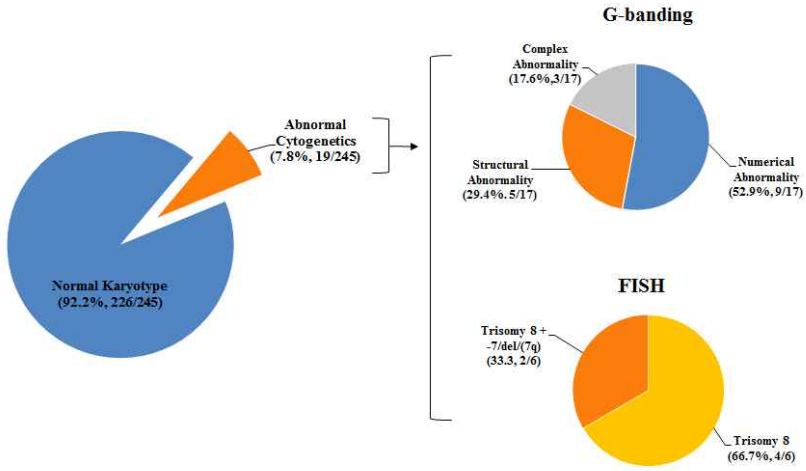

Figure 1. Abnormal cytogenetic features detected by conventional G-banding and/or fluorescent *in situ* hybridization (FISH).

3.2. Quantitative size of clonal fraction in AA

We compared the clone size measured by FISH and G-banding among AA, MDS and AML patients. The percentage of clonal cells was calculated as the percentage of BM nucleated cells with cytogenetic aberrations among total BM nucleated cells. We counted cells with aberrant FISH signals among 200 total bone marrow nucleated cells (% with aberrant signal among total BM nucleated cells) for a given FISH probe. The median percentage of cells with chromosome/FISH abnormalities (clone size) gradually increased from AA (21.4%/5.6%) to MDS (88.2%/51.0%) and to AML (95.0%/ 86.5%) (Fig 2).

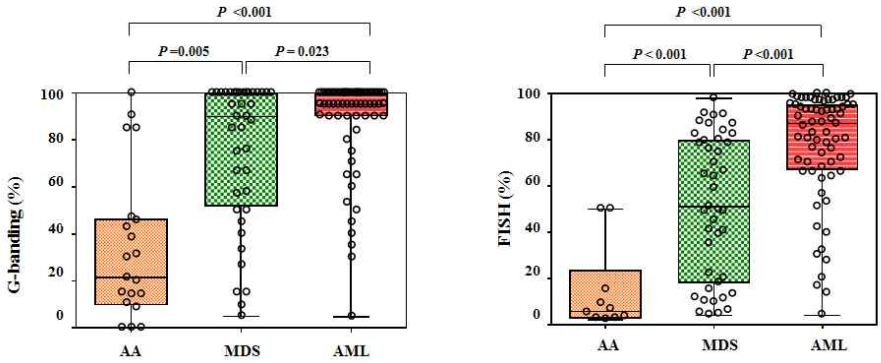

Figure 2. Proportions of clonal cells in patients with AA: A comparison with MDS and AML. AA, aplastic anemia; AML, acute myeloid leukemia; FISH, fluorescent *in situ* hybridization; MDS, myelodysplastic syndrome

3.3. Patients with disease progression

Among 393 AA patients, 4.6% showed disease progression to MDS (n = 13), AML (n = 3) and myelofibrosis (n = 2). One patient showed post-transplant lymphoproliferative disease (PTLD; plasma cell myeloma) after allogeneic bone marrow transplantation. The time to disease progression (the period taken to evolve into other diseases) ranged from 1 month to 135 months, with an average of 43 months. In the disease evolution group, 44.4% of the patients (8/18) showed abnormal cytogenetics that were not observed at the first medical examination. Among the abnormal cytogenetics, -7/7-q (50.0%) was the most common, followed by +8 (37.5%), 1q gain (12.5%). On the other hand, 7.0% (16/230) of the patients showed abnormal cytogenetics in the group without disease evolution (Table III, Fig 3).

Comparison of the AA patient group with aberrant cytogenetics detected by the MDS panel FISH results and the AA patient group with normal karyotypes, the aberrant cytogenetics group had a significantly higher disease progression rate ($P = 0.044$), and those that showed disease progression also showed poor survival ($P < 0.001$) (Fig 4).

Table 3. Clinical and cytogenetic features of AA Patients with disease progression

N	Age (yrs)	Sex	Severity	Cytogenetic Study		Disease progression		Cytogenetic Study	
				G-banding	FISH for -5/5q-, -7/7q-, +8, -20/20q-,1q gain	Time to progression (month)	Disease progression	G-banding	FISH for -5/5q-, -7/7q-, +8, -20/20q-,1q gain *
1	42	M	Non-severe	46,XY[20]	NT	46	MDS, REAB1	45,XY,-7[4]/46,XY[8]	Monosomy 7 87%
2	27	M	Severe	NT	NT	135	MDS, REAB1	46,XX,trp(1)(q21q32.3)[19]/46,XX[1]	Trisomy 1 57%
3	55	F	Non-severe	NT	NT	64	MDS, REAB1	46~47,XX,+8,15ps+[cp20]	Trisomy 8 36%
4	38	M	Non-severe	46,XX[20]	NT	110	MDS, REAB1	45,XY,-7[8]/45,idem, del(1)(p?32p?34.1)[12]	Monosomy 7 88.7%
5	38	F	Severe	47,XX,+8[19] /46,XX[2]	NT	119	MDS, REAB1	47,XX,+8[23]	Monosomy 8 59%
6	40	F	Non-severe	NT	NT	101	MDS, REAB1	45,XX,-7[22]	Monosomy 7 94.5%
7	25	F	Non-severe	46,XX[17]	All negative	56	MDS, RCUD	NT	NT
8	49	F	Severe	No mitosis	All negative	3	with Extensive Myelofibrosis	No mitosis	NT
9	56	M	Non-severe	46,XY[3]	All negative	91	MDS,REAB2	45,XY,-7[17]/46,XY[3]	Monosomy 7 91%
10	27	M	Non-severe	46,XY[4]	All negative	1	Hypoplastic MDS	46,XY[4]	All negative
11	41	F	Severe	No mitosis	All negative	11	AML(M0)	46,XY,t(12;19)(q22;q13)[17]/46,XY[1]	All negative

12	21	M	Non-severe	46,XX[9]	NT	14	AML(M4)	46,XX[4]/46,XY[4]	All negative
13	30	M	Severe	46,XY[17]	All negative	11	Hypoplastic MDS	46,XY[15]	All negative
14	59	M	Severe	No mitosis	All negative	6	With focal Myelofibrotic change	46,XY[6]	NT
15	58	M	Non-severe	47,XY,+8[17] /46,XY[3]	Trisomy 8 50%	4	Hypoplastic MDS	47,XY,+8[21]	Trisomy 8 50%
16	42	M	Severe	NT	NT	3	AML(M2)	46,XY[13]	All negative
17	23	M	Non-severe	NT	NT	43	MDS,RA	46,XY,inv(9)(p12q13)[20]	All negative
18	33	F	Non-severe	46,XX[16]	NT	60	Hypoplastic MDS	46,XX[20]	All negative

Abbreviations: MDS, myelodysplastic syndrome; RAEB, refractory anemia with excess blasts; RCUD, refractory cytopenia with unilineage dysplasia; RA, refractory anemia; AML, Acute myeloid leukemia; NT, not tested

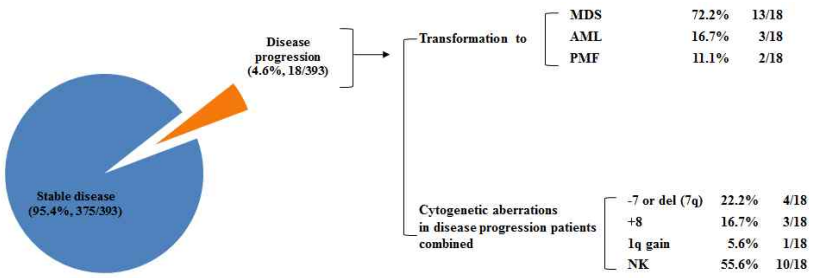

Figure 3. Frequencies of disease progression in aplastic anemia patients and their cytogenetics. AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; NK, normal karyotype; PMF, primary myelofibrosis

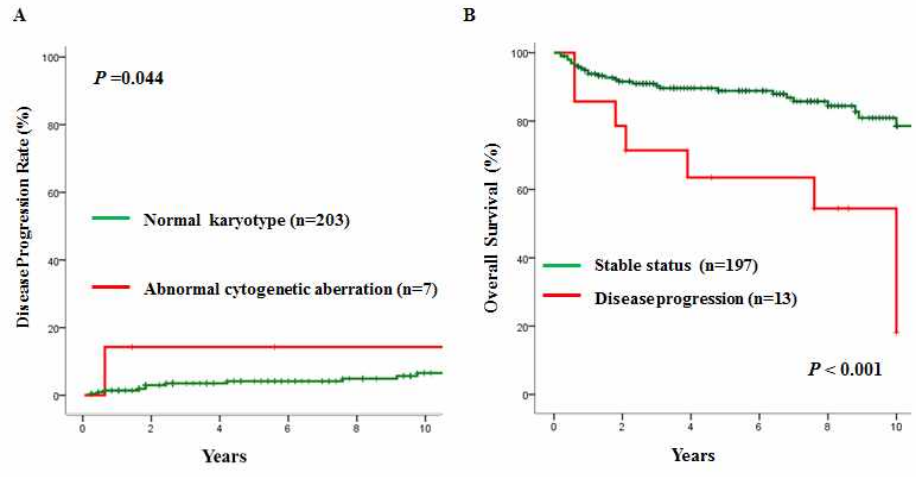

Figure 4. Kaplan-Meier analysis of disease free survival depending on cytogenetic aberrations in patients with AA. Patients with cytogenetic aberrations showed (A) higher disease progression and (B) adverse survival.

3.4. Treatment response according to cytogenetic aberrations and/or somatic mutation

Among a total of 245 patients with cytogenetic results, we selected 210 patients were followed up for more than 8 months to assess treatment response and the relationship between clinical courses, cytogenetic aberrations and/or somatic mutations (Table I). This subgroup consisted of 105 males and 105 females (median age: 42 years, ranges 20 - 83) excluding paediatric patients under 20 years of age.

We subgrouped AA into four groups according to treatment; bone marrow transplantation (BMT; (the group which only received BMT, in order to examine treatment response), IST, BMT after IST (the group which required a salvage BMT because of no response to IST) and supportive care (the group which only received supportive care, such as transfusion). Also, AA patients were subdivided into two groups according to cytogenetic aberrations: the normal karyotype group (NK) and the group with cytogenetic aberrations (CA) and/or somatic mutations (SM) [CA/SM]. The criteria of Camitta (2000) were adopted to determine IST response. Complete response is defined by normal hemoglobin for age, ANC $>1.5 \times 10^9/l$ and platelet count $>150 \times 10^9/l$, regardless of disease severity. Partial

response is defined as no longer meeting criteria for severe AA and transfusion independence, or doubling or normalization of at least one cell line or increase above baseline hemoglobin 30 g/l (if initially <60), ANC 0.5 × 10^9/l (if initially <0.5 × 10^9/l), and platelet 20 × 10^9/l (if initially <20 × 10^9/l) for mild AA. Complete and partial response to IST was 30.8% and 61.5%, respectively, at 8 months. The CA/SM patients (clonal group) tended to show an adverse response to IST compared to the patients group without CA/SM, but there was no significant statistical difference (p = 0.135) (Fig 5).

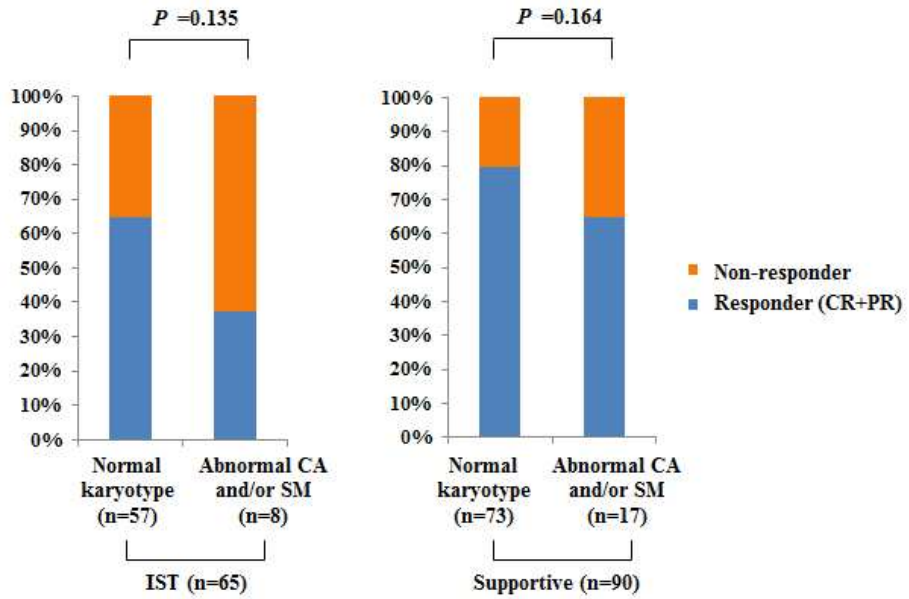

Figure 5. Comparison of the treatment response according to the presence of cytogenetic aberrations and/or somatic mutations in aplastic anemia patients with the immunosuppressive treatment group and the supportive group. CA, cytogenetic abnormality; CR, complete response; IST, immunosuppressive therapy; PR, partial response; SM, somatic mutation

3.5. Targeted sequencing

Among 70 patients that were examined by next generation sequencing with 88-gene panels, 23 patients were found to harbour somatic mutations (32.9%). The average age was 45 years, and the male : female ratio was 0.94. The mean age of the patients with SM was not different from that of patients without SM. Twenty-three patients had at least one somatic mutation, and 17 genes were involved. The detected genes were *NOTCH1* (4/23, 17.4%), *NF1* (3/23, 13.0%), *SCRIB* (3/ 23, 13.0%), *BCOR* (2/23, 8.7%), *DIS3* (2/23, 8.7%), *DNMT3A* (2/23, 8.7%), *MED12* (1/23, 4.3%), *CDKN2A* (1/23, 4.3%), *NPM1* (1/23, 4.3%), *NFKBIE* (1/23 4.3%), *LAMB4* (1/23, 4.3%), *SMARCA2* (1/23, 4.3%), *JAK2* (1/23, 4.3%), *RB1* (1/23, 4.3%), *POLG* (1/23, 4.3%), *U2AF1* (1/23, 4.3%) and *DDX3X* (1/23, 4.3%). Among the 70 patients, 4 patients showed disease progression. Somatic mutations identified in those 4 patients included *BCOR* mutation (1 patient) (Table IV).

The patients with SM did not show a difference in overall survival compared to those without SM (p = 0.749) (Fig 6A). Of note, the *DNMT3A* or *NOTCH1* was an independent adverse factor associated with short overall survival ($p < 0.001$) (Fig 6B). However, no significant differences in disease progression and response to IST were observed between patients with SM and

those without mutations (p = 0.653 and p = 0.579).

Table 4. Somatic mutations detected by targeted sequencing in patients with aplastic anemia

Gene	Chr.	Position	Ref.	Alt.	Nucleotide change	Protein change	Variant type	VAF	Number of Patients
CDKN2A	Chr2	21971170	A	T	c.188T>A	p.Leu63Gln	Nonsynonymous SNV	0.08	1
DNMT3A	Chr2	25468920	G	T	c.2577dupA	p.Trp860fs	Frameshift insertion	0.05	1
		25468920	G	T	c.1443C>A	p.Tyr481*	Stopgain SNV	0.29	1
NPM1	Chr5	170814993	C	A	c.41C>A	p.Pro14His	Nonsynonymous SNV	0.42	1
NFKBIE	Chr6	44233361	CG	C	c.139delC	p.Arg47fs	Frameshift insertion	0.43	1
LAMB4	Cjr7	107743602	C	T	c.1067G>A	p.Gly356Glu	Nonsynonymous SNV	0.49	1
SCRIB	Chr8	144886086	G	A	c.3145C>T	p.Arg1049Cys	Nonsynonymous SNV	0.31	2
								0.10	1
JAK2	Chr9	5077578	C	A	c.1990C>A	p.Leu664Ile	Nonsynonymous SNV	0.23	1
NOTCH1	Chr9	139399126	C	A	c.5017G>T	p.Gly1673Cys	Nonsynonymous SNV	0.27	1
		139412684	C	A	c.1160G>T	p.Cys387Phe	Nonsynonymous SNV	0.07	1
		139418178	C	T	c.394G>A	p.Gly132Ser	Nonsynonymous SNV	0.55	1
		139412706	G	T	c.1138C>A	p.Pro380Thr	Nonsynonymous SNV	0.09	1
SMARCA2	Chr9	2047427	C	A	c.989C>A	p.Pro330His	Nonsynonymous SNV	0.34	1
DIS3	Chr13	73337738	C	A	c.1978A>G	p.Asn660Asp	Nonsynonymous SNV	0.54	1
		73355766	C	A	c.205G>T	p.Asp69Tyr	Nonsynonymous SNV	0.14	1
RB1	Chr13	49039240	C	A	c.2318C>A	p.Ser773Tyr	Nonsynonymous SNV	0.13	1
POLG	Chr15	89866069	C	A	c.2330G>T	p.Gly777Val	Nonsynonymous SNV	0.13	1
NF1	Chr17	29528451	C	A	c.1208C>A	p.Pro403His	Nonsynonymous SNV	0.13	1
		29548880	C	A	c.1654C>A	p.Leu552Ile	Nonsynonymous SNV	0.13	1
		29676268	A G	A	c.7321_7321	p.Ala2441fs	Frameshift insertion	0.12	1

			G		+1delGG				
U2AF1	Chr21	44524456	G	A	c.101C>T	p.Ser34Phe	Nonsynonymous SNV	0.23	1
BCOR	ChrX	39914677	G	C	c.4685C>G	p.Ser1562*	Stopgain SNV	0.28	1
		39931847	G	A	c.2752C>T	p.Gln918*	Stopgain SNV	0.09	1
DDX3X	ChrX	90628317	C	A	c.121C>A	p.Pro41Thr	Nonsynonymous SNV	0.30	1
MED12	ChrX	70345274	C	A	c.2300C>A	p.Ala767Asp	Nonsynonymous SNV	0.11	1

Abbreviations: Chr., chromosome; Ref., reference sequence; Alt.,; alternative sequence; VAF, variant allele frequency; SNV, single nucleotide variant

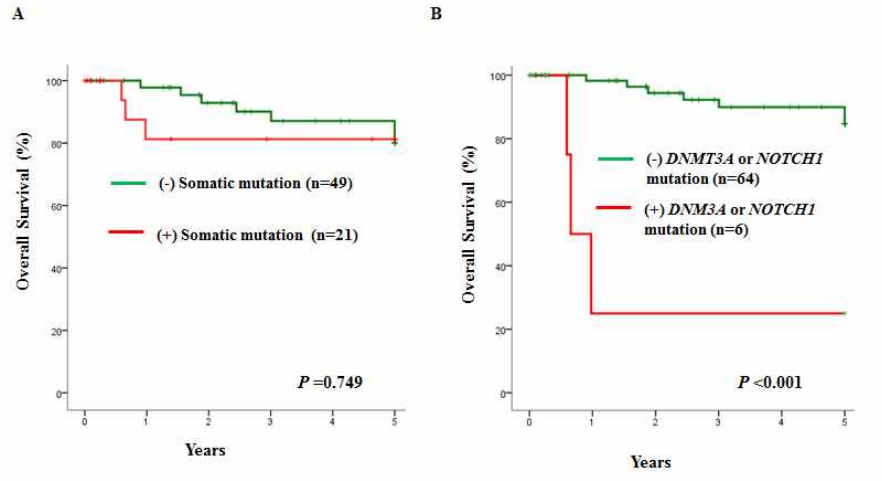

Figure 6. Comparison of overall survival according to (A) the presence of somatic mutations and (B) *DNMT3*A or *NOTCH1* mutation (log-rank test).

3.6. Distribution of telomere lengths in AA

The TLs in patients with AA (mean TL 6.73, SD 3.2; n = 135) were shorter than those of the normal healthy control group (mean TL 11.25, SD 4.46; n = 147) ($p < 0.001$) (Fig 7B). We defined the cell proportion with shorter TL as the proportion of cells belonging to the lower 10th percentile of the normal control group, and this proportion implicated the burden of short TL in one patient. Mean TL of shorter TL was 3.1 in AA, while the median TL in the normal control was 3.7 ($p < 0.001$). The median TL (5.55, n = 20) in AA patients with CA/SM was significantly shorter compared with AA patients without CA/SM (n = 115, median TL 6.10) ($p = 0.096$) and the normal control (n = 147, mean TL 11.25) ($p < 0.001$) (Fig 7B). The TL of AA patients decreased with age (Fig 7A). The age-adjusted TL was significantly shorter in patients with AA compared to normal control group ($p < 0.001$).

3.7. Treatment response to IST in correlation with Telomere length in AA

Interestingly, TL was associated with recovery of cytopenia after treatment. The mean TL of the bottom 5th percentile of the normal control group, i.e., the cell burden with severely attrite TL, was 5.9. Patients with TL < 5.9 had a poor response rate to IST (p = 0.048) (Fig 7C).

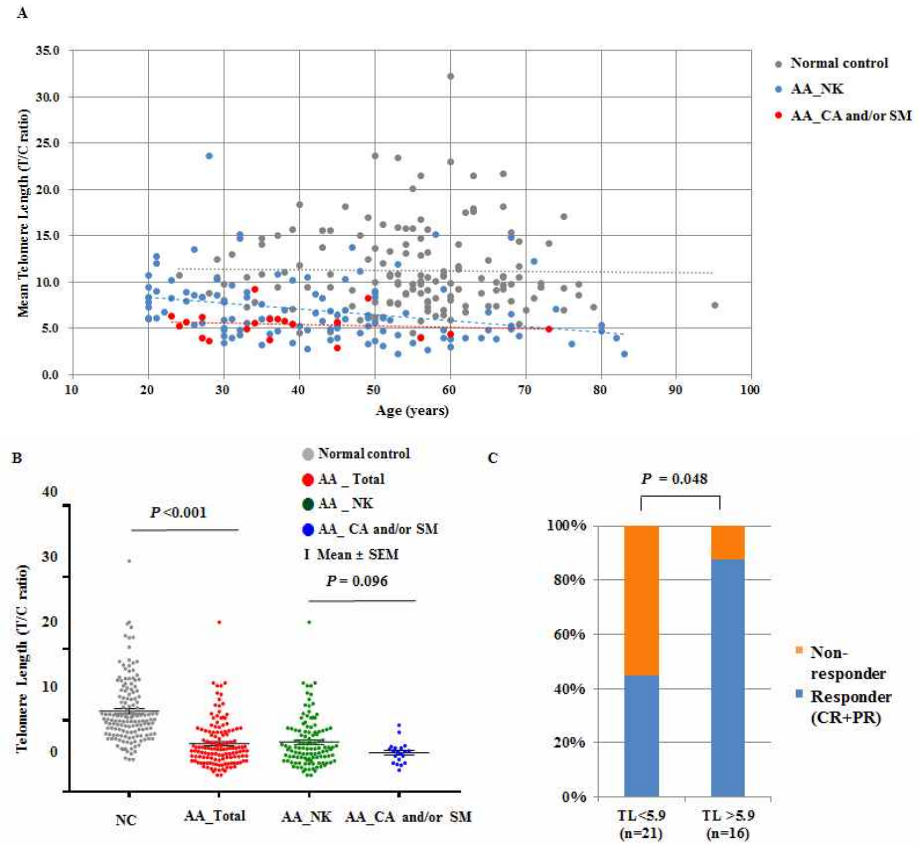

Figure 7. Comparison of the telomere length among AA patients according to their cytogenetic aberrations and somatic mutations. (A) Grey dots represent normal control (n=147), the blue dots represent AA patients without CA and/or SM (n=115), and red dots represent AA patients with CA and/or SM (n=20) (B) patients with CA and/or SM had shorter telomere length thatn patients with normal karyotype (C) AA patients with telomere length <5.9 T/C ratio showed poor response to IST.

3.8. The assessment of post-BMT endpoints in patients with AA

We evaluated post-BMT endpoints, including granulocyte/ platelet engraftment, disease progression, relapse, transplant-related mortality, time to disease progression (progression free survival) and time to relapse (Davies et al, 2000; Labopin et al, 2009; Ciceri et al, 2013). Granulocyte engraftment was defined as the first of three consecutive days with an ANC $\geq 0.5 \times 10^9$/l, and platelet engraftment was defined as the first of seven consecutive days with a platelet count $\geq 20 \times 10^9$/l without platelet transfusion. Time to disease progression meant disease-free survival to disease progression or relapse (Labopin et al, 2009; Ruggeri et al, 2015). Cytogenetic aberrations and/or somatic mutations were not associated with outcomes of post-BMT endpoints; engraftments, disease progression, relapse, transplant-related mortality (p = 0.702, 0.747, 0.492, 0.741, retrospectively) (Table V, Fig 8). TL also was not associated with days to granulocyte/platelet engraftment, disease progression, relapse and transplant- related mortality (p = 0.702, 0.067, 0.598, 0.802, retrospectively). TL was not associated with time to relapse or progression (p = 0.867 for progression-free survival; p = 0.314 for time to relapse). CA/SM were neither associated with time to relapse nor time to progression between

time to relapse or progression and CA/SM (p = 0.415, progression- free survival; p = 0.383; time to relapse).

Table 5. The assessment of post-bone marrow transplantation endpoints in patients with aplastic anemia

Characteristics	Total (n=55)	AA with normal karyotype (n=50)	AA with cytogenetic aberration and/or somatic mutation (n=5)	*p*-value
No. of patients with engraftment at first transplant, n (%)	43 (78.2)	39 (78.0)	4 (80.0)	0.702
Median days to neutrophil engraftment (days) *	11 (9-17)	12 (9-16)	10 (10-34)	0.743
Median days to platelet engraftment (days)	12 (9-21)	13 (9-20)	10 (5-67)	0.985
No. of patients with relapse	7 (12.7)	7 (14.0)	0 (0.0)	0.492
Times to relapse (days)*	174 (77-439)	174 (77-439)		
No. of patients with disease progression, n (%)	3 (5.5)	3 (6.0)	0 (0.0)	0.747
No. of patient with transplant related mortality, n (%)	11 (20.0)	10 (20.0)	1 (20.0)	0.741

* The values presented as the median and interquartile range in parentheses.

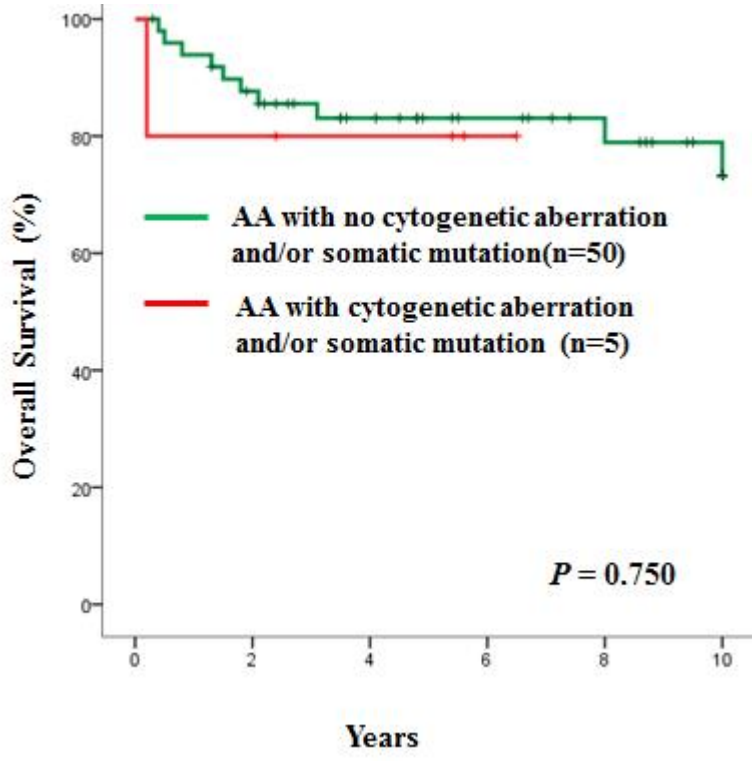

Figure 8. Overall survival according to the presence of cytogenetic aberrations and/or somatic mutations in aplastic anemia patients who received bone marrow transplantation

4. Discussion

This study examined the mean level of TL along with the distribution of TLs at a single cell level. Most of the recent studies regarding TL of AA patients used PCR techniques to measure the mean TL. Regarding the influence of telomere on cellular fate, there have been reports that cells with the shortest telomere, rather than the mean TL, determine the life and cancerous transformation of the cell population, by paracrine effect. The present study revealed that patients with AA have not only shorter TL, but also a much higher proportion of cells with a critically short telomere, compared to a normal healthy group. The proportion of cells with a TL below the 5th percentile of the normal control was 56.8% in AA. Those cells with critically short TLs are presumably susceptible to chromosomal aberrations and DNA damage and can be potential candidate cells for malignant transformation.

One of the novel findings in the current study is that the median age (36 years) of patients with SM is young, compared to the patients without SM, and 52.1% of patients with SM are aged less than 40 years. Furthermore, the median age (36 years) of patients with SM in Korea was significantly younger than the median age of patients with SM reported by Kulasekararaj et al (2014) (median age 45 years, p = 0.059).

A difference between the current study and the other studies is that we performed a MDS FISH panel (5 probes) on almost of the patients who underwent G-banding study (n = 186). It is possible that we might have been able to detect aberrant cytogenetics more sensitively than the conventional G-banding technique. We assume that FISH was particularly useful for the detection of cytogenetic aberrations in AA, as patients with AA usually harbour a small clonal fraction. On the other hand, the patient groups with and without somatic mutations did not differ in age.

We observed that the AA patient group with chromosome aberrations had a lower clonal cell percentage compared to patients with MDS and AML, which are evolutionary diseases from AA. The clonal cell percentage was defined as the percentage of cells that show cytogenetic aberrations among total BM cells. When we compared patients with AA and CA (n = 20), MDS (n = 43) and AML (n = 70), the median percentage of clonal cells by G-banding was the lowest in AA with 21.4%, followed by 88.2% in MDS and 95.0% in AML. Similarly, the allele burdens with SM in AA showed a lower frequency than that in MDS in the current study (The mean variant allele frequency was 23.3% in AA and 36% in MDS) (MDS data not published).

In the present study, adverse prognostic factors were abnormal cytogenetics, *NOTCH1* or *DNMT3A* mutations and short TL.

Meanwhile, Yoshizato et al (2015) reported *DNMT3A* and *ASXL1* as adverse prognostic mutations. Considering that the patients included in the study reported by Yoshizato et al (2015) were mostly Caucasian, we can presume that an ethnic difference might be present in prognostic factors. The AA patients with CA showed a tendency to disease progression and a significantly lower treatment response rate to IST. On the other hand there was no difference in parameters of outcome between AA patients with CA and without CA in the BMT-treated group. Based on the theory that AA caused by the autoimmune system will respond to IST, we can predict that the AA patient harbouring clonal cells will not respond to IST. When we consider that AA is a heterogeneous disease group, we can speculate that patients with SM or CA would be better treated with BMT rather than IST.

In summary, the CA and SM frequencies were 7.8% and 32.9%, respectively, and the disease progression rate was 4.6%. CA correlated with disease transformation, while short TLs were associated with poor response to IST. The unique findings of the present study are (i) the median age of AA patients with CA were significantly younger (36 years), and (ii) AA showed a smaller percentage of clonal cells with aberrant G-banding or FISH, compared to AML and MDS.

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Supplementary Table

Table S1. List of 88 genes for target sequencing

Gene	Function	*Gene*	Function
ASXL1	Chromatin modification	*DNMT3A*	DNA methylation
ATM	DNA repair	*EEF1E1*	Other
ATRX	Chromatin modification	*EGR2*	Transcription
BARD1	DNA repair	*ETV6*	Transcription
BCOR	Transcription	*EZH2*	Chromatin modification
BIRC3	Receptor/Kinase	*FAM46C*	Other
BRAF	RAS pathway	*FAT4*	Other
BRCC3	DNA repair	*FBXW7*	Receptor/Kinases
BRD2	Transcription	*FLT3*	Receptor/Kinases
BRD4	Other	*GATA1*	Transcription
CALR	Transcription	*GATA2*	Transcription
CARD6	Other	*HIST1H1E*	Other
CBL	RAS pathway	*IDH1*	DNA methylation
CCND1	Cell cycle	*IDH2*	DNA methylation
CDKN2A	Cell cycle	*IKZF1*	Transcription
CEBPA	Transcription	*ITPKB*	Signaling
CHD2	Other	*JAK2*	Receptor/Kinases
CSF1R	Receptor/Kinase	*KIAA0355*	Other
CSF3R	Receptor/Kinase	*KIT*	Receptor/Kinases
DAP3	Other	*KLHL6*	Other
DDX3X	Other	*KRAS*	RAS pathway
DIS3	Other	*LAMB4*	Other
LRP1B	Other	*SAMHD1*	Other
MAPK1	Signal/Kinase	*SCRIB*	Other
MED12	Other	*SETBP1*	Other
MPL	Receptor/Kinases	*SF1*	Splicing
MYD88	Signaling	*SF3A1*	Splicing
NF1	RAS pathway	*SF3B1*	Splicing
NFKBIE	Other	*SH2B3*	Signaling
NOTCH1	Receptor/Kinases	*SMARCA2*	Other
NPM1	Transcription	*SMC1A*	Cohesin
NRAS	RAS pathway	*SMC3*	Cohesin

PHF6	Transcription	*SRSF2*	Splicing
PLEKHG5	Other	*STAG2*	Cohesin
POLG	Other	*TCF12*	Transcription
POT1	Other	*TET2*	DNA methylation
PRKD3	Signaling	*TGM7*	Other
PRPF40B	Splicing	*TP53*	Transcription
PTEN	Other	*U2AF1*	Splicing
PTPN11	RAS pathway	*U2AF2*	Splicing
RAD21	Cohesin	*WT1*	Transcription

국문 초록

이번 연구는 383명의 재생불량빈혈을 진단받은 환자를 대상으로 흔히 나타나는 세포유전학적 변이와 체세포 변이를 알아보고, 예후와의 관련성을 살펴보고자 하였다. 245명 환자에게서 염색체검사(G-banding)와 혹은 형광동소보합법(FISH, Fluorescence in situ hybridiation)을 시행하였고, 70명의 환자에게서 88개의 혈액종양과 관련된 유전자를 타겟 염기서열 분석(Targeted sequencing)을 시행하여, 클론성(Clonality)을 규명하였다. 또한 추가적으로 135명의 환자에게서 단일세포(Single cell) 수준에서 텔로미어(Telomere) 길이를 측정하였다. 총 383명의 환자 중 8명 (4.6%) 환자가 골수형성증후군 (Myelodisplastic syndrome) 혹은 급성골수성백혈병(Acute myeloid leukemia) 혹은 골수섬유증 (parimary myelofibrosis)으로의 악성 혈액 종양질환으로 진행되었다. 악성 혈액 종양질환으로 진행한 환자들에서 관찰된 세포유전학적 변이는 홑염색체 7 (monosomy 7)이 50.0%로 가장 많이 관찰되었다. 타겟 염기서열 분석 (Targeted sequencing)을 시행한 70명의 환자 중 3분의 1 (32.9%)에서 1개 이상의 돌연변이가 관찰되었다. 빈도 순 으로는 *NOTCH1, NF1, SCRIB, BCOR*, 그리고 *DNMT3A* 순이었다. 클론성이 확인된 환자들은 전체의 30.7% 이었으며, 이들 환자들은 클론성이 없는 환자들에 비해서 면역억제제치료(immunosuppressive treatment)에 불량한 반응을 보였으나, 통계학적으로는 유의하지 않았다. 재생빈혈환자들의 텔로미어 길이는 정상 대조군에 비해서 통계적으로 유의하게 짧았으며, 클론성이 검출된 환자들은 검출되지 않은 환자들에 비해서

더 짧았다. 텔로미어 길이가 5.9 이상을 보인 환자들은 그렇지 않은 환자들에 비해서 면역억제제치료에 더 치료 반응이 좋았다. 결론적으로 클론성 변화 혹은 짧은 텔로미어 길이를 가진 재생불량빈혈 환자들은 면역억제제치료에 불량한 치료 반응을 나타내었다. 따라서 짧은 텔로미어 길이는 면역억제제치료에 반응을 예측할 수 있는 예측인자 및 바이오마커(Bio-marker)로 사용될 수 있을 것으로 기대된다.

주요어: 재생불량빈혈, 텔로미어 길이, 체세포 돌연변이, 면역억제요법

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