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Sex-Biased CD3ζ 3′-UTR SNP Increased Incidence Risk in Aplastic Anemia

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Purpose: Aplastic anemia (AA) is a bone marrow failure syndrome with an unclear pathogenesis. Abnormal T cell immunity is one of the mechanisms involved in AA, and CD3ζ is an important signaling molecule for T cell activation. Single-nucleotide polymorphisms (SNPs) in CD3ζ 3′-untranslated region (3′-UTR) were associated with some immune-related disease occurrence and affect CD3ζ protein level. In this study, our aim was to analyze whether CD3ζ 3′-UTR SNPs were associated with AA susceptibility and had influence on CD3ζ protein level and provide new research data for exploring the pathogenesis of aplastic anemia.

Patients and Methods: We screened the genotypes of SNPs in 101 healthy individuals and 91 AA patients by PCR-RFLP and sequencing. In addition, the effect of specific CD3ζ 3′-UTR SNPs was analyzed by flow cytometry and dual luciferase assay.

Results: Four SNPs of CD3ζ 3′-UTR, 1184 C >G (rs3738212), 1292 delG (rs3831958), 1403 G >C (rs1052230) and 1410 A >T (rs1052231) were identified from Chinese healthy individuals and AA patients in which rs3738212 was not previously reported. Increased risk of AA was observed in female AA who with heterozygous genotype of linkage disequilibrium SNP (rs3831958, rs1052230 and rs1052231). Different genotypes of rs3738212 have sex-biases feature in AA, higher 1184 CC frequency in male AA and higher 1184 CG frequency in female AA. Furthermore, rs3738212 could upregulate CD3ζ protein level.

Conclusion: This study first identified sex-specific CD3ζ 3′-UTR SNPs that were associated with risk of AA. Our data also demonstrated that rs3738212 could upregulate CD3ζ protein level.

Keywords: aplastic anemia, CD3ζ 3′-untranslated region, single-nucleotide polymorphisms, CD3ζ protein level

Introduction

Aplastic anemia (AA) is a bone marrow failure syndrome, which characterized by pancytopenia and dysfunction hematopoietic stem cell.[1] The pathophysiology mediated by T cell immune remains unclear in most cases. Previous studies have shown aberrant T cell activation signal molecules in AA, such as increased CD3ζ mRNA expression level in AA.[2] As one of the molecules of T cell receptor (TCR)-CD3 complex, CD3ζ plays a central role in T cell activation signal transduction. The human CD3ζ gene comprises eight exons.[3,4] The spliced mRNA product is 1,492 bp long, comprises a 492 bp coding region and a 3′-untranslated region (3′-UTR) spanning 906 bp. CD3ζ gene expression is regulated at the transcriptional, posttranscriptional, and posttranslational levels.[5] Recently, increasing evidences suggested that posttranscriptional regulation is an important component of CD3ζ gene regulation.[6] Since 3′-UTRs of mRNAs play a key role in posttranscriptional gene regulation by affecting mRNA stability, localization and transport.[7] Several recent studies on the effects of alternatively spliced 3′-UTR variants on CD3ζ mRNA expression have strengthened this concept.[8] Even our studies showed that different distribution of CD3ζ 3′-UTR splice variants in healthy individuals and CML patients,[9] and AA patients.[10] In addition, CD3ζ

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3′-UTR isoforms may influence the CD3ζ mRNA expression level in T cells from severe aplastic anemia (SAA). Thus, the regulation of CD3ζ expression is an important issue, which is needed to characterize.

The association between CD3ζ expression and CD3ζ 3′-UTR polymorphisms/mutations was studied in systemic lupus erythematosus (SLE).[11] Results have shown that two single nucleotide polymorphisms (SNPs: rs1052230 and rs1052231) in strong disequilibrium were the causal variants associated with low CD3ζ expression.[12] Family-based association analysis showed that a haplotype with low expression of SNP, rs1052231 predisposes to SLE incidence.[13] However, little is known about the characteristics of the polymorphisms/mutations in the CD3ζ 3′-UTR in healthy Chinese individuals and AA patients. Moreover, it is important to address whether the polymorphisms/mutations reported above can alter the CD3ζ expression in individuals other than SLE patients. In this study, we reported for the first time the characteristics of polymorphisms/mutations in the CD3ζ 3′-UTR and their potential function in regulating CD3ζ expression in healthy Chinese individuals and AA patients.

Materials and Methods

Samples from AA Patients and Healthy Individuals

One hundred and one healthy volunteers (HIs) including 55 males (median age: 27 years, range: 11–67 years) and 46 females (median age: 36.5 years, range: 15–71 years), ninety-one AA patients including 49 males (median age: 25 years, range: 8–73 years) and 42 females (median age: 27.5 years, range: 10–67 years) were selected for this study.

The AA group consisted of 28 patients with newly diagnosed AA, 19 AA patients receiving IST (8 cases with NSAA, 7 cases with SAA, and 4 cases with VSAA) and 44 AA patients have been treated with IST (11 cases with NSAA, 25 cases with SAA, and 8 cases with VSAA). The information and clinical data of the patients are described in Table 1. AA diagnoses were established by bone marrow biopsy and peripheral blood counts. All of the procedures were approved by the Ethics Committee of School of Medicine of Jinan University.

Polymerase Chain Reaction (PCR) for CD3ζ 3′-UTR

Peripheral blood mononuclear cells (PBMCs) isolation was performed with lymphocytes separation medium (TBDscience, Tianjin, China), and DNA extraction was performed with QIAamp DNA Blood Mini Kit (Qiagen, Duesseldorf, Germany). The primers (CD3ζ 3′-UTR-F and CD3ζ 3′-UTR-R) for PCR amplification are listed in Additional file (Table S1), all primers were synthesized by Invitrogen (Guangzhou, China). Genomic DNA of PBMCs from AA and HIs were used as templates, and PCR procedures were done with Universe High-fidelity Hot Start DNA Polymerase (BioTools, Jupiter, FL, USA) according to the manufacturer's instruction. After completion of PCR, 50 μL of the PCR products were electrophoresed in 1% agarose gel and purified with Universal DNA Purification Kit (Tiangen, Beijing, China). Purified PCR products were sequenced to examine CD3ζ SNPs 1403 G >C (rs1052230) and 1410 A >T (rs1052231) by Invitrogen (Guangzhou, China).

Table 1 Characteristics of AA Patients

Characteristics	NSAA (N=26)	SAA (N=41)	VSAA (N=24)
Age (year)	24 (8–67)	27 (10–73)	27.5 (14–59)
Sex			
Male	13 (50.0)	21 (51.2)	15 (62.5)
Female	13 (50.0)	20 (48.8)	9 (37.5)
Hematological parameters			
Hemoglobin (g/L)	67.55 (10.97)	66.61 (9.50)	65.09 (6.69)
Platelet count (× 10^9/L)	27.49 (16.30)	30.11 (20.19)	24.83 (17.40)
Absolute neutrophil count (× 10^9/L)	1.92 (1.40)	1.32 (1.43)	0.39 (0.81)
Absolute lymphocyte count (× 10^9/L)	1.32 (0.57)	0.93 (0.50)	0.75 (0.44)
Absolute reticulocyte count (× 10^9/L)	29.20 (18.67)	20.96 (27.91)	7.03 (7.91)

Note: The data are presented as means (standard deviation) for most variables, age is presented as median (range) and sex is presented as n (%).

PCR and Restriction Fragment Length Polymorphism (RFLP)

NCBI accession number J04132.1 was used as reference sequence for CD3ζ SNP description. PCR-RFLP assays were performed to examine CD3ζ SNPs, 1184 C >G (rs3738212) and 1292 delG (rs3831958). Restriction enzymes EcoNI (for 1184 C>G identification) and AflIII (for 1292 delG identification) were purchased from New England Biolabs (Beverly, MA, USA). The primers sequence for 1184 C >G identification (1184 C>G-F and 1184 C>G-R) and 1292 delG identification (1292 delG-F and 1292 delG-F) by PCR-RFLP were listed in Additional file (Table S1). PCR product was digested to completion according to the manufacturer's instructions. The digestion products were electrophoresed in 3% agarose gel. The different genotypes of CD3ζ SNPs 1184 C >G (rs3738212) and 1292 delG (rs3831958) were successfully confirmed by sequencing. Both orientations of the fragments were sequenced with the PCR primer pairs, respectively.

CD3ζ Protein Level Analysis by Flow Cytometry

Flow cytometry assays were performed right after the peripheral blood of AA patients and healthy individuals were collected. Antibodies FITC anti-human CD3 (Clone: HIT3a) and PE anti-CD247 (For CD3ζ protein level examination, Clone: 6B10.2) were purchased from Biolegend (San Diego, CA, USA).

The staining of CD3 was performed under the standard surface staining procedure, while the staining of CD3ζ was performed after fixation and permeabilization with Fixation Buffer and Intracellular Staining Permeabilization Wash Buffer (Biolegend, USA), according to the manufacturer's instruction.

Analysis was performed with BD FACS Verse flow cytometer (BD Biosciences, San Jose, CA, USA) and Flowjo V10.3 software (Flowjo LLC, Ashland, OR, USA). The expression of CD3ζ was measured via mean fluorescent intensity (MFI) and analyzed as MFI index.[13]

Dual Luciferase Assay

The PCR products amplified from CD3ζ 3′-UTR, which contains restriction sites for enzymes XhoI and MssI (Thermo Fisher Scientific, Waltham, MA, USA) were cloned into psiCHECK™ −2 vector (Promega, Madison, WI, USA), as the 3′-UTR of Renilla reniformis luciferase's coding sequence. The primer pair (RV-F and RV-R) for amplification was listed in Additional file (Table S1), and DNA samples from 2 healthy individuals with known CD3ζ 3′-UTR sequences were used as PCR templates. The recombined vector CD3ζ -L-C contains CD3ζ 3′-UTR segments in accordance with CD3ζ NCBI reference sequence, while CD3ζ -L-G contains 1184 C>G (rs3738212) mutation compared to CD3ζ -L-C. Both recombined vectors were sequenced for confirmation.

The recombined vectors were then transfected into HEK293T cells with Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). The cells were maintained in DMEM medium (Thermo Fisher Scientific, USA) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). 48 hours after transfection, Dual-Glo® Luciferase Assay System (Promega, USA) and Synergy 4 Microplate Reader (Bio Tek, Biotek Winooski, Vermont, USA) were used for luciferase activity measurements of both Firefly luciferase and Renilla luciferase as LF and LR. LR was then normalized to LF and derived the value of relative luciferase activity for further analysis. The experiment was performed in duplicate groups and repeated for 3 times.

Statistical Analysis

Variables like patient age are shown as median with range, whereas CD3ζ MFI index and relative luciferase activities were represented as means ± standard deviation (SD).

The two-tailed Student's *t*-test was performed to compare the relative luciferase activities and CD3ζ MFI index in different groups. The chi-square test was used to compare genotype, allelic, haplotype and diplotype frequencies between different groups. A $p < 0.05$ was considered to be statistically significant.

Results

Characteristic of Genotype and Allele Frequencies of SNPs in the CD3ζ 3′-UTR from Chinese Healthy Individuals and AA Patients

In this study, four SNPs, 1184 C >G (rs3738212), 1292 delG (rs3831958), 1403 G >C (rs1052230) and 1410 A >T (rs1052231) were identified from Chinese healthy individuals and AA patients (Figure 1A–H). Among these SNPs, 1292 delG (rs3831958), 1403 G >C (rs1052230) and 1410 A >T (rs1052231) had been only reported in SLE patients and healthy individuals,[13,14] but 1184 C >G (rs3738212) was not previously reported.

We further analyzed the genotype and allele frequencies of these SNPs (Table 2) in different sex and different severities of AA. There was no significant difference between AA and HIs. However, interestingly, in female AA the

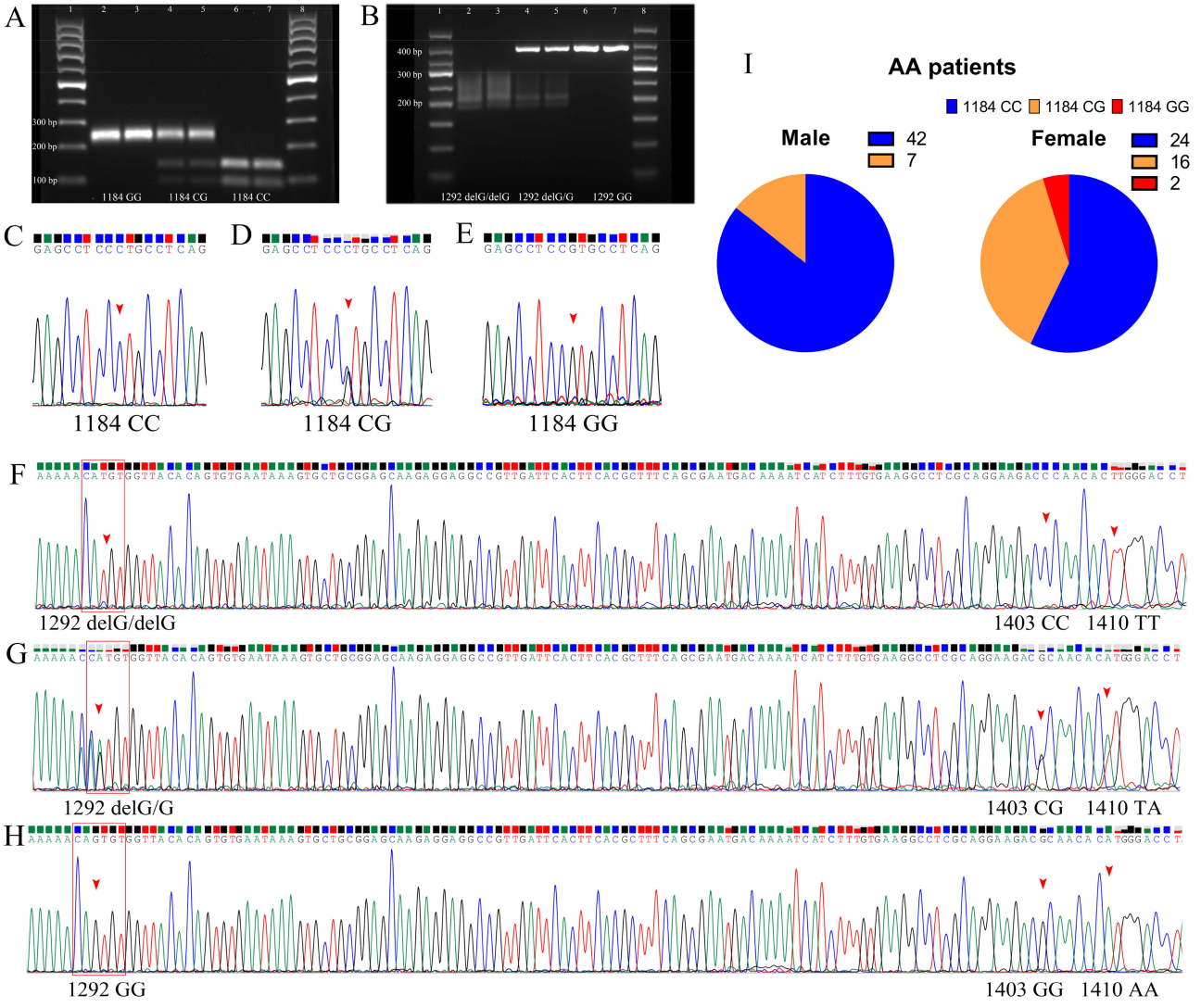

Figure 1 Characteristic of SNPs in CD3ζ 3′-UTR from Chinese HIs and AA.

Notes: (**A**) Agarose gel electrophoresis results for 1184 C>G (rs3738212), lane 1 and 8 are 100 bp scale DNA ladders, lane 2 and 3 are the 1184 GG genotype (single band, 236 bp), lane 4 and 5 are the 1184 CG genotype (3 bands: 236 bp, 143 bp and 93 bp), lane 6 and 7 are the 1184 CC genotype (2 bands: 143 bp and 93 bp). (**B**) Agarose gel electrophoresis results for 1292 delG (rs3831958), lane 1 and 8 are 50 bp scale DNA ladders, lane 2 and 3 are the 1292 delG/delG genotype (2 bands: 195 bp and 182 bp), lane 4 and 5 are the 1292 delG/G genotype (3 bands: 378bp, 195 bp and 182 bp), lane 6 and 7 are the 1292 GG genotype (single band: 378 bp). (**C–E**) Sequencing results of 1184 C>G (rs3738212), there are 1184 CC, 1184 CG and 1184 GG genotype, respectively. (**F–H**) Sequencing results of SNPs, 1292 delG (rs3831958), 1403 G>C (rs1052230) and 1410 A>T (rs1052231), there are dominant genotype (1292 delG/delG, 1403 CC, 1410 TT), heterozygous genotype (1292 delG/G, 1403 CG, 1410 TA) and minor genotype (1292 G/G, 1403 GG, 1410 AA), respectively. All sequencing results are presented from 5′ to 3′ corresponding to the mRNA sequences. Red arrows pointed the sites of SNPs. Red frames indicated the area where SNP 1292 delG (rs3831958) take place. In figure G the shifted signal upstream to 1292 site was caused by 1292 site's heterozygosity. (**I**) The genotype characteristic of 1184 C>G (rs3738212) in different sex of AA.

Table 2 The Genotype and Allele Frequencies of CD3ζ 3′-UTR SNPs from Chinese Healthy Individuals and AA Patients

SNP	Number (%)			Number (%)			Number (%)		
	AA (M)	HI (M)	*P*	AA (F)	HI (F)	P	AA	HI	P
rs3738212 Genotype									
CC	42 (85.7%)	39 (70.9%)	0.069	24 (57.1%)	32 (69.6%)	0.226	66 (72.5%)	71 (70.3%)	0.733
GG	0	0	–	2 (4.8%)	1 (2.1%)	0.504	2 (2.2%)	1 (1.0%)	0.501
CG	7 (14.3%)	16 (29.1%)	0.069	16 (38.1%)	13 (28.3%)	0.327	23 (25.3%)	29 (28.7%)	0.592
Allele									
C	91 (92.9%)	94 (76.4%)	0.089	64 (76.2%)	77 (83.7%)	0.213	155 (85.2%)	171 (84.7%)	0.889
G	7 (7.1%)	16 (14.6%)	0.089	20 (23.8%)	15 (16.3%)	0.213	27 (14.8%)	31 (15.3%)	0.889
rs3831958 Genotype									
delG/delG	32 (65.3%)	35 (66.1%)	0.938	20 (47.6%)	31 (64.6%)	0.105	52 (57.1%)	66 (63.0%)	0.244
GG	2 (4.1%)	4 (7.5%)	0.457	3 (7.2%)	5 (10.4%)	0.586	5 (5.5%)	9 (8.6%)	0.363
delG/G	15 (30.6%)	14 (36.4%)	0.639	19 (45.2%)	12 (25.0%)	0.044	34 (37.4%)	26 (28.4%)	0.074
Allele									
delG	79 (80.6%)	84 (79.2%)	0.808	59 (70.2%)	74 (77.1%)	0.297	138 (75.8%)	168 (79.2%)	0.416
G	19 (19.4%)	22 (20.8%)	0.808	25 (29.8%)	22 (22.9%)	0.297	44 (24.2%)	44 (20.8%)	0.416
rs1052230 Genotype									
CC	32 (65.3%)	35 (66.1%)	0.938	20 (47.6%)	31 (64.6%)	0.105	52 (57.1%)	66 (63.0%)	0.244
GG	2 (4.1%)	4 (7.5%)	0.457	3 (7.2%)	5 (10.4%)	0.586	5 (5.5%)	9 (8.6%)	0.363
CG	15 (30.6%)	14 (36.4%)	0.639	19 (45.2%)	12 (25.0%)	0.044	34 (37.4%)	26 (28.4%)	0.074
Allele									
C	79 (80.6%)	84 (79.2%)	0.808	59 (70.2%)	74 (77.1%)	0.297	138 (75.8%)	168 (79.2%)	0.416
G	19 (19.4%)	22 (20.8%)	0.808	25 (29.8%)	22 (22.9%)	0.297	44 (24.2%)	44 (20.8%)	0.416
rs1052231 Genotype									
TT	32 (65.3%)	35 (66.1%)	0.938	20 (47.6%)	31 (64.6%)	0.105	52 (57.1%)	66 (63.0%)	0.244
AA	2 (4.1%)	4 (7.5%)	0.457	3 (7.2%)	5 (10.4%)	0.586	5 (5.5%)	9 (8.6%)	0.363
TA	15 (30.6%)	14 (36.4%)	0.639	19 (45.2%)	12 (25.0%)	0.044	34 (37.4%)	26 (28.4%)	0.074
Allele									
T	79 (80.6%)	84 (79.2%)	0.808	59 (70.2%)	74 (77.1%)	0.297	138 (75.8%)	168 (79.2%)	0.416
A	19 (19.4%)	22 (20.8%)	0.808	25 (29.8%)	22 (22.9%)	0.297	44 (24.2%)	44 (20.8%)	0.416

heterozygous genotype frequencies of SNPs, rs3831958, rs1052230 and rs1052231 were significantly higher than those in female HIs (p = 0.044, p = 0.044, p = 0.044, respectively). Furthermore, 1184 GG genotype was only detected in female AA and HIs. The genotype frequency of 1184 CC in male AA was significantly higher compared with female AA (p = 0.002), while 1184 CG frequency in male AA was significantly lower compared with female AA (p = 0.009, Figure 1I).

This feature was also found in different status AA. The genotype frequency of 1184 CC in male SAA was significantly higher compared with female SAA (p = 0.014), while 1184 CG frequency in male SAA was significantly lower compared with female SAA (p = 0.014, Figure 2A). However, we did not find a correlation between 1184 C >G (rs3738212) genotypes and IST treatment responses in AA patients. And although the difference was not statistical significance, the heterozygous genotype frequencies of SNPs, rs3831958, rs1052230 and rs1052231 in female VSAA was about 26% higher than that in male VSAA (Figure 2B).

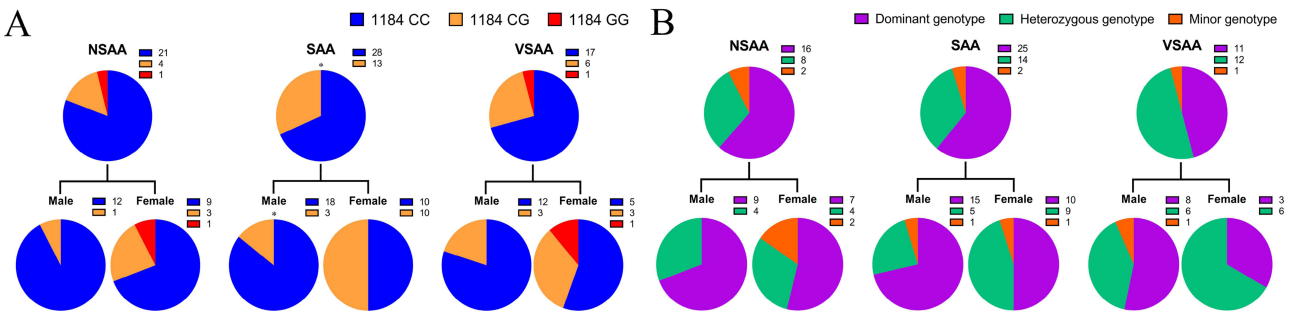

Figure 2 The genotype characteristic of SNPs, rs3738212, rs3831958, rs1052230 and rs1052231 among NSAA, SAA and VSAA.
Notes: (**A**) Distribution of the genotype of rs3738212 among NSAA, SAA and VSAA, 1184 CG frequency in male SAA was significantly lower compared with female SAA, p=0.014, while 1184 CG frequency in male SAA was significantly lower compared with female SAA, p=0.014. (**B**) Distribution of the genotype of SNPs rs3831958, rs1052230 and rs1052231 among NSAA, SAA and VSAA, the difference was not statistical significance.

Sex-Biased CD3ζ 3′-UTR SNPs in AA Patients

According to the definition of D′-value and r2-value, we identified three SNPs, rs3831958, rs1052230 and rs1052231 have strong linkage disequilibrium (LD), while rs3738212 with these 3 SNPs has no strong LD (Figure 3A). The strong LD suggested the 3 SNPs could be analyzed as a whole, while rs3738212 to be considered as a standalone factor. Thus, samples were allowed to be separated into a limited number of groups. Following identification of LD, healthy individuals and AA patients were analyzed according to their different haplotypes and diplotypes of these identified SNPs. The results of haplotypes and diplotypes of CD3ζ 3′-UTR SNPs were shown in Table 3.

The distribution of the haplotypes and diplotypes showed divergence in AA patients with different sex. In female AA, the haplotype Z1 frequency was significantly lower compared with female HIs (p = 0.039), and diplotype Z2ZM frequency was significantly higher in female AA compared with that in female HIs (p = 0.009). Meanwhile in male AA, the haplotype Z1 and diplotype Z1Z1 frequencies showed to be significantly higher compared with each of those in female AA (p = 0.000, p = 0.002, respectively). The haplotype ZM frequency and diplotype Z2ZM frequency were significantly higher in female AA compared with each of those in male AA (p=0.002, p = 0.023, respectively, Figure 3B and C).

The sex-bias distribution of haplotypes displayed were also observed in different severity AA patients. The haplotype Z1 frequency was significantly higher in male NSAA or SAA compared with that in female NSAA or SAA (p = 0.020, p = 0.007, respectively), while haplotype ZM frequency was significantly lower in male SAA compared with that in female SAA (p = 0.003). The diplotypes Z1Z1 frequency was significantly higher in male SAA compared with that in female SAA (p = 0.007, Figure 4A and B).

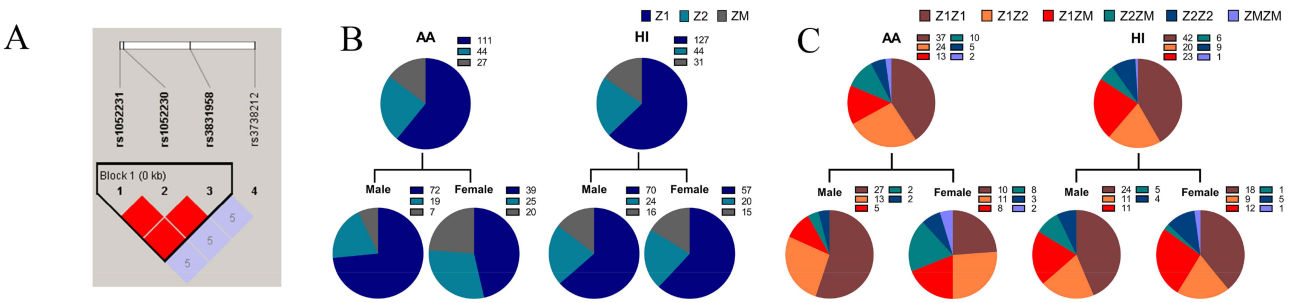

Figure 3 The feature of CD3ζ 3′-UTR SNPs in different sex from HIs and AA.
Notes: (**A**) Linkage disequilibrium analysis of the SNPs, rs3738212, rs3831958, rs1052230 and rs1052231. The D′ Chart generated from 91 AA patients or 101 healthy individuals resulted to be the same. The D′-values of all plots are 1. The r2-values of red plots are 1, and the r2-values of cyan plots are 0.05 as zeros are omitted in the figures. (**B** and **C**) The haplotype and diplotype distribution of CD3ζ 3′-UTR SNPs in different sex of AA and HIs.

Table 3 Haplotypes and Diplotypes Analysis of the CD3ζ 3′-UTR SNPs Detected from Chinese Healthy Individuals and AA Patients

SNPs	1184 C>G rs3738212	1292 delG rs3831958	1403 G>C rs1052230	1410 A>T rs1052231	Frequencies (%)		
					AA	HI	P
Haplotypes							
Z1	C	delG	C	T	111 (61.0%)	127 (62.9%)	0.704
Z2	C	G	G	A	44 (24.2%)	44 (21.8%)	0.577
ZM	G	delG	C	T	27 (14.8%)	31 (15.3%)	0.889
Diplotypes							
Z1Z1	CC	delG/delG	CC	TT	37 (40.7%)	42 (41.6%)	0.897
Z1Z2	CC	delG/G	CG	TA	24 (26.4%)	20 (19.8%)	0.279
Z1ZM	CG	delG/delG	CC	TT	13 (14.3%)	23 (22.8%)	0.133
Z2ZM	CG	delG/G	CG	TA	10 (11.0%)	6 (5.9%)	0.206
Z2Z2	CC	GG	GG	AA	5 (5.5%)	9 (8.9%)	0.363
ZMZM	GG	delG/delG	CC	TT	2 (2.1%)	1 (1.0%)	0.501

The Effect of SNPs in CD3ζ 3′-UTR on CD3ζ Protein Level

It has been reported that LD SNPs, rs1052230 and rs1052231 downregulated CD3ζ protein level in healthy control from UK (62.5% of the healthy controls were female). In the current study, rs3831958, rs1052230, and rs1052231 were found to be in complete LD. In HIs group, the heterozygous genotype of SNPs (rs3831958, rs1052230 and rs1052231) showed a trend of downregulated CD3ζ protein level compared with dominant genotype (p = 0.079). In female HIs subgroup, this trend was more obvious (p=0.055). However, the same trend of CD3ζ protein level was not found in male HIs subgroup (Figure S1).

The CD3ζ protein level in HIs with 1184 CG genotype was significantly higher (28.09 ± 2.65) than HIs with 1184 CC genotype (22.72 ± 1.16, p = 0.034), and this obvious effect of 1184 CG on CD3ζ protein level was also found in male HIs subgroup (p = 0.025). Nevertheless, the difference was not found in female HIs (Figure 5A). According to the diplotype features of SNPs in CD3ζ 3′-UTR, we further analyzed CD3ζ MFI index among different diplotypes. The CD3ζ protein level in HIs with Z1ZM diplotype was significantly higher (29.82 ± 11.71) than HIs with Z1Z2 (20.94 ± 6.32, p = 0.022), while CD3ζ protein level in male HIs with Z1ZM diplotype was significantly higher (29.71 ± 8.05) than male HIs with Z1Z1 (21.43 ± 6.17, p = 0.017, Figure 5B).

To examine whether the rs3738212 variants affect the CD3ζ protein level, we established luciferase reporter vectors containing the CD3ζ 3′-UTR with different rs3738212 alleles. These vectors were designated as CD3ζ -L-G and CD3ζ -L-C, respectively, containing the rs3738212-C or -G alleles (Figure 5C). These vectors were transfected into HEK293T cells. The luciferase activity of CD3ζ -L-G group increased by 35% compared with CD3ζ -L-C group (p = 0.000, Figure 5D).

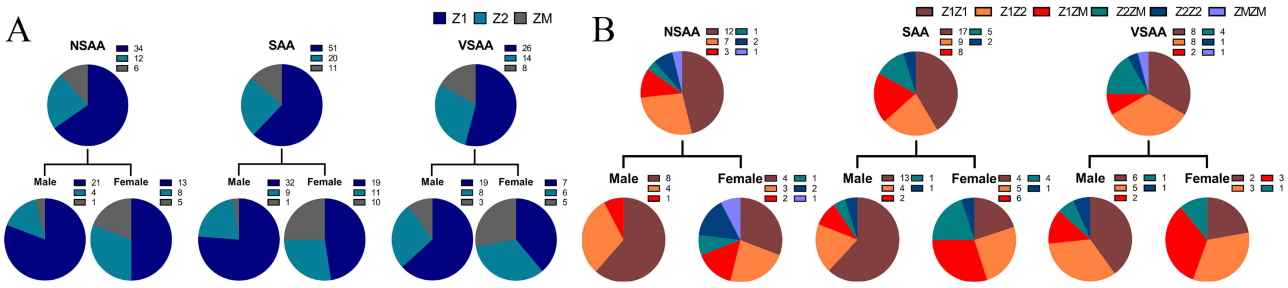

Figure 4 The feature of CD3ζ 3′-UTR SNPs in different sex of AA with different severities.
Notes: (**A** and **B**) The haplotype and diplotype distribution of CD3ζ 3′-UTR SNPs in different sex of AA with different severities.

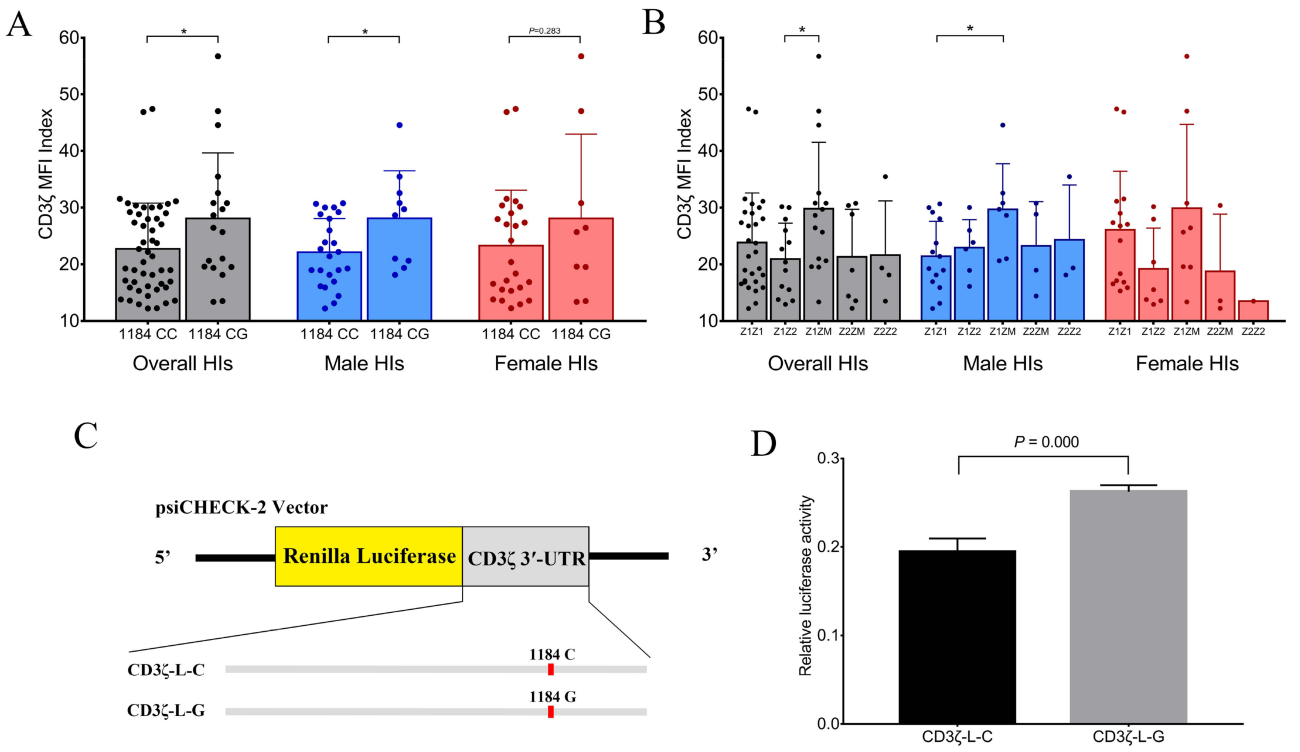

Figure 5 The effect of rs3738212 on CD3ζ protein level.
Notes: (**A**) CD3ζ protein level with different 1184 C>G (rs3738212) genotype from HIs. (**B**) CD3ζ protein level with different diplotypes in the CD3ζ 3′-UTR from HIs. (**C**) The construction of luciferase reporter vectors containing different rs3738212 alleles. (**D**) Dual luciferase assay result showed the effect of rs3738212 on luciferase activity. *p<0.05.

Discussion

AA is a bone marrow hematopoietic failure syndrome caused by various etiologies, and the pathogenesis of AA is still unclear.[1] The incidence of AA shows geographical variability, which is 2- to 3-fold higher in Asia than in the West. This variability in incidence rates may reflect differences in exposure to environmental factors, genetic background, and so on. The sex-specific incidence is slightly higher in males than in females (ratio:1.18:1). A biphasic age distribution feature was present in previous studies, one among young adults and another in the elderly.[15,16]

The previous results indicated that TCR signal pathway involved T cell immune pathogenesis of AA, highlighting CD3ζ as an important T cell activation molecule involved in this pathogenesis process. It has been reported that the initiation and development of SLE is related to inappropriate regulation of B cells, while hyperactivity of T cells also plays a pathogenic role in SLE.[17] Recent studies have suggested that post-transcriptional regulation plays an important role in regulating the expression of CD3ζ gene, and SNP in the CD3ζ 3′UTR region in SLE patients downregulate CD3ζ protein levels and are associated with disease susceptibility.[13] However, there are few studies on the distribution feature of SNP in CD3ζ 3′-UTR in Chinese healthy individuals and AA. In this study, we analyzed the loci of rs3738212, rs3831958, rs1052230 and rs1052231 from CD3ζ 3′-UTR and examined the association of four SNPs with AA.

Sex Biased SNP in CD3ζ 3′-UTR Increased the Incidence Risk of AA

Firstly, we identified CD3ζ 1292 delG, 1403 G>C and 1410 A>T has strong LD. The female AA with heterozygous genotype of these LD SNPs exhibited a 2.48-fold risk of AA (95% CI: 1.006 to 5.974, P = 0.044), while we did not observe an increased risk for the heterozygous in male AA. Several significant SNPs are associated with an increased risk of coronary endothelial dysfunction. These associations appear to be sex specific, PON1 in women and KIF6 and NFKB1 in men.[18] To date, this is the first epidemiological study to document the genotype frequency of rs3831958, rs1052230 and rs1052231 among Chinese population and assess the association between these SNPs and the risk of female AA incidence. Previous data from SLE patients (93% female) indicated that rs1052230 and rs1052231 were predisposed to

SLE.[13] Combining these results implied that these SNPs might increase susceptibility to some autoimmune diseases in female. In the future, it will be necessary to analyze the distribution characteristics of these SNPs for other autoimmune diseases.

Secondly, we also identified that different genotypes of CD3ζ 1184 C>G have sex-biases feature, higher 1184 CC frequency in male AA and higher 1184 CG frequency in female AA, while the phenomenon was also found in SAA. As we know, the sex ratio of AA had been close to 1:1 in most study. Hence, the sex-biases of 1184 C >G might be not associated with AA susceptibility. Further studies are required to assess the significance of sex-biases of 1184 C >G in AA.

Finally, we noticed Z2ZM diplotype has a higher risk of female AA (Odds ratio: 10.59; 95% CI: 1.428 to 119.8, P = 0.009). The interactions of multiple SNPs were involved in the occurrence and development of disease.[19–21] These results suggested that the four SNPs did not have the effect alone but might be in synergy with each other in affecting female AA risk. In addition, diplotype Z1Z1 frequency showed decline trend with AA severity (NSAA: 50%, SAA: 41% and VSAA: 30%, respectively). SNPs in 3′-UTR are very well correlated with disease status in several instances.[22,23] Although the frequency among different AA severity was not statistically significant, the results gave us a hint of diplotype Z1Z1 might be an SNP marker associated with AA severity rather than AA risk markers.

SNP rs3738212 Up-Regulate CD3ζ Protein Level in Chinese Healthy Individuals

1184 CG genotype upregulated CD3ζ protein level in Chinese HIs, this effect was also found in male HIs. The CD3ζ protein level trends to raise in female HIs with 1184 CG genotype than those with 1184 CC genotype. To substantiate this observation, luciferase assay clearly evidenced that luciferase activity was significantly upregulated in HEK293T cells transfected with the 1184 GG variant than 1184 CC. Polymorphic variants that cause luciferase activity change were usually accompanied by a similar increased protein level. These finding show that CD3ζ 3′-UTR carrying allele G of rs3738212 could up-regulate CD3ζ protein level in Chines HIs.

It is interesting as there is little research in the literature of molecular research of this SNP. However, it is still unclear as to how CD3ζ expression levels are increased in Chinese healthy individuals with 1184 CG genotype. One possible explanation is that miRNAs can regulate gene expression via the partial or total hybridization of specific sequences located in target mRNAs.[24,25] The SNP is not located in the miRNA binding site currently predicted in the CD3ζ 3′-UTR, but 30 bases downstream to the miRNA-504 binding site (1147–1153 bp). Obviously, SNPs within or proximal to miRNA-binding sites in target genes have the potential to either create or destroy binding sites, which affects the efficiency of miRNA binding to target sequences.[26–29] The 1184 C>G variant may potentially interfere with this site, consequently leading to an increase in CD3ζ protein levels. Therefore, the underlying mechanisms of 1184 C>G affecting CD3ζ protein levels still require further study.

There were limitations in this study. Almost 70% AA patients in this study are receiving or have received IST. Considering that IST might be affecting T cell immune status and clinical parameters, we were unable to analyze the correlation between these CD3ζ 3′-UTR SNPs and CD3ζ protein levels or clinical parameters in AA patients.

In AA patients, we did not find a correlation between 1184 C>G (rs3738212) genotypes and IST treatment responses. In future work, we will primarily analyze the SNPs and CD3ζ expression in newly diagnosed AA patients, and track the IST treatment responses.

Conclusion

To our knowledge, this is the first study to report the association characteristics of the CD3ζ 3′-UTR SNP in Chinese healthy individuals and AA patients. The sex skewing of heterozygous genotype SNPs (rs3831958-rs1052230-rs1052231) is associated with AA incidence risk in female AA. We also identified genetic factors, rs3738212 that could up-regulate CD3ζ protein level in Chinese healthy individuals. Through these findings, let us have a more comprehensive understanding of the immune characters in Chinese health individuals and further studies are required to confirm the effect of 1184 C >G (rs3738212) mutations on CD3ζ expression in AA patients.

Abbreviations

AA, aplastic anemia; SAA, severe aplastic anemia; VSAA, very severe aplastic anemia; NSAA, non-severe aplastic anemia; SNPs, single-nucleotide polymorphisms; 3′-UTR, 3′-untranslated region; PCR-RFLP, polymerase chain reaction and restriction fragment length polymorphism; TCR, T cell receptor; SLE, systemic lupus erythematosus; HIs, healthy volunteers; IST, immunosuppressive therapy; PBMCs, peripheral blood mononuclear cells; LD, linkage disequilibrium; PON1, paraoxonase 1 gene; KIF6, Kinesin Family Member 6; NFKB1, nuclear factor of κ light polypeptide gene enhancer in B-cell 1 gene; SD, standard deviation.

Data Sharing Statement

The data used and/or analyzed during the current study are available from the corresponding author Bo Li on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of School of Medicine of Jinan University. All adult participants provided written informed consent. For the minor participants, written informed consent was obtained from their parents/guardians and assent was obtained from the minors. Our study complied with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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