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ORIGINAL ARTICLE

Influence of genetic polymorphisms of *IL23R*, *STAT3*, *IL12B*, and *STAT4* on the risk of aplastic anemia and the effect of immunosuppressive therapy

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Abstract
Studies have suggested that IL-23/STAT3 and IL-12/STAT4 signaling pathways associate with aplastic anemia (AA) occurrence. Polymorphisms in pathway-related genes may contribute to AA risk. In the current study, we investigated the association between polymorphisms in genes of *IL23R*, *STAT3*, *IL12B*, and *STAT4* and occurrence, severity, and immunosuppressive outcome of AA in the Han population in southwest China. In the current 164 AA cases and 211 controls study, we found T allele and TT genotype of *rs*7574865 were more frequent in the cases than that in the controls. In the additive model, individual carrying *rs*7574865 T allele demonstrated a 37% (OR (95% CI) = 1.37 (1.02–1.85), *P*per = 0.036) increased AA risk. In the recessive model, carrier with *rs*7574865 TT genotype showed a 2.08-fold increased AA risk (OR (95% CI) = 2.08 (1.14–3.70), *P*per = 0.017). Additionally, we showed that G allele and GG genotype of *rs*11209032 were more frequent in the 88 non-severe AA cases than that in the 76 severe AA ones. Our study also found G allele and GG genotype of *rs*11209032, and GG-genotype of *rs*744166 associated with the immunosuppressive therapy outcome in AA patients. Current study results support that functional *STAT4* (*rs*7574865), *IL23R* (*rs*11209032), and *STAT3* (*rs*744166) variants may associate with occurrence, severity, and immunosuppressive outcome of AA in the Han population in southwest China.

Keywords Aplastic anemia · Genetic polymorphisms · IL12B · IL23R · STAT3 · STAT4 · Meta-analysis · Immunosuppressive therapy

Introduction

Aplastic anemia (AA) is the paradigm of human bone marrow (BM) failure syndromes, characterizing BM hypoplasia and pancytopenia in the peripheral blood (PB). Epidemiological studies suggest the prevalence of AA is ~2 per million persons in Western countries, and it is 2–3 times higher in East Asia, of which the incidence is ~7 per million persons in China [1–4]. Although its specific pathogenesis has not been completely elucidated, immune-mediated suppression of hematopoiesis and genetic vulnerability are considered to play vital roles.

Accumulating evidence has suggested that BM hematopoietic stem cells of most AA patients are attacked by autoreactive cytotoxic T cells, such as interferon-γ (IFN-γ)-secreting $CD4^+$Th1 cells and interleukin-17 (IL-17)-secreting $CD17^+$Th17 cells [5–8]. Studies suggest interleukin-12 (IL-12) could induce the activation of signal transcription and transducer factor 4 (STAT4), which promotes the secretion of IFN-γ of Th1 cells [5]. A high concentration of IFN-γ could affect hematopoietic stem cell proliferations through inducing apoptosis and decreasing self-renewal [6]. The IL-12/STAT4/IFN-γ signaling pathway, as an important signaling pathway in mediating T cells immunity, contributes to pathogenesis of AA. Besides, current studies also demonstrate

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the absolute number of $CD4^+$Th17 cells and the level of IL-17 are elevated in the AA patients and elucidate the specific pathological mechanism pathway of Th17 cells affecting AA risk [7]. Interleukin-23 (IL-23), as a proinflammatory and immunoregulatory agent, activates the transcription of STAT3, and increases the secretion of IL-17 of Th17 cells. The IL-23/STAT3/IL-17 signaling pathway is reported involving in the occurrence and progress of various autoimmune diseases, including AA [8].

Immunosuppressive therapy (IST), consisting of antithymocyte globulin (ATG)/antilymphocyte globulin (ALG), and cyclosporine (CsA), is the preferred treatment modality for AA patients. About 70% of AA patients could reach hematological recovery with IST, through suppressing the over-activation of T cells, regulating T cells subsets balance, inhibiting the over-production of myelosuppressive cytokines [9]. However, some patients with IST could not reach hematological response and some responders relapse. It is not fully understood which factors could affect patients' response and relapse.

Numerous studies have reported that single-nucleotide polymorphisms (SNPs) of some immunoregulatory cytokines may have significant association with the occurrence, development, and therapy outcome of some diseases [10–12]. Therefore, we wondered whether genetic polymorphisms of cytokines in the IL-12/STAT4/IFN-γ and IL-23/STAT3/IL-17 signaling pathways were associated with inclination and IST outcome of AA individuals. Herein, in the current study, we performed an analysis to detect which SNPs in *IL23R* (*rs*11209032, *rs*1884444); *STAT3* (*rs*1053005, *rs*4796793, and *rs*744166); *IL12B* (*rs*3212227); and *STAT4* (*rs*7574865, *rs*897200) were associated with AA susceptibility, severity, and whether the significant SNPs affected response to IST of AA patients in the Han population in southwest China.

Materials and methods

Ethics statement

The study protocols were approved by the Ethics Review Board of West China Hospital of Sichuan University. The study conformed to the principles outlined in the declaration of Helsinki. All patients provided written informed consent before participating in the study.

Study subjects

From Sep. 2013 to Dec. 2014, consecutive patients with AA were recruited from the inpatient and outpatient units of the department of hematology in West China Hospital of Sichuan University. Patients were excluded if they fulfilled the following criteria: (1) Ethnicity other than Han-Chinese, (2) patients with bone marrow transplantation, (3) patients with AA/PNH syndrome, and (4) Patients with pure red cell aplastic anemia (PRCA). The controls were sex- and age-matched healthy unrelated individuals who were selected from those coming to the hospital for regular health examinations. At recruitment, each participant donated approximate 2–4 ml of blood for genomic DNA extraction.

We collected the basic characteristics of AA patients through the hospital information system and laboratory information system, including sex, age, red blood cells counts (RBCs), hemoglobin (HGB), platelet counts (PLTs), white blood cells counts (WBCs), absolute lymphocyte counts (ALCs), absolute neutrophil counts (ANCs), absolute reticulocyte counts (ARCs), mean corpuscular volume (MCV), and red cell distribution width (RDW). We divided the recruited AA patients into severe aplastic anemia (SAA) and non-severe aplastic anemia (NSAA), according to the criteria by Camitta for diagnosis in aplastic anemia [13, 14].

We followed up the recruited AA patients and recorded the therapeutic regimens and outcomes with IST. Therapeutic regimens of IST included standard ATG/ALG, ATG/ALG + CsA, and only regular CsA administration. We followed up the IST outcomes of AA patients more than 3 months before 31 Jan. 2015. Response was confirmed by two or more blood counts at least 4 weeks apart. Specific criteria of therapeutic effect was shown in Table 1 [14].

SNP sites selection and genotyping

We screened polymorphic sites in genes *IL23R*, *STAT3*, *IL12B*, and *STAT4* by reading articles. We searched the tag SNPs in genes *IL12B*, *STAT4*, *IL23R*, and *STAT3* through Hapmap database and Haploview software. We hunted for the functional SNPs in genes *IL23R*, *STAT3*, *IL12B*, and *STAT4* through the

Table 1 Criteria of response to IST in AA

Response	Severe AA	Non-severe AA
None	Still severe or even worse	Worse or not fulfill the following criteria
Partial	Transfusion-independent; no longer meeting criteria for severe diseases	Transfusion independence (if previous dependent) or doubling or normalization of at least one cell line or increase of baseline hemoglobin of > 30 g/l (if previously < 60 g/l), neutrophils of > 0.5×10^9/l (if initially < 0.5×10^9/l), platelet > 20×10^9/l (if previously < 20×10^9/l)
Complete	Hemoglobin normal for age; Neutrophils > 1.5×10^9/l; Platelet > 150×10^9/l	Same criteria for severe AA

Table 2 The primers of reference alleles studied

SNP ID	Forward primer	Reverse primer	Product length (bp)
*rs*11209032	5′-TCCCTACATCACCC TCTTTGC-3′	5′-GGTGTTTTCTTNSAA GCTGAATTGC-3′	74
*rs*1884444	5′-GSAACAGTCTTTTC CTGCTTCCA-3′	5′-TSAAGGGCTATTACTG CATCCCA-3′	70
*rs*1053005	5′- GGGGGAGACGAC CTTCTCTA-3′	5′-ACACATNSAACTGTCTC CAGGC-3′	70
*rs*744166	5′-GCNSAACATTGAG AGGGNSAAT-3′	5′-TGCTGTGGCTGTAATGT CTTG-3′	101
*rs*4796793	5′-TAACTACGCTATCC CGTGCG-3′	5′-GSAACCAGGCAGGGAG TGTA-3′	117
*rs*3212227	5′-AGACANSAACGGA ATAGACCCA-3′	5′-CTGATTGTTTNSAATGA GCATTTAGC -3′	12 8
*rs*7574865	5′- TGGTGTGGATGGA GGTAAGG −3’	5′-SAATCCCCTGSAATTCC ACTG-3′	185
*rs*897200	5’-AAGGACATGTSAA ACCAGTGTGA −3’	5′-CTAGTGGCTGCCGTGTTG-3′	100

online software SNPinfo (http://snpinfo.niehs.nih.gov/). As a result, we picked up eight SNPs in our current study, including *IL23R* (*rs*11209032, *rs*1884444); *STAT3* (*rs*1053005, *rs*744166, and *rs*4796793); *IL12B* (*rs*3212227); and *STAT4* (*rs*7574865, *rs*897200).

Genomic DNA was extracted using a QIAamp DNA Blood mini kit (QIAGEN, Germany) and diluted to 10 ng/μl with buffer, according to the manufacturer's instructions. The target fragments containing reference alleles from all of the study subjects were amplified by PCR with corresponding specific primers (Table 2). The specific PCR amplification and corresponding genotyping of the eight SNPs were performed by the method of high-resolution melting (HRM) in the LightCycler® 480 (Roche Diagnostics) and direct sequencing.

Statistical analysis

Continuous variables conforming to normal distribution were described as mean ± standard deviation (SD); otherwise, by median and interquartile range (P_{25}–P_{75}). Categorical variables were described as frequency and percentage (%). Variables conforming to normal distribution and homogeneity between two groups were compared by *t* test; otherwise, by Mann-Whitney *U* test.

Hardy-Weinberg equilibrium was evaluated by chi-square test for each SNP. $P < 0.05$ was considered statistically significant.

Allele and genotype case-control association analysis was conducted using all genotype data. For each SNP, we calculated empirical significance value on the basis of 10,000 permutations. This ensures that deviation from small sample size will not cause false positives. All the statistical analysis was performed by the software PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink). Two-sided $P < 0.05$ was considered statistically significant.

To assess which factors affected AA IST outcome, we utilized single-sample *t* test to screen the significant variables, then logistic method to adjust the variables effect. The statistical analysis was conducted by SPSS 17.0. Two sided $P < 0.05$ was considered statistically significant.

Meta-analysis

For further assessment of the association between genetic polymorphisms in genes *IL23R*, *STAT3*, *IL12B*, and *STAT4* and AA susceptibility, we searched MEDLINE, EMBASE, Cochrane library, and Chinese databases (CNKI, CQVIP, and Wan-fang Databases) to collect the related literatures published in English and Chinese till Jan 2015, utilizing the theme words of "aplastic anemia," "SNP," "*rs*11209032," "*rs*1884444,", "*rs*744166," "*rs*4796793," "*rs*1053005," "*rs*3212227," "*rs*897200," and "*rs*7574865." We extracted the relative data from each eligible article and conducted a meta-analysis by Review Manager Version 5.3 (http://www.cc-ims.net/RevMan).

The inclusion criteria were (1) studies evaluating the association between "*rs*11209032," "*rs*1884444," "*rs*744166," "*rs*4796793," "*rs*1053005," "*rs*3212227," "*rs*897200," and "*rs*7574865" polymorphisms and AA risk. (2) Available data for calculating allelic odds ratios (ORs) with corresponding 95% confidence interval (95% CI). (3) Genotypes in controls conforming to Hardy-Weinberg equilibrium ($P > 0.05$). Reviews and case reports were excluded.

The following data were extracted from each eligible study: first author's name, year of publication, study design, geographic location or ethnicity of study population, sample size, and frequency of allele in cases and controls. Heterogeneity across all the eligible studies was estimated by the Cochran's *Q* statistic. Heterogeneity was considered evident at $P < 0.05$ for the *Q* statistic. Random-effect model was used when heterogeneity among studies existed; otherwise, fixed-model was utilized. The allele model with combined ORs with 95% CIs

Table 3 Basic characteristics of study population

Variables	Patients ($n = 164$)
Female, *n* (%)	83 (50.61)
Age, year, *X* (SD)	33.80 (16.10)
SAA, *n* (%)	76 (46.30)
NSAA, *n* (%)	88 (53.70)
Patients with IST, *n* (%)	54 (32.90)
Patients with over 3-month follow-up, *n* (%)	40 (24.40)
RBCs ($\times 10^{12}$/l), *X* (SD)	2.24 (0.84)
ARCs ($\times 10^{12}$/l), *M* (P_{25}–P_{75})	0.08 (0.01–0.50)
MCV (fl), *X* (SD)	99.40 (13.64)
RDW-CV, *M* (P_{25}–P_{75})	16.10 (14.10–17.60)
HGB (g/l), *X* (SD)	75.17 (27.29)
WBCs ($\times 10^{9}$/l), *X* (SD)	2.45 (1.39)
ALCs ($\times 10^{9}$/l), *X* (SD)	1.20 (0.70)
ANCs ($\times 10^{9}$/l), *M* (P_{25}–P_{75})	1.51 (0.36–1.54)
PLT ($\times 10^{9}$/l), *M* (P_{25}–P_{75})	26.80 (9.00–31.00)

was used to assess the association between the investigated eight SNPs and AA risk. *P* value was two sides and $P < 0.05$ was considered statistically significant.

Results

Characteristics of the study population

During our study period, we consecutively recruited a total of 180 AA patients. Out of the 180 patients, 4 patients were not Han nationality, 3 patients suffered from PRCA, 3 patients were diagnosed AA/PNH syndrome, and 4 patients were because of specimen failure of DNA extraction. After excluding these ineligible patients, a total of 164 patients were included in the current study, including 88 NSAA and 76 SAA. The characteristics of the patients were summarized in Table 3.

The association between genetic polymorphisms of *IL23R* (*rs*11209032, *rs*1884444); *STAT3* (*rs*1053005, *rs*744166, and *rs*4796793); *IL12B* (*rs*3212227); and *STAT4* (*rs*7574865, *rs*897200) and AA risk

The genotypic distribution did not deviate from the Hardy-Weinberg equilibrium for the eight target SNPs in the cases and controls (Table 4). The minor allele frequency of the eight SNPs were similar to the ones reported by NCBI database and some articles in Chinese population [10, 12, 15]. There was no statistically significant difference in genotype and allele frequency for the SNPs of *rs*11209032 (C > A), *rs*1884444 (G > T), *rs*1053005 (C > T), *rs*744166 (G > A), *rs*4796793 (G > C), *rs*3212227 (G > T), *rs*897200 (C > T) in the 164 AA cases and 211 controls. Our current study showed that T allele and TT genotype of *rs*7574865 variant were more frequent in the 164 cases than in the 211 controls (42.7 vs 34.6%; 18.9 vs 10.5%). In the additive model, individual carrying the *rs*7574865 T allele demonstrated a 37% (OR (95% CI) = 1.37 (1.02–1.85), *P*per = 0.036) increased AA risk. In the recessive model, carrier with *rs*7574865 TT genotype had an increased AA risk with an OR of 2.08 (OR (95%CI) = 2.08 (1.14–3.70), *P*per = 0.017). In the

Table 4 The results of HWE of the genotypic distribution of 8 SNPs (*rs*1884444 (G > T), *rs*11209032 (G > A), *rs*7574865 (T > G), *rs*897200 (C > T), *rs*3212227 (G > T), *rs*744166 (G > A), *rs*1053005 (C > T), and *rs*4796793 (G > C)) in the cases and controls

SNP ID	Genotype	Cases (*n*, 164)	Controls (*n*, 211)	χ^2	*P* value	SNP ID	Genotype	Cases (*n*, 164)	Controls (*n*, 211)	χ^2	*P* value
*rs*1884444	TT	64	84	0.20*	0.66*	*rs*11209032	AA	42	57	0.02*	0.88*
	GT	79	101	0.21**	0.61**		AG	82	99	0.80**	0.37**
	GG	21	26				GG	40	55		
*rs*7574865	TT	55	87	0.13*	0.72*	*rs*897200	TT	62	82	2.83*	0.09*
	TC	78	102	0.98**	0.32**		TC	69	93	1.17**	0.27**
	CC	31	22				CC	33	36		
*rs*3212227	TT	57	74	0.99*	0.32*	*rs*744166	AA	56	67	0.197	0.69
	GT	74	90	3.72**	0.06**		AG	82	97	1.100	0.29
	GG	33	47				GG	26	47		
*rs*1053005	TT	66	84	0.19*	0.66*	*rs*4796793	CC	64	72	0.35*	0.55*
	TC	74	90	2.21**	0.14**		CG	74	93	2.32**	0.13**
	CC	24	37				GG	26	46		

**P* value of HWE in the cases; ***P* value of HWE in the controls

Table 5 The association between *rs*1884444 (G > T), *rs*11209032 (G > A), *rs*7574865 (T > G), and *rs*897200 (C > T) polymorphisms and AA risk

SNP ID	Genotype/allele	Cases (164, %)	Controls (221, %)	Additive model		Recessive model		Dominant model	
				OR (95% CI)	P_{per}	OR (95% CI)	P_{per}	OR (95% CI)	P_{per}
rs1884444	TT	64 (39.0)	84 (39.8)	1.01 (0.75–1.37)	0.926	1.06 (0.56–2.02)	0.889	0.99 (0.63–1.55)	0.972
	GT	79 (48.2)	101 (47.9)						
	GG	21 (12.8)	26 (12.3)						
	T	207 (63.1)	269 (63.7)						
	G	121 (36.9)	153 (36.3)						
rs11209032	AA	42 (25.6)	57 (27.1)	0.99 (0.75–1.33)	0.948	0.93 (0.57–1.52)	0.773	1.09 (0.66–1.80)	0.734
	AG	82 (31.2)	99 (46.8)						
	GG	40 (15.2)	55 (26.1)						
	A	166 (50.6)	213 (50.5)						
	G	162 (49.4)	209 (49.5)						
rs7574865	GG	55 (33.5)	87 (41.2)	1.37 (1.02–1.85)	0.036	2.08 (1.14–3.79)	0.017	1.42 (0.93–2.19)	0.108
	GT	78 (47.6)	102 (48.3)						
	TT	31 (18.9)	22 (10.5)						
	G	188 (57.3)	276 (65.4)						
	T	140 (42.7)	146 (34.6)						
rs897200	TT	62 (37.8)	82 (38.9)	1.08 (0.08–1.44)	0.627	1.20 (0.70–2.05)	0.512	1.03 (0.67–1.58)	0.891
	TC	69 (42.1)	93 (44.0)						
	CC	33 (20.1)	36 (17.1)						
	T	193 (58.8)	257 (60.9)						
	C	135 (41.2)	165 (39.1)						

P_{per} denoted empirical significance values on the basis of 10,000 permutations in the three models, respectively

Table 6 The association between *rs*3212227 (G > T), *rs*744166 (G > A), *rs*1053005 (C > T), and *rs*4796793 (G > C) polymorphisms and AA risk

SNP ID	Genotype/allele	Cases (164, %)	Controls (221, %)	Additive model		Recessive model		Dominant model	
				OR (95% CI)	P_{per}	OR (95% CI)	P_{per}	OR (95% CI)	P_{per}
*rs*3212227	TT	57 (34.8)	74 (35.1)	0.96 (0.72–1.29)	0.801	0.89 (0.53–1.49)	0.651	1.04 (0.67–1.61)	0.859
	GT	74 (45.1)	90 (42.7)						
	GG	33 (20.1)	47 (22.2)						
	T	188 (57.3)	238 (56.4)						
	G	140 (42.7)	184 (43.6)						
*rs*744166	AA	56 (34.1)	67 (31.8)	0.84 (0.63–1.12)	0.227	0.81 (0.31–2.17)	0.679	1.27 (0.58–2.78)	0.554
	AG	82 (50.0)	97 (46.0)						
	GG	26 (15.9)	47 (22.2)						
	A	194 (59.2)	231 (54.7)						
	G	134 (40.8)	191 (45.3)						
*rs*1053005	TT	66 (40.2)	84 (39.8)	0.93 (0.69–1.25)	0.641	0.92 (0.47–1.83)	0.891	1.17 (0.67–2.02)	0.581
	TC	74 (45.2)	90 (42.7)						
	CC	24 (14.6)	37 (17.5)						
	T	206 (62.8)	258 (61.1)						
	C	122 (37.2)	164 (38.9)						
*rs*4796793	CC	64 (39.0)	72 (34.1)	0.801 (0.60–1.07)	0.135	0.80 (0.30–2.17)	0.665	0.60 (0.27–1.32)	0.201
	CG	74 (45.1)	93 (44.1)						
	GG	26 (15.9)	46 (21.8)						
	C	202 (61.6)	237 (56.2)						
	G	126 (38.4)	185 (43.8)						

P_{per} denoted empirical significance values on the basis of 10,000 permutations in the three models, respectively

Table 7 The association between *rs*1884444 (G > T); *rs*11209032 (G > A); *rs*7574865 (T > G), *rs*897200 (C > T), *rs*3212227 (G > T), *rs*744166 (G > A), *rs*1053005 (C > T), and *rs*4796793 (G > C) polymorphisms and AA risk (NSAA vs controls)

SNP ID	Genotype/ allele	NSAA (88, %)	Control (211, %)	χ^2	*P* value	SNP ID	Genotype/ Allele	NSAA (88, %)	Controls (211, %)	χ^2	*P* value
*rs*1884444	TT	36 (40.9)	84 (39.8)	0.180	0.914	*rs*3212227	TT	30 (34.1)	74 (35.1)	0.490	0.783
	GT	40 (45.5)	101 (47.9)				GT	41 (46.6)	90 (42.7)		
	GG	12 (13.6)	26 (12.3)				GG	17 (19.3)	47 (22.2)		
	T	112 (63.6)	269 (63.7)	0.001	0.980		T	101 (57.4)	238 (56.4)	0.049	0.824
	G	64 (36.4)	153 (36.3)				G	75 (42.6)	184 (43.6)		
*rs*11209032	AA	19 (21.5)	57 (27.1)	1.796	0.407	*rs*744166	AA	32 (36.3)	67 (31.8)	0.896	0.639
	AG	40 (45.5)	99 (46.8)				AG	40 (45.5)	97 (46.0)		
	GG	29 (33.0)	55 (26.1)				GG	16 (18.2)	47 (22.2)		
	A	78 (44.3)	213 (50.5)	1.884	0.170		A	104 (59.1)	231 (54.7)	0.955	0.329
	G	98 (55.7)	209 (49.5)				G	72 (40.9)	191 (45.3)		
*rs*7574865	GG	23 (26.1)	87 (41.2)	7.448	0.024	*rs*1053005	TT	33 (37.5)	84 (39.8)	0.378	0.828
	GT	49 (55.7)	102 (48.3)				TC	37 (42.0)	90 (42.7)		
	TT	16 (18.2)	22 (10.5)				CC	18 (20.5)	37 (17.5)		
	G	95 (54.0)	276 (65.4)	6.885	0.009		T	103 (58.5)	258 (61.1)	0.355	0.551
	T	81 (46.0)	146 (34.6)				C	73 (41.5)	164 (38.9)		
*rs*897200	TT	36 (40.9)	82 (38.9)	0.255	0.880	*rs*4796793	CC	37 (42.1)	72 (34.1)	1.683	0.431
	TC	36 (40.9)	93 (44.0)				CG	34 (38.6)	93 (44.1)		
	CC	16 (18.2)	36 (17.1)				GG	17 (19.3)	46 (21.8)		
	T	108 (61.4)	257 (60.9)	0.011	0.916		C	108 (61.4)	237 (56.2)	2.206	0.137
	C	68 (38.6)	165 (39.1)				G	68 (38.6)	185 (43.8)		

stratified analysis, our study found that *rs*7574865 T allele and TT genotype were mainly associated with NSAA occurrence. Specific results are shown in Tables 5, 6, 7, and 8.

Table 8 The association between *rs*1884444 (G > T), *rs*11209032 (G > A), *rs*7574865 (T > G), *rs*897200 (C > T), *rs*3212227 (G > T), *rs*744166 (G > A), *rs*1053005 (C > T), and *rs*4796793 (G > C) polymorphisms and AA risk (SAA vs controls)

SNP ID	Genotype/ allele	SAA (76, %)	Control (211, %)	χ^2	*P* value	SNP ID	Genotype/ allele	SAA (76, %)	Control (211, %)	χ^2	*P* value
*rs*1884444	TT	29 (38.2)	84 (39.8)	0.102	0.950	*rs*3212227	TT	27 (35.5)	84 (39.8)	3.412	0.182
	GT	38 (50.0)	101 (47.9)				GT	33 (43.4)	101 (47.9)		
	GG	9 (11.8)	26 (12.3)				GG	16 (21.1)	26 (12.3)		
	T	96 (63.2)	269 (63.7)	0.017	0.898		T	87 (57.2)	269 (63.7)	2.009	0.156
	G	56 (36.8)	153 (36.3)				G	65 (42.8)	153 (36.3)		
*rs*11209032	AA	23 (30.3)	57 (27.1)	4.269	0.118	*rs*744166	AA	24 (31.6)	57 (27.1)	0.446	0.800
	AG	42 (55.2)	99 (46.8)				AG	42 (55.3)	99 (46.8)		
	GG	11 (14.5)	55 (26.1)				GG	10 (13.1)	55 (26.1)		
	A	88 (57.9)	213 (50.5)	2.467	0.116		A	90 (59.2)	213 (50.5)	3.422	0.064
	G	64 (42.1)	209 (49.5)				G	62 (40.8)	209 (49.5)		
*rs*7574865	GG	32 (42.1)	87 (41.2)	5.037	0.081	*rs*1053005	TT	33 (43.4)	87 (41.2)	0.432	0.806
	GT	29 (38.2)	102 (48.3)				TC	37 (48.7)	102 (48.3)		
	TT	15 (19.7)	22 (10.5)				CC	6 (7.9)	22 (10.5)		
	G	93 (61.2)	276 (65.4)	0.866	0.352		T	103 (67.8)	276 (65.4)	0.278	0.598
	T	59 (38.8)	146 (34.6)				C	49 (32.2)	146 (34.6)		
*rs*897200	TT	27 (35.5)	82 (38.9)	1.066	0.587	*rs*4796793	CC	27 (35.5)	82 (38.9)	2.017	0.365
	TC	32 (42.1)	93 (44.0)				CG	40 (52.6)	93 (44.0)		
	CC	17 (22.4)	36 (17.1)				GG	9 (11.9)	36 (17.1)		
	T	86 (56.6)	257 (60.9)	0.868	0.352		C	94 (61.8)	257 (60.9)	0.042	0.838
	C	66 (43.4)	165 (39.1)				G	58 (38.2)	165 (39.1)		

Table 9 The association between *rs*1884444 (G > T), *rs*11209032 (G > A), *rs*7574865 (T > G), *rs*897200 (C > T), *rs*3212227 (G > T), *rs*744166 (G > A), *rs*1053005 (C > T) and *rs*4796793 (G > C) polymorphisms and AA severity (NSAA vs SAA)

SNP ID	Genotype/ allele	NSAA (88, %)	SAA (76, %)	χ^2	*P* value	SNP ID	Genotype/ allele	NSAA (88, %)	SAA (76, %)	χ^2	*P* value
*rs*1884444	TT	36 (40.9)	29 (38.2)	0.358	0.836	*rs*3212227	TT	30 (34.1)	27 (35.5)	0.176	0.916
	GT	40 (45.5)	38 (50.0)				GT	41 (46.6)	33 (43.4)		
	GG	12 (13.6)	9 (11.8)				GG	17 (19.3)	16 (21.1)		
	T	112 (63.6)	96 (63.2)	0.008	0.929		T	101 (57.4)	87 (57.2)	0.001	0.978
	G	64 (36.4)	56 (36.8)				G	75 (42.6)	65 (42.8)		
*rs*11209032	AA	19 (21.5)	23 (30.3)	7.693	0.021	*rs*744166	AA	32 (36.3)	24 (31.6)	1.707	0.426
	AG	40 (45.5)	42 (55.2)				AG	40 (45.5)	42 (55.3)		
	GG	29 (33.0)	11 (14.5)				GG	16 (18.2)	10 (13.1)		
	A	78 (44.3)	88 (57.9)	6.014	0.014		A	104 (59.1)	90 (59.2)	0.000	0.982
	G	98 (55.7)	64 (42.1)				G	72 (40.9)	62 (40.8)		
*rs*7574865	GG	23 (26.1)	32 (42.1)	5.786	0.055	*rs*1053005	TT	33 (37.5)	33 (43.4)	5.150	0.076
	GT	49 (55.7)	29 (38.2)				TC	37 (42.0)	37 (48.7)		
	TT	16 (18.2)	15 (19.7)				CC	18 (20.5)	6 (7.9)		
	G	95 (54.0)	93 (61.2)	1.732	0.188		T	103 (58.5)	103 (67.8)	0.051	0.821
	T	81 (46.0)	59 (38.8)				C	73 (41.5)	49 (32.2)		
*rs*897200	TT	36 (40.9)	27 (35.5)	0.677	0.713	*rs*4796793	CC	37 (42.1)	27 (35.5)	3.652	0.161
	TC	36 (40.9)	32 (42.1)				CG	34 (38.6)	40 (52.6)		
	CC	16 (18.2)	17 (22.4)				GG	17 (19.3)	9 (11.9)		
	T	108 (61.4)	86 (56.6)	0.773	0.379		C	108 (61.4)	94 (61.8)	0.008	0.929
	C	68 (38.6)	66 (43.4)				G	68 (38.6)	58 (38.2)		

The association between genetic polymorphisms of *IL23R* (*rs*11209032, *rs*1884444); *STAT3* (*rs*1053005, *rs*744166, and *rs*4796793); *IL12B* (*rs*3212227) and *STAT4* (*rs*7574865, *rs*897200) and AA severity

To assess the association between these eight SNPs polymorphisms and AA severity, we compared the frequency distribution of alleles and genotypes of these eight SNPs between SAA and NSAA cases. Consequently, there was no statistically significant difference in allele and genotype frequency for the SNPs of *rs*1884444 (G > T), *rs*1053005 (C > T), *rs*744166 (G > A), *rs*4796793 (G > C), *rs*3212227 (G > T), *rs*897200 (C > T) in the 76 SAA cases and 88 NSAA ones. Our current study demonstrated

Table 10 The results of *rs*1884444 (G > T), *rs*11209032 (G > A), *rs*7574865 (T > G), *rs*897200 (C > T), *rs*3212227 (G > T), rs744166 (G > A), *rs*1053005 (C > T) and *rs*4796793 (G > C) polymorphisms and IST outcome of AA patients

Variables	Genotype			Allele		χ^2	*P* value	Variables	Genotype			Allele		χ^2	*P* value
*rs*744166	AA	AG	GG	A	G			*rs*1053005	TT	TC	CC	T	C		
Non-response	8	5	6	21	17	8.254*	0.012*	Non-response	9	7	3	25	13	2.280*	0.320*
Response	4	15	2	23	19	0.002**	0.964**	Response	8	12	1	28	14	0.007**	0.934**
*rs*7574865	GG	GT	TT	G	T			*rs*897200	TT	TC	CC	T	C		
Non-response	7	7	5	21	17	0.508*	0.776*	Non-response	7	9	3	23	15	0.902*	0.637*
Response	6	10	5	22	20	0.067**	0.796**	Response	9	7	5	25	17	0.008**	0.927**
*rs*1884444	TT	GT	GG	T	G			*rs*3212227	TT	GT	GG	T	G		
Non-response	9	9	1	27	11	2.777*	0.249*	Non-response	5	10	4	20	18	3.601*	0.165*
Response	5	13	3	23	19	2.259**	0.133**	Response	8	5	8	21	21	0.055**	0.814**
*rs*11209032	AA	AG	GG	A	G			*rs*4796793	CC	CG	GG	C	G		
Non-response	7	11	1	25	13	6.124*	0.047*	Non-response	10	6	3	26	12	2.907*	0.234*
Response	4	10	8	18	26	5.061**	0.024**	Response	6	12	3	24	18	1.083**	0.298**

* SNP genotype is associated with the IST outcome of AA patients; ** SNP allele is associated with the IST outcome of AA patients

Table 11 The results of the basic characteristic and IST outcome of AA patients

Variables	Non-response (*n*, 19)	Response (*n*, 21)	χ^2/F/Z value	*P* value
Onset age	29.84 ± 16.52	33.67 ± 15.62	− 0.753	0.456
Sex (men)	9	14	1.520	0.218
PLT (× 10^9/l), *M* (P_{25}–P_{75})	14 (6–31)	13 (8.5–37)	− 0.244	0.807
RBCs (× 10^{12}/l), *X* (SD)	2.23 ± 0.83	2.49 ± 1.12	− 0.818	0.419
HGB (g/l), *X* (SD)	74.16 ± 25.28	82.00 ± 33.29	− 0.832	0.411
WBCs (× 10^9/l), *X* (SD)	2.33 ± 1.95	2.48 ± 1.16	− 0.296	0.769
ALCs (× 10^9/l), *X* (SD)	1.11 ± 0.69	1.08 ± 0.75	0.110	0.913
ANCs (× 10^9/l), *X* (SD)	1.10 ± 1.40	1.25 ± 0.77	− 0.422	0.676
ARCs (× 10^{12}/l), *M* (P25–P75)	0.029 (0.007–0.054)	0.027 (0.012–0.052)	− 0.176	0.860
RDW-CV *X* (SD)	15.36 ± 2.49	16.08 ± 2.50	− 0.901	0.373
MCV (fl), *X* (SD)	94.80 ± 13.85	99.52 ± 9.31	− 1.277	0.209

that G allele and GG genotype of *rs*11209032 variant were more frequent in the 88 NSAA cases than in the 76 SAA ones (42.1 vs 55.7%; 14.5 vs 33.0%). Specific results are shown in Table 9.

The results of affected factors of IST outcome of AA patients

During our follow-up period, a total of 54 patients received IST. Out of the 54 patients, 13 patients were treated with ATG/ALG, 3 patients with ATG combined with CsA, and 38 patients with CsA administration. A total of 14 patients lost follow-up or the follow-up time was less than 3 months. Finally, we assessed the 3-month immunosuppressive treatment outcome of the 40 AA patients; 21 patients achieved hematological response and 19 ones did not. The response rate was 52.5%. Our study found G allele and GG genotype of *rs*11209032 variant, and GG genotype of *rs*744166 site were associated with the IST outcome of AA patients. Our current study did not show that these factors of patient onset age, sex, PLTs, RBCs, HGB, WBCs, ALCs, ANCs, ARCs, RDW, and MCV were associated with IST outcome of AA patients. Specific results are shown in Tables 10 and 11.

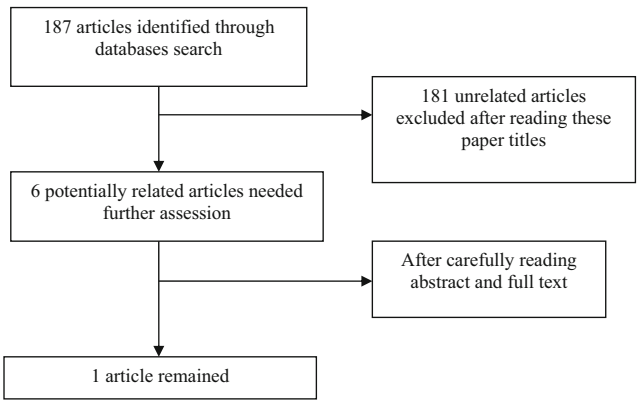

Fig. 1 Specific screening flow diagram of articles in meta-analysis

The results of meta-analysis

As shown in Fig. 1, a total of 187 articles were retrieved. After reading the titles, 181 unrelated studies were excluded. After further reading the abstract and full text of the remaining six papers, only one paper which focused on the association between *rs*7574865 polymorphism and AA risk, met the inclusion criteria [16]. Our meta-analysis included the Feng's and our current study, consisting of 366 AA patients and 406 controls. The basic information of included studies was shown in Table 12. We found there was no heterogeneity between studies; therefore, we utilized fixed-model to assess the association between *rs*7574865 polymorphism and AA risk in the combined population. Our meta-analysis also showed T allele and TT genotype increased AA risk. In the additive model, individual carrying the *rs*7574865 T allele demonstrated a 44% (OR (95% CI) = 1.44 (1.17–1.78), $P = 0.001$) increased AA risk. In the recessive model, carrier of *rs*7574865 TT genotype had an increased AA risk with an OR (95% CI) of 1.81 (1.15–2.85, $P < 0.001$). Specific results are summarized in Figs. 2 and 3.

Discussion

STAT4, as a critical immune regulation factor in some signal pathways, was first found in GWAS that *rs7574865* (T > G)genetic polymorphism involved in rheumatic arthritis and systemic lupus erythematosus occurrence [17]. Studies have shown similar autoimmune diseases share the same susceptibility genes [18]. Since then, plenty of researchers also reported that *STAT4* genetic polymorphisms were associated with some other

Table 12 The results of Meta-analysis of *rs*7574865 polymorphism and AA risk

Variables	Cases (GG/GT/TT)	G/T	Controls (GG/GT/TT)	G/T	P_{hetero}	C_{OR}	Cp
S1	55:78:31	188:140	87:102:22	276:146	0.81*	1.44 (1.17, 1.78)*	< 0.001*
S2	66:114:22	246:158	91:90:14	272:118	0.61**	1.81 (1.15–2.85)**	< 0.001**
Sc	121:192:53	434:298	178:192:36	548:264			

*P value of heterogeneity test under the additive model;**P value of heterogeneity test under the recessive model. S1represented the current study; S2 denoted Feng's study. C_{OR}* denoted the OR value under the additive model; C_{OR}** denoted the OR value under the recessive model; Cp* represented the P value under the additive model; Cp** represented the P value under the recessive model

autoimmune diseases [19–21]. Our current data showed T allele and TT genotype of rs*7574865* increased AA risk. In the additive model, individual carrying the *rs7574865* T allele demonstrated a 37% increased AA risk. In the recessive model, carrier of *rs7574865* TT genotype had an increased AA risk with an OR of 2.08. In the stratified analysis, we found that T allele and TT genotype of *rs7574865* were mainly associated with NSAA occurrence. Our current study results are consistent with the Feng's [16]. To avoid false positive results caused by small sample size, we performed the Meta-analysis by combining our current study and Feng's, finding T allele and TT genotype of *rs7574865* really increased AA risk. Previous study results have demonstrated *rs7574865* T allele was associated with high expression of gene of *TBX21*. Gene *TBX21* could regulate the differentiation of transcript factor of Th1 cells [22]. However, due to our current limited study condition, we did not perform further research about how *rs7574865* affect AA occurrence.

IL-23R mainly mediates the intracellular signal transduction of IL-23, which could promote various cells differentiation and development and inflammatory cytokines secretion through STAT3 signal pathways [23]. Our current data showed individuals carrying *rs*11209032 G allele and GG genotype could protect them from NSAA to SAA development. Maybe, due to our current small sample size, we did not find the association between *rs*11209032 genetic polymorphisms and AA occurrence, but AA severity. Although, many researchers have reported *rs*11209032 genetic polymorphisms related to some T lymphocyte cell-mediated autoimmune diseases, few studies explore the specific function mechanism of *rs*11209032 site. We predict *rs*11209032 location is the transcription factor binding site by SNPinfo software, indicating a possible role in affecting the transcription factor action and mRNA expression, even the translation of related protein, finally impacting the diseases occurrence and development. However, our current study results need to be further verified by larger sample, multiple nationalities, and multi-area studies.

In our evaluation of IST outcome of AA patients, the hematological response rate was 52.5%, different with those reported by Scheinberg [24] and Atta [25]. We found G allele and GG genotype of *IL23R-rs*11209032 were associated with better IST outcome of AA patients. By contrast, GG genotype of *STAT3-rs*744166 was associated with IST failure of AA patients. Previous researchers have reported that the onset age and ARCs, and ALCs of AA patients could be the predictive factors of IST outcome [26]. Our current study was unable to show the association. These different study findings may be caused by following reasons. Firstly, different treatment regimens (ATG/ALG, ATG/ALG + CsA, just CsA), recruited study subjects, and follow-up time maybe the main reasons of different response rates. Secondly, studies suggest that the treatment effect of horse ATG is better than rabbit ATG; ATG from different sources may have different response rates [24, 25]. Thirdly, different drug dose and response of study population may lead to different response effect. Lastly, our current study retrospectively collected the data of AA patients, some patients

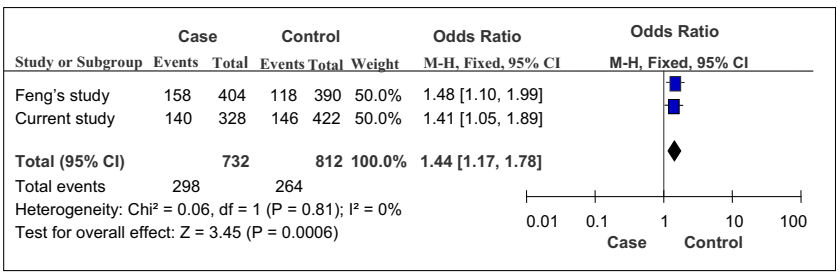

Fig. 2 The results of meta-analysis of *rs7574865* polymorphisms and AA risk under additive model

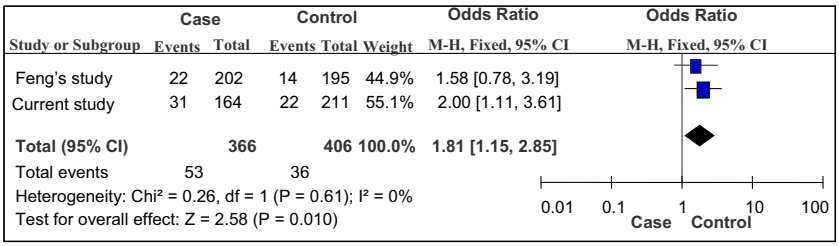

Fig. 3 The results of meta-analysis of *rs7574865* polymorphisms and AA risk under recessive mode

did not have ARCs tests in the first clinic appointment, maybe, causing the result difference of many researches.

Author contributions Li Zhao and Chunxia Chen performed the research. Li Zhao and Li Qin designed the research study. Xiaojun Lu, Huanling Zhu, and Yuming Sun contributed essential reagents or tools. Li Zhao, Lixin Wang, Bing Han, and Bin Tan analyzed the data. Li Zhao and Chunyan Huang wrote the paper.

Compliance with ethical standards All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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