Immuno-Related Gene Polymorphisms associated with Acute Myeloid Leukemia

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Short Title: SNPs-AML

Abbreviations

AML	acute myeloid leukemia
SNPs	single-nucleotide polymorphisms
WBC	white blood cell
OS	overall survival
Tregs	regulatory T cells
Th	T helper
GWAS	genome-wide association studies
ALL	acute lymphocytic leukemia
MM	multiple myeloma
MAF	minor allele frequency
BMMCs	bone marrow mononuclear cells
HWE	Hardy–Weinberg Equilibrium
ORs	odds ratios
CI	confidence interval
K-W	Kruskal-Wallis

CR	complete remission
HGB	hemoglobin
PLT	platelet
LDH	lactate dehydrogenase
IL	Interleukin
WHO	World Health Organization

Summary

Though the pathogenesis of acute myeloid leukemia (AML) is still unknown, accumulating evidence has revealed that immune response plays a vital part in the pathogenesis. Here, we investigated the involvement of 24 single-nucleotide polymorphisms (SNPs) of immuno-related genes, including cytokines (*IL2, IL4, IL9, IL-12A, IL-22, IFNG,* and *TGFB1*), transcriptional regulatory genes (*TBX21, STAT1, STAT3, STAT5B, STAT6, GATA3, FOXP3,* and *IRF4*), and others (*IL2RA IL6R NFKBIA*), in 269 AML inpatients and 200 healthy controls. Furthermore, we analyzed the relationship between the SNPs and clinical characteristics. Immuno-related SNP genotyping was performed on the Sequenom MassARRAY iPLEX platform. All the SNPs in healthy controls were consistent with Hardy–Weinberg equilibrium. All final *p* values were adjusted by Bonferroni multiple testing. Our results showed that IL-22 (rs2227491) was significantly associated with the white blood cell (WBC) counts. STAT5B (rs6503691) showed a close relationship with the recurrent genetic abnormalities in patients with AML. We verified the negatively independent effect of age and risk of cytogenetics on overall survival (OS). More importantly, the GG genotype of IL-12A (rs6887695) showed a negative impact on AML prognosis independently. Furthermore, the relative expression of IL-12 was decreased in GG genotype, no matter under codominant or recessive model. However, no correlation was observed between the SNPs mentioned above and disease susceptibility, risk stratification, and survival. Our findings suggest that immuno-related gene

polymorphisms are associated with prognosis in AML, which may perform as novel inspection targets for AML patients.

1 Introduction

Acute myeloid leukemia (AML) is a clonal, abnormally differentiated malignant hematopoietic disease, and is characterized by recurrent cytogenetic abnormalities[1, 2]. The enormous molecular heterogeneity of the disease has become increasingly apparent over the past 15 years, including mutations in *FLT3*, *NPM1*, *KIT*, *CEBPA*, and *TET2*[3]. Fortunately, some therapies that target the mutations have been applied to patients with AML[4]. Although the innovative formulation in AML therapy has made progress, the overall treatment efficacy in AML patients is disappointing. Therefore, it is of vital importance to explore its pathological mechanism and find novel therapeutic targets for AML.

Abundant studies have reported that immune imbalance exists in the development of malignant tumors[5]. Immune system impairment exists in AML patients, and T cells, the most important component of the immune system, are found to be numerically and functionally defective[6, 7]. These defects have been reported to influence the effect of regulatory T cells (Tregs) that suppress the proliferation and function of T helper (Th) cells[8, 9]. In addition, numerous cytokines exert profound effects in the progression of hematopoietic malignancies, especially AML[10]. More importantly, targeting the immune molecules or cells has preliminarily been shown to be an effective therapy against AML,

both experimentally and clinically. Therefore, further investigation in the function of the immune system in the pathological mechanism or therapy is required.

Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variants and their functions have been gradually uncovered in many fields of biology, especially in human diseases[11-13]. The roles of inflammatory or immuno-related SNPs have been investigated in AML patients and susceptibility, prognosis, and survival related-SNPs were determined[14, 15]. IL-1β (rs16944) GA genotype contributed to the cytogenetics favorable-risk and the GT genotype of IL18 (rs1946518) statistically led to a poorer AML-specific survival[15]. Zhu et al. reported that G single mutant and GG mutation homozygote of IL-17F were related with AML susceptibility[14]. Nursal et al. suggested that functional variants of the TNF-α, IL-10, and TGF-β1 may have a significant association with the etiopathogenesis of AML[16]. Therefore, we further investigated the roles of SNPs of immuno-related cytokines or transcription factors in AML. The nominated gene variants, summarized in Table 2, have different functions and implicate some yet-unanticipated functions in AML pathogenesis, including pro-inflammatory or anti-inflammatory cytokines and key regulatory factors of T cell subsets. However, some of them have been investigated in other malignant blood diseases. G allele of rs4487645 that modulates IRF4-binding affinity and regulates CDCA7L expression might confer susceptibility to multiple myeloma (MM)[17]. Two genome-wide association studies (GWAS) showed that rs3824662 of GATA-3 was identified as a susceptibility

locus for acute lymphocytic leukemia (ALL) with independent validation[18, 19]. In addition, our group has previously reported the associations between NLRP3 inflammasome and certain kinds of diseases in the hematopoietic system, including AML, ALL, and MM[15, 20].

To better understand the potential unique etiology in AML, we performed genotyping by primer-extension mass spectrometry using the Sequenom MassARRAY platform[21] at 24 candidate SNPs to provide genotyping accuracy in 269 AML cases and 200 healthy controls. In this study, Hardy-Weinberg equilibrium and minor allele frequency (MAF) were used to evaluate the applicability of the 24 candidate SNPs. Given that immune disorder may be involved in AML, we hypothesized that these SNPs were also associated with AML. Therefore, we analyzed the relationship among disease susceptibility, routine blood, risk stratification or survival analysis, and SNPs. Furthermore, combined with mRNA expression and functional analysis, we further determined the possible signal pathways that showed the involvement of these SNPs in AML. These findings indicate that SNPs may contribute to the clinical relevance in AML.

2 Materials and methods

2.1 Characteristics of the Study Group

For the detection of genetic polymorphisms, 269 AML inpatients (147 males, 122

females) with a median age of 46 (16-80) years were recruited in the study at Qilu Hospital, Shandong University, China, from July 2010 to June 2017. Final diagnoses of patients with AML were confirmed by WHO classification system (version 2016) and NCCN guidelines (version 3.2017)[22, 23]. Accordingly, 200 healthy controls (108 females and 92 males) with a median age of 44.5 (20-75) years were enrolled in the study. The characteristics of AML patients and healthy controls were shown in Table 2. Furthermore, routine blood, risk stratification and survival analysis of patients with AML were also shown in the tables. All participants were informed about the purpose of the study. The study was realized with the approval of the institutional ethics committee. Informed consent was obtained from every patient before his/her enrollment in the study in accordance with the Declaration of Helsinki.

2.2 DNA extraction and genotyping

Genomic DNA was isolated from peripheral blood leukocytes by standard salting out method following the manufacturer's instruction of TIANamp blood DNA kit (Tiangen Biotech, Beijing, China). SNP genotyping used Sequenom iPLEX and MALDI-TOF-based Mass-ARRAY platform (BGI Tech., Beijing, China), which is based on a multiplex PCR reaction, a locus specific single-base extension reaction, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and allowed analysis of up to 40 SNPs in a single well reaction. Primers were designed using Assay Design Suite v2.0 (Agena Bioscience, USA) available from online tools of

Agena Bioscience (https://www.mysequenom.com/Tools). Six negative controls and six positive controls were interspersed with the study samples to ensure accuracy. Moreover, 16 samples selected from the study group were detected in two independent test panels, achieving 99% reproducibility.

2.3 RNA extraction and real-time RT-PCR

Total RNA of bone marrow mononuclear cells (BMMCs) was isolated by TRIzol™ Reagent (15596026#, Invitrogen, US) and converted into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A#, Takara, Japan) according to the manufacturer's instructions. The relative expression of mRNA was determined by Roche 480II Light Cycler System using SYBR Green (Toyobo, Japan) as a double-strand DNA-specific binding dye. The PCR reactions were cycled 40 times after the initial denaturation (95°C, 10 min) at 95°C for 15 s and at 60°C for 30 s. Primers in the study were designed by Primer premier 5.0 (PREMIER Biosoft. http: //www.premierbiosof- t.com/primerdesign) and were intron spanning. The primers for IL12 and β-actin are as follows: IL12 forward: GCCTCAGGTGAGATTCTCGG, IL12 reverse: ATAGGGCATCTTCCCCAGGT; β-actin forward: CACCAACTGGG-ACGACAT, β-actin reverse: GCACAGCCTGGATAGCAAC. The amplification efficiencies between the target genes and the reference control (β-actin) were compared for delta Ct (ΔCt) calculation.

2.4 Statistical analysis

All the 24 candidate SNPs were tested for Hardy–Weinberg Equilibrium (HWE) by Pearson's goodness-of-fit, chi-squared ($\chi 2$) test. SNPs within HWE ($p > 0.05$) and adequately common (Minor Allele Frequency, MAF > 5%) in the general population were included in the study. Relationships between the phenotype or allele frequency of SNPs and AML susceptibility, routine blood, risk stratification or survival analysis were determined statistical strategy as followed: Preliminary screening by a chi-squared ($\chi 2$) test or a Fisher's exact test; Univariate binary logistic regression analyses were used to analyze odds ratios (ORs) with a corresponding 95% confidence interval (95% CI) and adjust for the age and gender; The p value needs to be adjusted by Bonferroni multiple testing to counteract. In the study, p value was a stricter threshold; Multiple binary logistic regression was used to counteract interference from multiple independent variables. A Cox proportional hazards model was used to perform the multivariate analysis of AML prognosis. The gene expression was calculated by Kruskal-Wallis (K-W) test. A two-tailed $p < 0.05$ (or adjusted by Bonferroni multiple testing) was considered statistically significant. All statistical analyses were performed using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Study Population

The characteristics of AML inpatients and controls are shown in Table 1. There was no

statistical difference in the distribution of age (median 46, range 18-80 vs median 45.5 years, range 20-75 years) and gender (male/female: 147/108 vs 108/92) between AML patients and controls. The design was a hospital-based case-control study, and the possibility of selection bias cannot be ruled out. Therefore, rs2104286, rs2232365 and rs3761549 were not included in further analysis for the deviation of HWE in controls (Table 2). In addition, the MAF of rs1859430 was less than 5%, which did not suit the HapMap project (Table 2).

3.2 Association between immuno-related SNPs and AML susceptibility

Four genetic models were used to analyze the association between immuno-related SNPs and AML. We analyzed the relationship between every single locus and the susceptibility to AML. Preliminary screening by a chi-squared ($\chi 2$) test or a Fisher's exact test showed that genotypic frequencies of rs2070874 and rs2243250 in IL4 and rs1179251 in IL-22 under the dominant model were significantly associated with susceptibility to AML ($p < 0.05$). Univariate logistic regression analysis revealed that only rs2243250 in IL-4 under dominant model was significantly associated with susceptibility to AML after adjusting for age and gender (OR = 0.66, 95% CI = 0.45-0.96, p = 0.03). However, among the above SNPs, there was no SNP contributing to the susceptibility of AML after Bonferroni multiple correction. (Table 3)

3.3 Association between immuno-related SNPs and tumor burden of AML

Acute leukemia is characterized by increased cellularity of peripheral white blood cells (WBC), mild anemia, and thrombocytopenia. We analyzed the relationship between immuno-related SNPs and leukemia cell burden. As reported, the complete remission (CR) achievement and median OS were affected by BM blasts within AML patients [24]. Of the recruited cases, 89% has a high percentage (≥42%) of BM blasts at diagnosis, which was referred to hereafter as the "high BM blasts" group; 11% has a low percentage (＜42%) of BM blasts at diagnosis, which was referred to hereafter as the "low BM blasts" group. In the study, genotypic frequencies of rs2069762 in IL-2 under recessive model revealed statistically significant difference between high and low BM blasts groups ($p < 0.05$). When the AA/AC genotype was used as a reference, the CC genotype was significantly associated with BM blasts after adjusting for the age and gender ($p = 0.038$). However, rs2069762 in IL2 under recessive model showed no statistical significance with the BM blasts at diagnosis after Bonferroni multiple correction. (Table 4)

Marked hyperleukocytosis (WBC counts >100 × 10^9/L) confers to poor prognosis of de novo non-M3 AML[25, 26]. Th high level of peripheral infiltration of leukemia cell burden was defined as WBC counts ≥100 × 10^9/L, whereas the low level was defined as WBC counts < 100 × 10^9/L in this study. Preliminary screening showed that genotypic frequencies of rs2227491 in IL-22 under the codominant, dominant, and allelic models were significantly associated with peripheral infiltration of leukemia cell burden ($p <$

0.05). In addition, allelic frequencies of rs2243250 in IL-4 were significantly associated with peripheral infiltration of leukemia cell burden (p<0.05). After Bonferroni multiple correction, genotypic frequencies of rs2227491 in IL-22 under both dominant and allelic models were also significantly associated with peripheral infiltration of leukemia cell burden (pad just < 0.003). Next, we analyzed the peripheral infiltration of leukemia cell burden in different genotypes of rs2227491. We found that after adjusting for age and gender, AML patients carrying the TT genotype of IL-22 rs2227491 showed a 4.2-fold increased risk of high level of peripheral infiltration compared with patients carrying major genotype TC/CC in the dominant model (OR = 4.2, 95% CI = 1.91-9.28, p = 0.0004). Rs2227491 allelic distribution also showed a statistically significant difference (p = 0.002). (Table 4)

3.4 Association between hemoglobin, lactate dehydrogenase and SNPs in AML

Clinical variables may be associated with SNPs. We performed a chi-squared analysis on all cases to identify SNPs that may affect the clinical variables of AML patients. The association of hemoglobin (HGB), platelet (PLT) counts, and lactate dehydrogenase (LDH) with AML prognosis was studied. In this study, HGB 80 g/L or lower was defined as low HGB, and higher as high HGB. As for PLT counts lower than 30×10^9/L was defined as low PLT, and those higher than 80×10^9/L were defined as high or intermediate PLT. In addition, plasma LDH above the upper limit of institutional reference range was referred to as high LDH (≥230 U/L) and that below it was referred

to as low LDH (＜230 U/L). Preliminary analysis showed that genotypic frequencies of rs324011 in STAT-6 and rs2243115 in IL-12A under both codominant and dominant models revealed statistically significant difference between high and low HGB groups ($p < 0.05$ for all). In addition, allele frequency and genotypic frequencies under the codominant and recessive models of rs2069718 in IFN-γ were also significantly associated with HGB ($p < 0.05$ for all). After adjusting for age and gender, AML patients carrying the CC genotype of rs324011 in STAT-6 showed low level of HGB at diagnosis compared with patients carrying CT/TT (OR=0.55; 95%CI=0.33-0.91; p= 0.02). Only the genotypic frequency of rs2228145 in IL6R under dominant model was significantly associated with the high level of plasma LDH after adjusting for age and gender ($p < 0.05$). No statistical difference was found between other SNPs and PLT counts ($p > 0.05$). However, no SNPs were significantly associated with HGB or LDH after Bonferroni multiple correction ($p > 0.05$). (Table 5)

3.5 Association between immuno-related SNPs and WHO classification or risk stratification of AML

Cytogenetic and molecular genetic changes in AML patients play an important role in assessing prognosis or guiding treatment [22]. AML patients with or without recurrent genetic abnormalities were divided into two groups, which were used to analyze the association between the immuno-related SNPs and recurrent genetic abnormalities by chi-squared tests. Preliminarily, genetic frequency of rs6503691 STAT5 under codominant and recessive models was significantly associated with recurrent genetic

abnormalities (RGAs) ($p<0.05$ for all). The association between STAT5 rs6503691 and recurrent genetic abnormalities was also statistically significant after Bonferroni multiple correction ($p<0.0027$). Univariate logistic regression analyses adjusting for age and gender showed that when the CC genotype (under codominant model) or CC/CT genotype (under recessive model) was used as a reference, the TT or CT genotypes (under codominant model), and TT (under recessive model) were significantly associated with recurrent genetic abnormalities ($p<0.05$ for all). (Table 6)

Risk stratification of AML was based on cytogenetic and molecular genetic changes, and was used to assess the prognosis of AML[23]. Therefore, the association between SNPs and risk stratification (favorable, intermediate and adverse) was analyzed in the study. The genetic frequencies of rs1800469 under codominant, dominant and recessive models were significantly associated with risk stratification ($p<0.05$ for all). However, rs1800469 under codominant, dominant and recessive models lost the statistical difference after adjusting for age and gender factors by multiple logistic regression. (Table 6)

3.6 Association of SNPs and sensitivity of induction chemotherapy in non-M3 AML

Of the 269 enrolled cases, 226 cases were non-M3 FAB subtype patients with AML. Within the total non-M3 AML patients, 191 patients received full-intensity induction chemotherapy while 35 patients were excluded from our study because of death during

Cycles One of induction chemotherapy or rejection to further treatment. Of the 191 patients, 67.54% (129/191) achieved a morphological CR after one or two cycles of therapy, 32.46% (62/191) failed to achieve a CR after at least two cycles of therapy (the "refractory" group). Univariate analysis of the 191 patients examining the effect of response to induction therapy on median OS demonstrated a significant difference in the outcome between the CR and refractory groups (median OS of 39, and 18 months, respectively; $p<0.001$). (Figure 1)

SNPs may be associated with induction chemotherapy responses of non-M3 AML patients[27, 28]. In this study, the association between SNPs and treatment responses was evaluated in the 183 non-M3 AML patients. We found statistically significant associations between the immuno-SNPs and induction chemotherapy responses. Allelic frequencies and genotypic frequencies under the codominant and recessive models of rs3771300 revealed statistically significant difference among sensitive, intermediate, and refractory groups ($p<0.05$). In addition, allelic frequencies and genotypic frequencies under the codominant model of rs3824662 showed a significant association within the three groups. However, there was no SNP remaining significant after Bonferroni multiple correction.

3.7 Age, sex, risk of cytogenetics and SNPs and survival in AML

In patients with AML, median OS for age ≥60 (17 months) versus age <60 (40 months)

were significantly different (p<0.001). The median OS for favorable (56.0 months) versus intermediate (27.0 months) or unfavorable (22.0 months) was also significantly different (p<0.001 for all). There was no significant difference in the median survival between sex. Next, the association between SNPs and survival of patients with AML was analyzed in the four genetic models. Preliminary screening by Kaplan-Meier analysis showed that genotypic frequencies of rs744166 under the dominant and codominant models were significantly associated with survival in patients with AML (p < 0.05). In addition, rs6887695 under allelic, recessive, and codominant models was significantly associated with survival in patients with AML (p < 0.05). The remaining SNPs showed no significant difference in the median survival. Among the above SNPs, only rs6887695 under codominant and recessive models was significantly associated with survival of patients with AML after Bonferroni multiple correction. AML patients carrying CC genotype of IL-12A rs6887695 showed a decreased median OS (22 months) compared with patients carrying GG genotype under codominant model. In addition, rs6887695 showed a significantly shorter median survival with GC/CC (22.0 months) compared to GG (33.0 months) under recessive model.

Finally, multivariate analysis was performed using a Cox proportional hazards model of SNPs in combination with age, sex, and cytogenetic risk group. As shown in our study, intermediate (OR=3.17, 95%CI =2.17- 4.65) and unfavorable (OR= 3.12, 95%CI =1.88-5.18) cytogenetics, and age ≥60 years (OR=3.17, 95%CI=2.17- 4.65) showed an

independent negative effect on OS in multivariate analysis, which is consistent with previous studies[29]. After adjusted for age and gender, AML patients carrying the GG genotype of IL-12A rs6887695 showed an increased median OS compared with patients carrying major genotype GC (OR=0.398, 95%CI=0.247-0.641) or CC (OR=0.510, 95%CI=0.333-0.782) under codominant model (Figure 2). In addition, patients with AML carrying the GG genotype of rs6887695 showed an increased median OS compared with patients carrying major genotype GC/CC (OR=0.463, 95%CI=0.310-0.693) under recessive model. In summary, the GG genotype of rs6887695 showed an independent favorable impact on prognosis.

3.8 Association of IL-12A rs6887695 and mRNA expression level of IL-12

To further determine the role of IL-12A rs6887695 on the prognosis of AML patients, we explored the association of rs6887695 and the mRNA expression level of IL-12. The expression of IL-12 was analyzed in 51 AML patients which include 13 CC cases, 23 CG cases and 15 GG cases. Under codominant model (Figure 3A), patients with CC genotype showed a higher level of IL-12 mRNA expression than those with GG genotype ($p = 0.028$). Furthermore, the level of IL-12 was also elevated in patients with heterozygous CG compared with patients with GG genotype. Under recessive model (Figure 3B), we found that the IL-12 expression was significantly lower in samples with pooled GG genotype compared with the CG/GG genotype ($p = 0.006$; Figure 3B). However, under dominant model, there was no difference of IL-12 mRNA expression

between CC/CG genotype and GG genotype.

4 Discussion

In this study, SNP genotyping was analyzed by Mass-ARRAY System in a 269 AML patients-cohort and a 200 healthy controls-cohort. We focused on highlighting the high throughput sequencing to identify immuno-related SNPs that may contribute to disease susceptibility and prognosis of AML patients. This study showed that 18 valid immuno-related SNPs were analyzed in AML patients and healthy controls; after strictly statistical analysis, three SNPs were significantly correlated with clinical outcomes, including WBC counts, WHO classification, and median OS; GG genotype of rs6887695, along with other known risks, including age, risk of cytogenetics, and WBC counts were confirmed as an independent poor prognostic factor; however, no immune-related SNP contributed to the susceptibility of patients with AML.

Previous studies showed that the abundance of SNPs are associated with disease susceptibility and prognosis in AML patients[30-32]. For example, Wagner et al. reported that IDH1 rs11554137 was associated with an inferior outcome in AML[30]. Recently, some of our studies had identified the association between NLRP3 inflammasome-related SNPs and the pathogenesis of hematologic malignancies, including AML, multiple myeloma, acute lymphoblastic leukemia as well as chronic myeloid leukemia [15, 20, 33, 34]. Our study showed that IL-1β (rs16944) was correlated with

the cytogenetical risk and the GT genotype of IL-18 (rs1946518) led to statistically poorer AML-specific survival[15]. We found that the AT genotype of CARD8 (rs2043211) was significantly higher compared to TT genotype in high and intermediate risk patients with CML[33]. Furthermore, our study showed that the CARD8-C10X (rs2043211) AT genotype contributed to the susceptibility of multiple myeloma[34]. However, in contrast to multiple autoimmune diseases, only a few studies had identified the association between immuno-related SNPs and the pathogenesis of AML[14.] Our study comprehensively, assessed the associations between 18 immuno-related SNPs and the disease susceptibility and prognosis of AML patients in the Chinese Han population. Of these SNPs, (1) peripheral infiltration of leukemia cells was more severe in samples with TT genotype of rs2227491 compared with the TC, CC genotype (codominant model), or compared with TC/CC (dominant model); (2) As for STAT5 rs6503691, the CC genotype (under codominant model) or CC/CT genotype (under recessive model) was used as a reference; the TT or CT genotypes (under codominant model), and TT (under recessive model) were significantly associated with recurrent genetic abnormalities. (3) In addition, rs6887695 under codominant and recessive models was associated with survival of patients with AML.

A few studies reported that the SNPs of IL-22 were correlated with the development of some inflammatory diseases and cancers, such as inflammatory bowel disease, chronic rhinosinusitis, and colon cancer[35-38]. In our study, rs2227491 in IL-22 was significantly

associated with peripheral infiltration of leukemia cell burden. The results showed that patients with AML carrying TT genotype or T allele displayed severe peripheral infiltration, indicating leukemia cell burden. As reported, marked hyperleukocytosis (WBC counts >100 × 10^9/L) confers a poor prognosis of newly-diagnosed AML[26], which has been adopted in the Chinese guidelines of AML[25]. In addition, overload of WBCs results in different kinds of clinical manifestations, tumor lysis syndrome, thrombosis, as well as intracranial bleeding, which may cause early death before induction chemotherapy[39]. However, the mechanism of rs2227491 in IL-22 causing the high level of WBCs is still unclear. As reported, IL-22 stimulates signaling pathways re involved in the regulation of cell growth, proliferation, and cell cycle control in non-immune tissue such as skin, small intestine, colon, lung and liver[40, 41]. Eun et al. reported that rs2227485 SNP in IL-22 might be associated with the risk of papillary thyroid cancer[42]. It was also reported that rs2227485 in IL-22 was associated with bladder cancer risk[43]. In addition, the relationship between IL-22 and hyperleukocytosis or its induced clinical manifestations is worthy of further study.

The WHO continues to define specific AML disease entities by focusing on significant cytogenetic and molecular genetic subgroups that are recurrent genetic abnormalities, which may benefit diagnosing disease, assessing prognosis, providing treatment, and developing new drugs[44]. STAT5 rs6503691 was reported to be associated with recurrent genetic abnormalities of patients with AML[45]. Kreil et al. reported that rs6503691 is

associated with the response of patients with CML to IFN-α[46]. In addition, STAT3 expression level was strongly related to rs6503691 genotype, which may result from the fact that rs6503691 falls in a region of strong linkage disequilibrium at 17q21 which includes the entire STAT5A gene as well as the 5′ end of STAT5B and the 3′ end of STAT3. Therefore, polymorphic differences resulting in the difference of STAT3 expression may play an important role in the pathogenesis of AML. STAT3 is crucial for transcriptional regulation in human AML and enhances germline mutations, which contribute to tumor extrinsic immune evasion[47]. Han et al. reported that Th17 cells may play a crucial role in the pathogenesis of AML through Jak/Stat3 signaling pathway[48]. Yoshida et al. reported that Gö6976, a FLT3 kinase inhibitor, exerts a potent antiproliferative activity against leukemia cells via the suppression of STAT3/5 pathway[49]. However, because of the complexity of recurrent genetic abnormalities, the role of STAT5 rs6503691 in patients with AML needs to be further studied.

Recent studies highlighted the ability of IL-12 cytokines as potent agents that act on immunotherapy of AML, thus opens new perspectives for AML treatment[50]. The mechanism of the relationship between genetic changes of IL-12 and its function in anti-leukemia remained unclear[51]. In our study, GG genotype of rs6887695 in IL-12A increased the median OS of patients with AML. Thus, we hypothesized that the polymorphism of rs6887695 in IL-12A may influence its expression, and then regulate the internal anti-leukemia activity. Furthermore, Kupsa et al. found that elder age is associated with lower levels of IL-12[52]. Old age (> 60 years) is a well-known poor

prognostic marker[53]. In our study, we found that the mRNA expression of IL-12 was decreased in AML patients with GG genotype, no matter under codominant or recessive model. In both solid tumors and hematologic malignancies, IL-12 addition is an effective antitumor therapy[54, 55]. Hence, we speculated that the negative impact on prognosis of GG genotype of AML patients is related to the low level of IL-12 expression. Nevertheless, novel research needs to be carried out in order to understand the molecular mechanisms of genetic polymorphisms of rs6887695 in IL-12A.

In conclusion, our results find new prognostic markers and even therapeutic targets for AML, especially rs6887695, which may be have an independent negative impact on prognosis of AML. However, an accurately functional mechanism of the functions of SNPs in the pathogenesis of AML needs to be understood. Furthermore, there are limitations to the analysis between SNPs and AML in this study. Firstly, our methodology did not evaluate all the known SNPs or mutations, especially those related to the survival of AML, and did not detect other SNPs that are of unclear clinical significance in AML. In addition, no uniform therapy of induction chemotherapies was used in this study, and differences in specific therapies may influence the study. Furthermore, the limited size of the cases and controls enrolled may influence the effective and comprehensive analysis of all the variables in the multivariate analysis.

Author contributions

Daoxin Ma and Ming Hou designed the research and analyzed the data; Qinqin Liu and Mingqiang Hua wrote the manuscript; Shuxin Yan prepared the samples; Qinqin Liu, Mingqiang Hua, Ruiqing Wang, Xinyu Yang and Fengjiao Han performed the research, analyzed the data and provided scientific suggestions; Chen Zhang performed the research and analyzed the data.

Declaration of Competing Interest

None.

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Table 1 The demographic and clinical characteristics of AML patients and controls.

Variable	Case n (%)	Control n (%)
Gender		
Male	147(55)	108(54)
Female	122(45)	92(46)
Age (years, median, range)	46(18-80)	44.5(20-75)
＜60	209(78)	155(78)
≥60	60(22)	45(22)
WBC *		
median(×109/L)	14.6	NA
＜100×109/L	206(89)	NA
≥100×109/L	26(11)	NA
PLT		
median(×109/L)	40	NA
＞50×109/L	106(39)	NA
≤50×109/L	163(61)	NA
HGB		
median(g/L)	76	NA
＞60g/L	222(83)	NA
≤60g/L	47(17)	NA
Bone marrow blast		
median(%)	84	NA
＜70%	76(28)	NA
≥70%	193(72)	NA
WHO classification		
Yes	94(38)	NA
No	153(62)	NA
Risk stratification		
Favorable	64	NA
Intermediate	153(85)	NA
Adverse	27(15)	NA
Response		
CR	130(66)	NA
PR+NR	68(34)	NA

WBC: white blood cell; PLT: platelet; HGB: hemoglobin;
WHO: World Health Organization; CR: complete remission; PR: partial remission; NR: not remission; NA:not applicable.

Table 2 Selected genes and SNPs.

Gene	SNP	Variant	Variant allele	MAF	HWE (*p* value)
IL22	rs1179251	68251271C>G	G	30.78%	0.862824762
TGFB1	rs1800469	41354391A>G	G	49.25%	0.68368554
IL9	rs1859430*	135894824G>A	A	4.66%	0.267037328
IFNG	rs2069718	68156382A>G	G	13.57%	0.109238589
IL2	rs2069762	122456825A>C	C	32.13%	0.254557576
IL4	rs2070874	132674018C>T	T	22.68%	0.120462667
IL2RA	rs2104286#	6057082T>C	C	15.24%	2.60914E-10
IL22	rs2227485	68253933G>A	A	29.17%	0.999987112
IL22	rs2227491	68252741T>C	C	48.51%	0.317313626
IL6R	rs2228145	154454494A>C	C	37.90%	0.573226547
FOXP3	rs2232365#	49259429T>C	C	35.87%	1.44642E-14
NFKBIA	rs2233406	35405593G>A	A	10.78%	0.650233303
IL12A	rs2243115	159988493T>G	G	9.11%	0.251522315
IL4	rs2243250	132673462C>T	T	22.35%	0.616005203
STAT6	rs324011	57108399C>T	T	24.72%	0.959573615
FOXP3	rs3761549#	49260888G>A	A	17.91%	1.0757E-10
STAT1	rs3771300	190970870T>G	G	30.48%	0.705059208
GATA3	rs3824662	8062245C>A	A	31.60%	0.849079812
TBX21/TBET	rs4794067	47731462T>C	C	24.81%	0.165599198
STAT5B	rs6503691	42242072C>T	T	42.72%	0.895093917
IL12A	rs6887695	158822645G>C	C	38.43%	0.344100628
STAT3	rs744166	42362183A>G	G	38.43%	0.420304111

SNP: single-nucleotide polymorphisms; HWE: Hardy–Weinberg equilibrium; MAF: minor allele frequency (<5%); #SNP was not included in further analysis for the deviation of HWE; *SNP did not suit the HapMap project.

Table 3 Association between selected SNPs and susceptibility of AML patients.

Gene	SNPs	Model	Genotype/ Allele	Cases(n)	Controls(n)	OR (95% CI)	Adjusted *p* valve
IL22	rs1179251	Codominant	CC	134	82		
			CG	96	93	0.78(0.43-1.41)	0.414
			GG	32	25	1.24(0.68-2.25)	0.483
		Dominant	CC	134	82		
			CG/GG	128	118	0.66(0.45-0.96)	0.03
		recessive	CC/CG	230	175		
			GG	32	25	0.79(0.60-1.04)	0.097
		allele	C	364	257		
			G	160	143		0.104
IL4	rs2070874	Codominant	CC	17	5		
			CT	84	73	0.39(0.14-1.08)	0.072
			TT	162	122	1.16(0.78-1.72)	0.454
		Dominant	CC	17	5		
			CT/TT	246	195	0.37(0.13-1.02)	0.055
		recessive	CC/CT	101	78		
			TT	162	122	1.03(0.71-1.51)	0.877
		allele	C	118	83		
			T	408	317	0.91(0.66-1.24)	0.539
IL4	rs2243250	Codominant	CC	17	5		
			CT	80	71	0.38(0.14-1.07)	0.067
			TT	161	123	1.17(0.79-1.74)	0.44
		Dominant	CC	17	5		
			CT/TT	241	194	0.37(0.13-1.01)	0.051
		recessive	CC/CT	97	76		
			TT	161	123	1.03(0.70-1.51)	0.879
		allele	C	114	81		
			T	402	317	0.90(0.65-1.24)	0.527

SNP: single-nucleotide polymorphisms; CI: confidence interval; OR: odds ratio.

Table 4 Association between selected SNPs and tumor burden of AML patients.

Gene	SNPs	Model	Genotype/ Allele	＜42% n(%)	≥42% n(%)	OR（95%IC）	adjust *p* value
IL2	rs2069762	Codominant	AA	12	103		
			AC	12	104	2.95(097-8.93)	0.056
			CC	6	18	2.95(0.98-8.9)	0.055
		Dominant	AA	12	103		
			AC/CC	18	16.5	0.91(0.58-2.79)	0.534
		recessive	AA/AC	24	207		
			CC	6	18	2.95(1.06-8.2)	0.038
		allele	A	36	310		
			C	24	140	0.91(0.53-1.56)	0.158
Gene	**SNPs**	**Model**	**Genotype/ Allele**	**＜100 n(%)**	**≥100 n(%)**	**OR（95%IC）**	**adjusted *p* value**
IL22	rs2227491	Codominant	TT	43	16		
			TC	110	10	4.47(1.39-14.4)	0.012
			CC	48	4	1.09(0.33-3.65)	0.888
		Dominant	TT	43	16		
			TC/CC	158	14	4.2(1.91-9.28)	0.0004
		recessive	TT/TC	153	26		
			CC	48	4	2.04(0.68-6.14)	0.205
		allele	T	196	42		
			C	206	18	2.45(1.37-4.41)	0.003
IL4	rs2243250	Codominant	CC	15	0		
			CT	62	7	null	null
			TT	121	23	null	null
		Dominant	CC	15	0		
			CT/TT	183	30	null	null
		recessive	CC/CT	77	7		
			TT	121	23	2.09(.86-5.11)	0.105
		allele	C	92	7		
			T	304	53	2.29(1.01-5.21)	0.048

SNP: single-nucleotide polymorphisms; CI: confidence interval; OR: odds ratio.

Table 5 Association between selected SNPs and hemoglobin, lactate dehydrogenase of AML patients.

Gene	SNPs	Model	Genotype/ Allele	HGB(g/L) n 80≤	HGB(g/L) n ＞80	OR(95%CI)	adjusted *p* value
STAT6	rs324011	Codominant	CC	95	60		
			CT	36	48	0.99(0.39-2.48)	0.982
			TT	13	9	2.13(0.81-5.60)	0.127
		Dominant	CC	95	60		
			CT/TT	49	57	0.55(0.33-0.91)	0.02
		recessive	CC/CT	131	108		
			TT	13	9	1.29(0.53-3.17)	0.576
		allele	C	226	168		
			T	62	66	0.95(0.49-1.79)	0.864
Gene	**SNPs**	**Model**	**Genotype/ Allele**	**LDH(IU/L) n ＜230**	**LDH(IU/L) n ≥230**	**OR(95%CI)**	**adjusted *p* value**
IL6R	rs2228145	Codominant	AA	26	63		
			AC	17	87	0.63(0.24-1.63)	0.339
			CC	7	27	1.33(0.49-3.54)	0.57
		Dominant	AA	26	63		
			AC/CC	24	114	0.51(0.27-0.96)	0.038
		recessive	AA/AC	43	150		
			CC	7	27	0.91(0.37-2.22)	0.829
		allele	A	69	213		
			C	31	141	0.68(0.42-1.09)	0.11

SNP,single-nucleotide polymorphisms; HGB: hemoglobin; LDH: lactic dehydrogenase; CI:confidence interval; OR: odds ratio.

Table 6 Association between selected SNPs and WHO classification or risk stratification of AML patients.

Gene	SNPs	Model	Genotype/ Allele	RGAs YES	RGAs NO	OR（95%CI）	adjusted *p* value
STAT5B	rs6503691	Codominant	CC	44	88		
			CT	30	63	9.13(1.91-43.85)	0.005
			TT	10	2	9.77(2.00-47.69)	0.004
		Dominant	CC	44	88		
			CT/TT	40	65	1.17(0.68-2.01)	0.564
		recessive	CC/CT	74	151		
			TT	10	2	9.39(1.99-44.31)	0.004
		allele	C	118	239		
			T	50	67	1.46(0.95-2.25)	0.084

Gene	SNPs	Model	Genotype/ Allele	Risk stratification favorable	intermediate	adverse
TGFB1	rs1800469	Codominant	AA	25	33	4
			AG	23	73	17
			GG	15	40	3
				OR（95%CI）	1.48(0.96-2.27)	1.24(0.63-2.41)
				adjusted *p* value	0.071	0.535
		Dominant	AA	25	33	4
			AG/GG	38	113	20
				OR（95%CI）	0.31(0.09-1.01	0.71(0.29-1.71)
				adjusted *p* value	0.053	0.516
		recessive	AA/AG	48	106	21
			GG	15	40	3
				OR（95%CI）	2.27(0.59-8.80)	2.67(0.75-9.47)
				adjusted *p* value	0.234	0.127
		allele	A	73	139	25
			G	53	153	23
				OR（95%CI）	0.80(0.41-1.58)	1.20(0.65-2.22)
				adjusted *p* value	0.526	0.556

SNP: single-nucleotide polymorphisms;WHO: World Health Organization; CI:confidence interval; OR: odds ratio.