Genetic variation in Interleukin-17A is associated with increased risk and severity of pneumococcal meningitis in children

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Abstract

Background Streptococcus pneumonia is a leading cause of bacterial meningitis in children. Interleukin-17 (IL-17) promotes the host's defense against pneumococcal infections. We investigated whether single nucleotide polymorphisms (SNPs) of IL -17A were associated with pneumococcal meningitis (PM) in Chinese children.Methods Three SNPs of IL-17A promoter rs4711998 (-877A>G), rs8193036 (-737C>T) and rs2275913 (-197G>A) were studied in 95 laboratory-confirmed PM pediatric patients and 330 healthy controls by PCR-based sequencing. Luciferase assays were used to determine the effects of IL-17A haplotypes on expression. Serum concentrations of IL-17A and C-X-C motif ligands 1 (CXCL-1) were determined.Results Frequency of the haplotype A-C-A differed significantly between cases and controls (43.6% vs. 35.7%, OR: 1.397, 95% CI: 1.004-1.942, P =0.047). The haplotype had an increased expression of IL-17A compared to wild haplotype A-C-G (P<0.001). Moreover, it produced a higher level of serum C-reactive protein (>10mg/L) (46.6% vs. 28.4%, OR: 2.276, 95%CI: 1.011-5.126, P =0.043). This haplotype was associated with low level of cerebrospinal fluid (CSF) glucose (≤1.5mmol/L) (52.8% vs.25.1%, OR: 3.350, 95%CI: 1.787-6.280, P <0.001) and high level of CSF protein (45.3% vs.29.2%, OR: 2.010, 95%CI: 0.991-4.076, P =0.05).Conclusions Our result indicated that haplotype A-C-A of IL-17A was associated with susceptibility and severity of PM in Chinese children.

Background

Bacterial meningitis (BM) among the young children is one major public health concern around the world. *Streptococcus pneumoniae* (*S. pneumoniae*) is a leading cause of BM. Although pneumococcal conjugate vaccines have introduced in industrial countries, the global case-fatality ratio of PM in children younger than 5 years was 59% despite the mortality rate of 10 (4–13) per 100,000[1]. Cognitive impairments, hearing loss and psychological distress are the major sequelae among survivors of PM [2]. In China, during 2012-2017, of all the 1138 children with invasive pneumococcal disease, 39.2% (n=446) were diagnosed with meningitis. [3].

The pathogenesis of bacterial meningitis involves a complex interplay between pathogens and the host defense. Interleukin-17A (IL-17A) is an important pro-inflammatory cytokine, which is mainly produced by a subset of CD4+T cells named Th17 cells [4, 5]. It plays an important role in host innate immune responses against bacterial colonization and infection. Studies in animal models have demonstrated that IL-17A can promote production and recruitment of neutrophils to infection sites via inducing the expressions of various chemokines, inflammatory factors and adhesion molecules [6-8]. In most cases, nasopharyngeal colonization of *S. pneumoniae* is followed by its invasion into bloodstream. After crossing the blood–brain barrier (BBB), bacteria multiply and trigger activation of host immune defense [9, 10]. This response includes expression of inflammatory cytokines such as IL-6 and IL-8 through MyD88 signaling. However, these cytokines are not capable of clearing the pathogens effectively, only when IL-17A recruits effector cells such as neutrophils and macrophages can the pathogens be cleaned [10, 11]. A recent study demonstrated that downstream chemokines of IL-17A signaling pathway were significantly increased in cerebrospinal fluid (CSF) of BM patients which might serve as a neurological

prognostic index [12]. Another recent study reported that IL-17 signaling could recruit neutrophils by regulating the secretion of CXCL-1 in mycobacteria-infected mice [13]. IL-17 could be induced by heat-killed pneumococci and pneumococcal peptidoglycan (PGN) when co-cultured with CD4+ T cells and monocytes isolated from healthy volunteers, suggesting a Th17 response to *S. pneumonia* [14]

Single nucleotide polymorphisms of IL-17A may lead to altered production of IL-17A. Gene encoding human IL-17A is located on chromosome 6. Studies have shown that SNPs in IL-17A are associated with a majority of allergic and autoimmune diseases such as asthma, rheumatoid arthritis and ankylosing spondylitis [15-17]. SNPs of IL-17A rs4711998 (-877A>G), rs8193036 (-737C>T), rs2275913 (-197G>A) are found in the upstream of the transcriptional factor binding site (TFBS). Theoretically, these SNPs can influence the expression of nuclear factor of activated T cells (NFAT), which is known to regulate transcription activity of IL-17[18, 19]. Rasouli *et al* [20] reported that Iranian people with IL-17A rs4711998 (-877A>G) AA genotype had an increasing risk to brucellosis. Stappers *et al* [21] found that Eastern European individuals with IL-17A rs8193036(-737C>T) TT genotype were less susceptible to complicated skin and skin structure infections caused by *Staphylococcus aureus, β*-haemolytic *Streptococcus* group A–G, *Enterococcus faecalis* or *Escherichia coli*. Another study carried out in Finland showed that healthy children at two years of age who had undetectable or low serum concentration of IL-17A with rs2275913 (-197G>A) AA genotype were more likely to be colonized by *S. pneumoniae* [22]. These studies suggest that genetic variations of IL-17A can influence susceptibility and outcome of bacterial colonization and infections.

The aim of this study was to explore the possible associations between gene polymorphisms in IL-17A and susceptibility, severity and prognosis of PM in Chinese children. A total of 3 SNPs: IL-17A rs4711998 (-877A>G), rs8193036 (-737C>T) and rs2275913 (-197G>A) (Figure 1) were analyzed. The selection of these functional SNPs was based on already published data of *in vitro* and *in vivo* studies [19, 21, 22].

Methods

Study subjects From January 2014 to December 2018, 95 laboratory confirmed PM patients, comprising 62 males and 33 females in Department of Infectious Diseases, Beijing Children's Hospital, Beijing, China were enrolled as the case group. The median age was 19 months (range, 2 months to 13 years). All PM patients met the standards PM diagnosis as established by European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [23]. In addition, 330 healthy adults (168 males and 162 females) who attended annual medical examination in June 2015 were randomly selected as the control group. The age of the control group ranged from 21 to 60 years with a median age of 38 years. All study subjects were Chinese from different families and thus considered as unrelated individuals.

Clinical data collection Clinical characteristics of the study subjects were summarized in Table 1. According to electronic medical records (EMR), Chinese Han descendants were selected. Clinical information including fever, consciousness and seizures were obtained by an experienced pediatrician at

the Department of Infectious Diseases, Beijing Children's Hospital. Other clinical information on blood routine, cerebrospinal fluid (CSF) routine and biochemical examination, imaging examination, hospitalization time and other clinical information for treatment were all from EMR.

Sample collection and bacterial culture In the first three days of admission, peripheral blood and CSF samples were taken aseptically. To identify the pathogens, bacterial culture, Gram staining, acid fast staining and *S. pneumoniae* antigen test were performed in addition to the routine biochemical analysis. All pathogen classification and identification follow the standard laboratory identification method.

DNA extraction Genomic DNA was extracted from 200μL of whole blood samples in EDTA tubes using a DNA purification kit (QIAamp DNA Blood Mini Kit; Qiagen, Germantown, MD, USA) according to manufacturer's instructions. The extracted DNA was kept at −20°C until use.

SNPs analysis The primers used in this study were designed using the Primer Premier 6.0 software. The forward primer used was 5'-TCATTCACCTCAGTGGGGGTA-3'

and the reverse primer was 5'-ATGGATGAGTTTGTGCCTGCT-3'. The product size was 967bp. The total reaction volume of 50μL contained 5μL of 10×buffer, 1μL of each primer, 2μL (15-20ng/μL) of genomic DNA, 1μL of dNTP mixture, 0.4μL of Pfu DNA Polymerase and 40.6μL of deionized water. Amplification conditions consisted of initial denaturation at 95°C for 2min followed by 42 cycles of 95°C for 30s, 54°C for 30s, and 72°C for 2min, with a final extension for 5min at 72°C. The amplified products were examined by agarose gel electrophoresis and sequencing. Both positive and negative controls were used in each PCR run. The amplified products were sequenced at the Tsingke Biological Technology Co., Ltd, Beijing, China. The SNPs in interests were determined using Bioedit sequence alignment editor version 7.2.1. In order to ensure accuracy, one out of every 10 samples was selected for PCR and sequencing for the second time.

Construction of luciferase reporter plasmids Three luciferase reporter plasmids were constructed to explore whether IL-17A polymorphisms had an effect on IL-17A gene expression. The constructs of a 1663bp DNA fragment corresponding to the upstream region of TFBS of IL-17A, which were amplified from individual homozygous templates and were cloned into the pGL3-basic luciferase vector (Promega, Madision, USA). The vectors were then sequenced to confirm that there were no nucleotide errors.

Transient transfections and luciferase assays HEK293T cells were seeded in 24-well plates, and each well was transfected with 0.5μg of the vector DNA containing haplotype A-C-G, A-C-A or A-T-G of rs4711998

(-877A>G), rs8193036 (-737C>T) and rs2275913 (-197G>A), and 0.05μg of pGL4.74 which contains the Renilla luciferase gene by Viafect (Promega, Madision, USA), according to the manufacture's instruction. Cells were collected 24h after transfection, and luciferase activity was measured with a dual Luciferase reporter assay system (Promega, Madision, USA) and was normalized against the activity of the Renilla luciferase gene. Independent triplicate experiments were performed for each plasmid.

Serum cytokines measurement Of the 95 cases studied, 63 had sera available. The Luminext kit was used for measuring IL-17A and CXCL-1. The measurement run was done by Luminext MAGPIX system (Luminext) according to manufacturer's instructions. Duplicate wells for each sample were did and a volume of 50μL was used per well. Samples were diluted to 1:2 with sample diluent provided by the manufacturer before added into wells. The detection limit of IL-17A and CXCL-1 were 2.3pg/mL and 3.0pg/mL, respectively.

Statistical analysis Allelic and genotypic frequencies of each SNP studied were calculated. All of the SNPs analyzed were found to be in HWE (P>0.05). Categorical variables were analyzed by $\chi 2$ test or Fisher's exact test as appropriate. Univariate analysis was performed for continuous variables with Mann-Whitney *U* test or Student's *t*-test, The Odds Ratio (OR) and 95% confidence interval (CI) were calculated using unconditional binary logistic regression. Genotypes found to be statistically significant by univariate analysis were further analyzed by logistic regression. A two-tailed P value less than 0.05 was considered as significant. Bonferroni correction was employed for multiple testing correction and $P < 0.05/n$ (n =the number of comparisons) was considered significant. All the statistical analyses were conducted using the SPSS software, version 23.0 (IBM, Armonk, NY, USA). Estimation of haplotype frequencies and haplotype association analysis with the χ2 test were performed with SHEsis (http://analysis.bio-x.cn). The statistical analyses were carried out by using Graph-Pad Prism 6 (San Diego, CA).

Results

Demographic and clinical information During the study period 2014-2018, 95 cases hospitalized were laboratory-confirmed PM. Clinical and laboratory information was summarized in Table 1. Once the CSF or peripheral blood of *S. pneumoniae* culture and/or specific antigen were positive, *S. pneumoniae* infection was confirmed. Of 95 cases, 51 were CSF culture positive for *S. pneumoniae*, 35 were CSF positive for duplicate specific pneumococcal antigen and 9 were only blood culture positive for *S. pneumoniae*. All 95 PM patients had fever at admission.

Comparison of IL-17A promoter variants between patients and controls The location and distributions of genotypes and haplotype frequencies of three SNPs studied between PM patients and healthy controls (HCs) were summarized in Figure 1 and Table 2. The genotypes and allele frequencies of 3 SNPs between cases and controls did not differ significantly. However, a significant difference was found for haplotype

A-C-A, with 43.6% (83/190) and 35.7% (236/660) for patients and controls respectively. (OR: 1.397, 95% CI: 1.004-1.942, *P*=0.047) (Figure 1. b).

Expression of IL-17A by different haplotypes In order to evaluate whether the IL-17A haplotypes could influence its transcriptional activity, pGL3-basic vectors with haplotypes A-C-G, A-C-A and A-T-G were constructed. The above vectors were transfected into HEK293T cells. As shown in Figure 2, the vectors with haplotype A-C-A and A-T-G had significantly higher luciferase activities compared with the wild haplotype A-C-G (*P* < 0.001). These results showed that IL-17A haplotypes A-C-A or A-T-G in the promoter region were associated with an up-regulated transcriptional activity of IL-17A.

IL-17A promoter variants and serum IL-17A and CXCL-1 concentration IL-17A and the IL-17A signaling chemokine CXCL-1 were determined in 63 available serum samples. Of them, three (4.7%) had detectable IL-17A and 60 (95.3%) had detectable CXCL-1. The median concentration of IL-17A was 1.09 pg/ml (range, 0.85 to 3.3pg/ml). The median concentration of CXCL-1 was 10.3 pg/ml (range, 1.84 to 51.3 pg/ml). No difference in levels of CXCL-1 was found between subjects with different genotypes of the three IL-17A SNPs. However, higher frequency of the haplotype A-C-A was observed in the group with the highest concentrations (top 25% vs. bottom 75%: 58.5% vs.33.8%, OR: 2.22, 95%CI: 0.982-5.028, *P*=0.05) (Figure 3.).

IL-17A promoter variants and Clinical characteristics To study whether gene polymorphisms of the IL-17A could influence severity of PM, we analyzed data based on 11 clinical features that might be considered as predictors of PM severity: transient hearing impairment, CRP, WBC in CSF, glucose in CSF, protein in CSF and polynuclear cells proportion in CSF, WBC in blood, neutrophils proportion in blood [24, 25]. For IL-17A rs4711998 (-877A>G), frequencies of variant genotype GA was significantly lower in PM patients with an initial CSF glucose concentration of less than 1.5mmol/L compared with those with greater than 1.5mmol/L (26.9% vs. 47.6%, OR= 0.394, 95%CI: 0.161-0.963, *P*=0.039) (Table 3). For IL-17A rs8193036 (-737C>T), frequencies of variant genotype CT, the minor allele T carrier (CT+TT) were significantly lower in PM patients with lower CSF glucose concentration (less than 1.5mmol/L) (28.8% vs. 50.0%, OR=0.390, 95%CI: 0.162-0.938, *P*=0.033; 36.5% vs. 57.1%, OR=0.432, 95%CI: 0.188-0.992, *P*=0.046, respectively) (Table 3). However, after logistic regression analyses, significant difference observed between variant genotype GA of rs4711998 (-877A>G) and CSF glucose disappeared.

Frequency of haplotype A-C-A was significantly higher in PM patients with an initial CSF glucose concentration less than 1.5mmol/L compared with those with greater than 1.5mmol/L (52.8% vs.25.1%, OR= 3.350, 95%CI: 1.787-6.280, *P*<0.001), while haplotype A-T-G was significantly lower (11.2% vs.23.2%, OR= 0.426, 95%CI: 0.194-0.937, *P*=0.031) (Figure 4.a). For the proteins detected in CSF, there was a tendency that frequency of haplotype A-C-A was higher in patients with high level of CSF protein of more than 1000 mg/L compared with those with less than 1000 mg/L (45.3% vs. 29.2%, OR=2.010, 95%CI: 0.991-4.076, *P*=0.05), while frequencies of haplotype A-C-G and G-T-A were significantly lower (11.1% vs.24.3%, OR= 0.390, 95%CI: 0.167-0.910, *P*=0.003; 1.8% vs.17.4%, OR= 0.001, 95%CI: 0.000-0.017, *P*=0.002, respectively) (Figure 4.b). In peripheral blood, frequency of haplotype A-C-A was significantly

higher in cases with a CRP concentration of more than 10mg/L compared with those with less than 10 mg/L (46.6% vs. 28.4%, OR= 2.276, 95%CI: 1.011-5.126, *P*=0.043), while haplotype G-T-G frequency was significantly lower (8.4% vs.22.4%, OR= 0.321, 95%CI: 0.119-0.864, *P*=0.019) (Figure 4.c).

Discussion

In this study we showed for the first time the relationships between IL-17 SNPs and susceptibility and severity of Chinese children with laboratory-confirmed PM. Outcome of PM in Chinese children is associated with genetic variation of interleukin-17A. *S. pneumoniae* is an important commensal resident of the human nasopharynx. Although the carriers have no symptoms at first, *S. pneumoniae* can become invasive and spread from upper respiratory tract to the brain through bloodstream [26]. The mechanism of entering the blood-brain barrier is probably via platelet-activating factor receptor-mediated transcytosis across endothelial and epithelial cell layers [27]. In mouse experiment, *S. pneumoniae* can also transmigrate from nasopharyngeal epithelium to the central nervous system through olfactory nerves [28]. It is known that host defense has a crucial effect on the incidence and severity of infections caused by this particular pathogen [22]. The IL-17 family includes six members (IL-17A to F) and five receptors (IL-17RA to E) and plays an active role in host immune responses. IL-17A, expressed as a subset of CD4+ effector T (Th17) cells, has a strong biological activity. It has been shown that children with BM exhibit high fractions of γδ IL-17A producing cells in CSF [29]. When the IL-17A downstream signaling pathways which are common to nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling are activated, the pro-inflammatory factors like granulocyte colony-stimulating factor (G-CSF), IL-6 and CXCL-1 are up-regulated. As a result, the neutrophils are induced and recruited to the sites of inflammatory infiltration, where neutrophil cytotoxic and phagocytic activities are strengthened, thereby providing an amplification loop for neutrophil pro-inflammatory responses [30, 31]. Previously, Wright *et al* [32] showed that IL-17A production accelerated the clearance of Pneumococcal bacteria by enhancing the anti-pneumococcal response of human alveolar macrophages. Animal experiments also demonstrated that streptococcal-specific Th17 cells could migrate from nasal-associated lymphoid tissue to the brain by traveling along olfactory sensory axons [33]. All these suggest that IL-17A signaling has an effector role in pneumococcal immunity in humans.

The main result of the present study was that the haplotype A-C-A of IL-17A rs4711998 (-877A>G), rs8193036 (-737C>T) and rs2275913 (-197G>A) was more common in PM patients than in the Chinese population controls. And the haplotype may release excessive inflammatory factors by up-regulating the expression of IL-17, resulting in more severe illness. CRP, one of the acute-phase proteins, is primarily produced in the liver during episodes of acute inflammation or infection [34]. It is widely requested by the physicians in order to estimate the presence and severity of infectious and inflammatory diseases. IL-17A, however, plays a crucial role in the neutrophil expansion of progenitor cells and mature neutrophils in bone marrow, spleen and peripheral blood by inducing other inflammatory mediators such as IL-6, G-CSF, CXCL-1 and CXCL-2 [35]. In our study, although we failed to detect IL-17A in serum, we found that the frequency of haplotype A-C-A was higher in patients whose serum CXCL-1 was high (top 25%). Similar trend was also observed for serum CRP.

Multicenter studies have illustrated that low CSF glucose and high CSF protein are strong predictors for severity of patients with BM [36, 37]. It is known that the increased number of invasive *S. pneumoniae* destroy the cells in brain, thus the enzymes related to glycolysis are released, leading to an enhancement of glycolysis activity. Then the insufficient glucose in the CSF can greatly affect neuron energy supply and cause damage to the brain. Furthermore, low levels of glucose in the central nervous system have been associated with increased inflammation and cytokine levels in the CSF which may consequently results in the pathogenesis of meningitis-associated brain injury, leading to certain neurological sequelae [38, 39]. As for changes in CSF protein, it is often considered an indicator of blood-brain barrier permeability. Indeed, Asgari *et al* [40] have shown that high level of albumin was likely to originate from a barrier dysfunction.

It is known that IL-17A can damage BBB function by disrupting tight-junctions *in vitro* and *in vivo* through the generation of ROS in endothelial cells [41, 42]. In this study, we found a high frequency of PM patients who carry an IL-17A high-producing haplotype A-C-A. Our finding is consistent with those reported above. A recent study conducted among patients with tuberculosis in Argentina found that subjects with AA genotype of rs2275913 SNP, AA individuals displayed significantly higher concentration of serum IL-17A compared with those with GG genotype. Moreover, these subjects with AA genotype had higher disease severity such as displaying the highest bacilli burden, showing severe pulmonary conditions and exhibiting weak cell-mediated immunity against *Mtb*-Ag [43]. Our result is in agreement with the finding reported among TB patients.

There were certain limitations in this study. First, the gender distribution of patients with PM was not equal. We found the male gender might be a risk factor for susceptibility to PM (OR=1.834, 95%CI: 1.141-2.947, *P*=0.012). However, contradictory results have been reported. One post hoc analysis showed that gender had no significant effect on prognosis [44]. Another meta-analysis indicated the male gender might be one of the prognostic factors for sequelae and mortality after PM, but no explanations were provided[24]. In our study, we found frequency of haplotype A-C-A significantly increased in PM with lower CSF glucose in both male and female gender, indicating no gender differences in disease severity and prognosis (Supplementary Figure S1). It should be kept in mind that genes encoding IL-17 family members are not located on the sex chromosome of human. Secondly, the healthy adults were used as controls in this study. We recognize that the clinical characteristics and prognosis of PM in pediatric and adult patients are different. However, as stated above, the purpose to use adult as controls was to compare frequencies of different SNPs between patients and population controls and was not meant to compare the clinical characteristics of PM between the two groups. Genotypes do not alter with age, which is widely accepted. Thirdly, we did not find an association between SNPs of IL-17A and proportion of polynuclear cells in CSF or proportion of neutrophils in blood. The explanation could be that the patients with PM included in this study were caused by different serotypes of *S. pneumoniae*. As shown in animal experiments, IL-17 can have an adverse effect on *S. pneumoniae* with high capsular thickness and low invasive potential such as serotypes 3 and 6B [45]. Unfortunately, we did not have information on serotypes of pneumococcal strains isolated in our cohorts. On the other hand, in most cases a critical procedure for *S. pneumonia* to cause BM is to invade the blood-brain barrier through blood flow [46]. As

the highly encapsulated pneumococcal strains very seldom could traverse the alveolar epithelial barrier by neutrophil extracellular traps [45]. Fourthly, we did not determine level of CSF cytokines including IL-17A, because the samples were not available. Last, due to the limited sample size, we were unable to find out whether the less frequent haplotypes such as G-C-A, G-T-G, etc. were related to the susceptibility and severity of PM. In this study, serum IL-17A was not detected in most of patients. One explanation might be that these patients had already antibiotic treatments before admission to the hospital.

Conclusions

Our results indicate that the haplotype A-C-A of IL-17A was associated with susceptibility and severity of PM in Chinese children. The genetic variations could be used as a potential biomarker to evaluate outcome of PM in children. More association and functional studies are needed to reveal the exact mechanism causing the differences in clinical course of PM and to obtain genetic traits which can be used for patient profiling and management of CNS infections.

Abbreviations

IL-17: Interleukin-17; SNPs: single nucleotide polymorphisms; PM: pneumococcal meningitis; BM: Bacterial meningitis; S. pneumonia: Streptococcus pneumonia; BBB: blood–brain barrier; CSF: cerebrospinal fluid; EMR: electronic medical records; C-reactive protein: CRP; WBC: white blood cell; HC: healthy control; NF-κb: nuclear factor-kappa B; MAPK: mitogen-activated protein kinase; CNS: central nervous system.

Declarations

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Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Capital Medical University and the Beijing Children's Hospital, Beijing, China. The methods were carried out in accordance with the relevant guidelines, including any relevant details. Written informed consent was taken prior to the study enrollment from their guardians of children.

Consent for publication

Not applicable

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

KZ performed experiments, analyzed data and wrote the manuscript. XZ, YZ and LZ, were responsible for recruitment of patients and collection of clinical data. NZ performed experiments and analyzed data. NC performed experiments. GL supervised clinical part of this study. QH designed the experiments, supervised the project, analyzed data and finalized the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Clinical and laboratory information of study subjects

Characteristics	Patients with PM[a]
Clinical features	
Fever[b]	95(100%)
Seizures	48(50.5%)
Bacteremia	40(50.5%)
Hearing loss	34(35.8%)
Hospitalization time (days)	23 (16.0-34.5)
Level of consciousness at admission	
Normal consciousness	41 (43.2%)
Disturbed consciousness	54 (56.8%)
Laboratory variables	
CRP (mg/L)	74.9 (17.4-143.9)
Glucose in CSF (mmol/L)	1.1(0.3-2.5)
Proteins in CSF (mg/L)	1754.9 (1018.5-2695.3)
White cell count in CSF ($\times 10^6$/L) [c]	610.0 (124.3-1615.0)
Polymorphonuclear cells (%)	71.7(55.0-80.0)
Mononuclear cells or lymphocytes (%)	28.0 (20.0-45.0)
White blood cell count ($\times 10^9$/L) [c]	14.6 (8.7-20.5)
Neutrophils (%)	76.2 (51.7-88.6)
Lymphocytes (%)	20.1 (6.9-39.2)

Abbreviations: CRP, C-reactive protein; CSF, cerebrospinal fluid

[a]Absolute count(%) for categorical variables and median (IQR) for continuous data, unless otherwise stated.

[b]Axillary temperature >38.0°C

[c] For patients for whom CSF and blood samples were obtained in the first three days of hospitalization

Table 2. Genotype and allele frequencies of IL-17A in PM patients and controls

SNPs	Genotypes and allele frequencies	Cases, No. (%)	Controls, No. (%)
rs4711998	AA	51/95(53.7)	173/330(52.4)
	GA	34/95(35.8)	118/330(35.4)
	GG	10/95(10.5)	39/330(11.8)
	major allele A	136/190(71.6)	464/660(70.3)
	minor allele G	54/132(28.4)	196/660(29.7)
rs8193036	CC	52/95(54.7)	170/330(51.5)
	CT	36/95 (37.9)	114/330(34.5)
	TT	7/95(7.4)	46/330(14.0)
	major allele C	140/190(73.7)	454/660(68.8)
	minor allele T	50/190(26.3)	206/660(31.2)
rs2275913	GG	27/95(28.4)	103/330(31.2)
	GA	44/95(46.3)	149/330(45.2)
	AA	24/95(25.3)	78/330(23.6)
	major allele G	98/190(51.6)	355/660(53.8)
	minor allele A	92/190(48.4)	305/660(46.2)

There were no statistically significant differences in genotype and allele frequencies of the SNPs studied between cases and controls

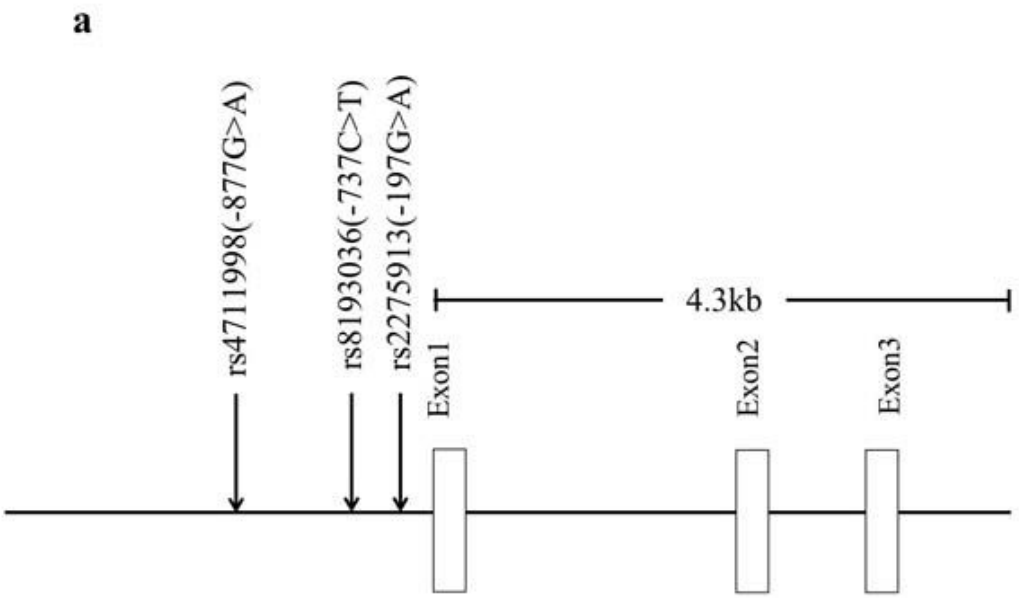
Table 3. Clinical characteristics in PM patients and genotypes and allele frequencies of IL-17A

Characteristics	Patients with symptoms or parameters defined (%)		OR (95% CI)	P value
	Glucose in CSF(mmol/L)			
rs4711998[a]	≤1.5	>1.5		
	(*n*=52)	(*n*=42)		
AA	32(0.615)	18(0.429)	1	Ref.
GA	**14(0.269)**	**20(0.476)**	**0.394(0.161-0.963)**	**0.039**
GG	6(0.116)	4(0.095)	0.844(0.210-3.390)	1.000
major allele A	78(0.75)	56(0.667)	1	Ref.
minor allele G	26(0.25)	28(0.333)	0.667(0.353-1.258)	0.209
rs8193036[a]				
CC	33(0.635)	18(0.429)	1	Ref.
CT	**15(0.288)**	**21(0.500)**	**0.390(0.162-0.936)**	**0.033**
TT	4(0.077)	3(0.071)	0.727(0.146-3.614)	1.000
major allele C	81(0.779)	57(0.679)	1	Ref.
minor allele T	23(0.221)	27(0.321)	0.599(0.313-1.150)	0.122

Abbreviations: OR, odds ratio; CI, confidence interval.

[a] Information was only available in 94 subjects.

Figures



b

Promoter region	Haplotype frequency PM/HC(%)
- - - A- - - C- - - G - - -	13.5/17.8
- - - A- - - C- - - A - - -	**43.6/35.7**
- - - A- - - T- - - G - - -	14.4/12.4
- - - A- - - T- - - A - - -	**0.0/4.4**
- - - G- - - C- - - G - - -	12.6/10.6
- - - G- - - C- - - A - - -	3.4/4.7
- - - G- - - T- - - G - - -	11.0/13.0
- - - G- - - T- - - A - - -	1.5/1.4

Figure 1

A Map of the IL-17 gene and the three-locus IL-17 promoter region haplotypes identified in 330 HCs and 95 patients with laboratory confirmed PM and their estimated frequencies. (a) Three SNPs of IL-17A rs4711998 (-877A>G), rs8193036 (-737C>T) and rs2275913 (-197G>A) are located on 5' upstream promoter region. The rectangles show the starting position of each of the three exons. (b) Linkage disequilibrium was determined with the use of x2 tests, and the estimated haplotype frequencies were

calculated as described above. Significant differences was found for haplotype A-C-A and A-T-A, with 43.6% (83/190) and 35.7% (236/660); 0.0% (0/190) and 4.4% (29/660) for patients and controls, respectively.

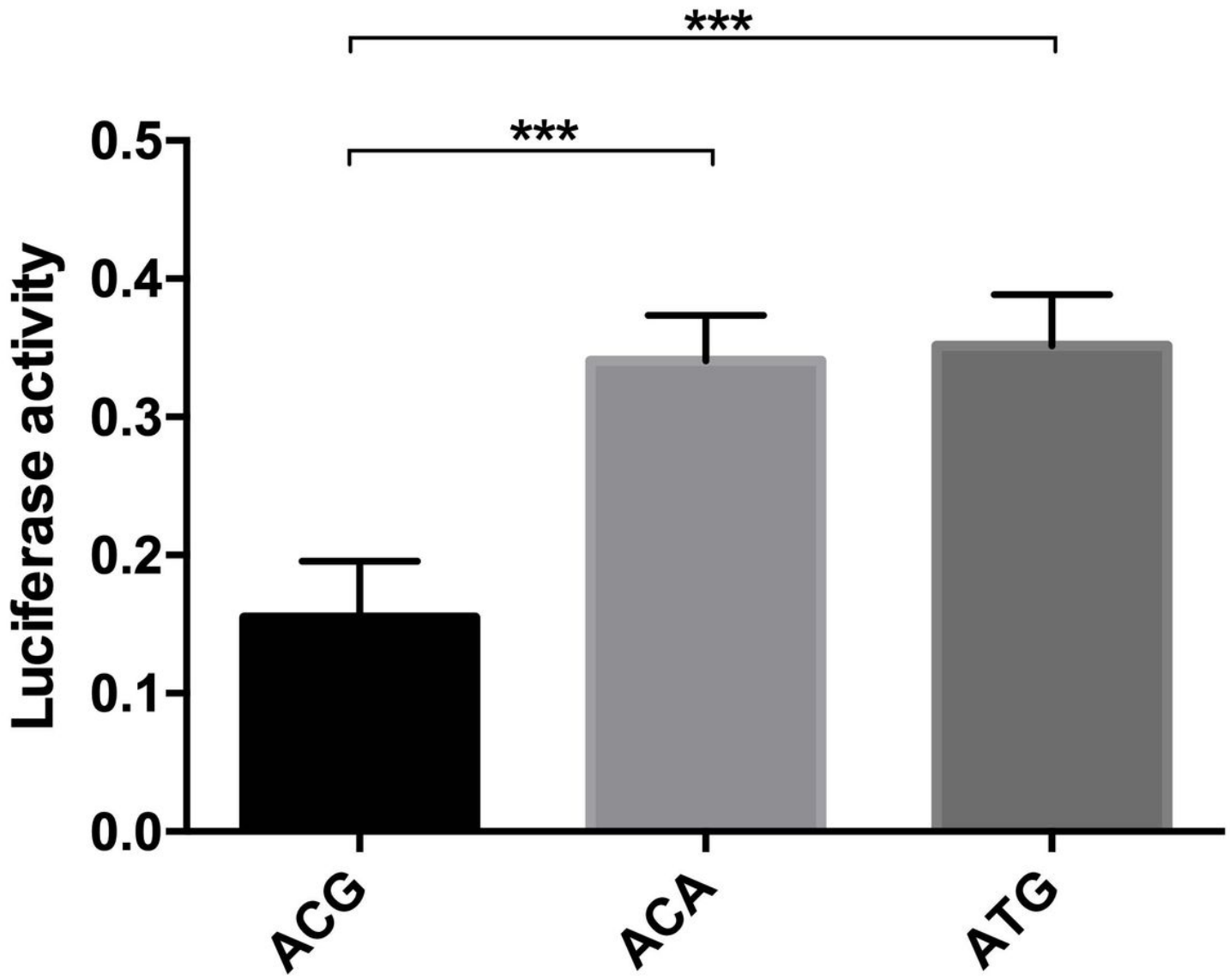


Figure 2

Three constructs were transiently transfected into HEK293T cells. Luciferase activity of each construct was normalized against internal control of Renilla luciferase. Data indicated mean values with SD from 3 independent experiments. Student- t test was used for group comparison (***P <0.001).

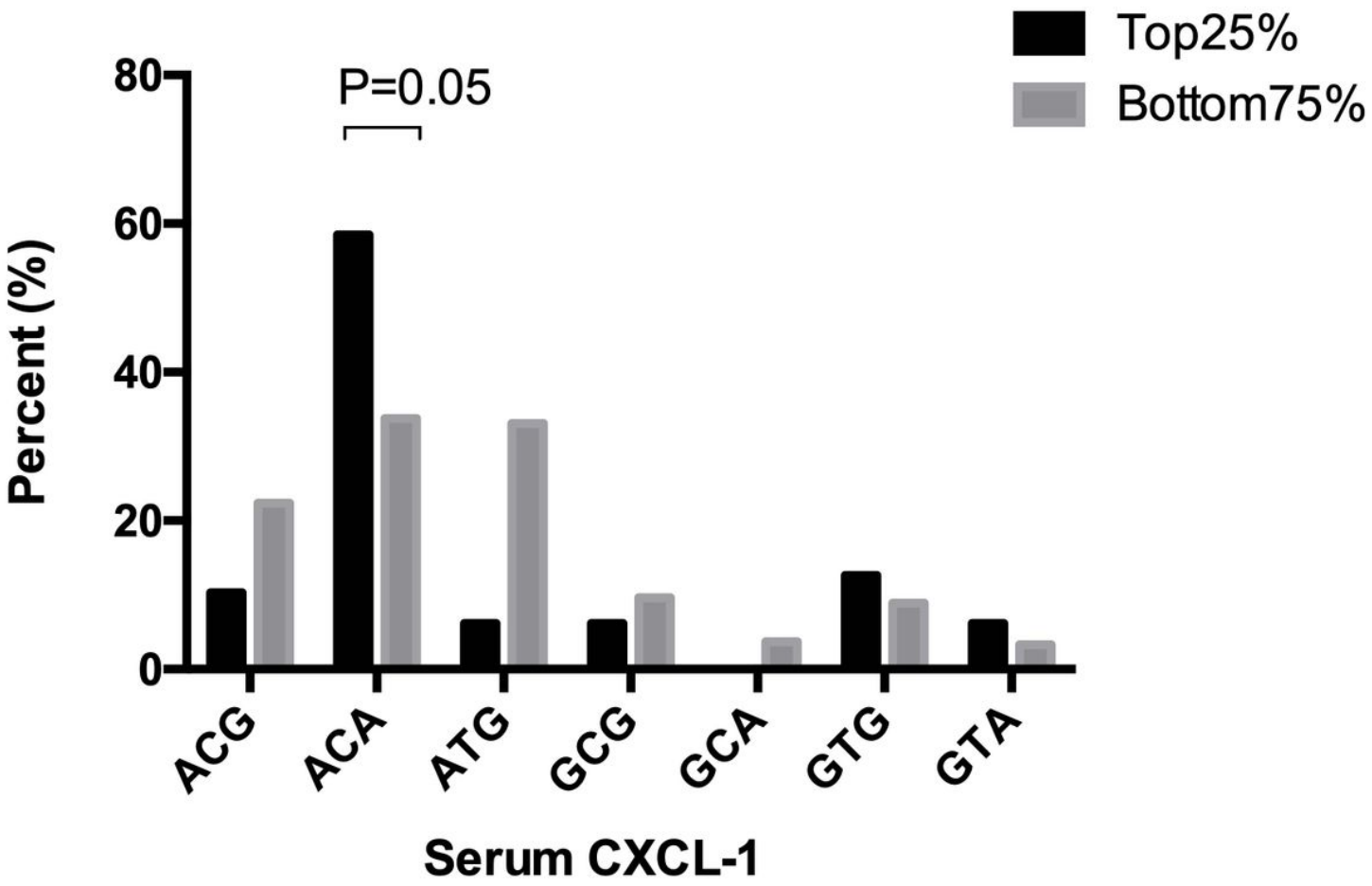


Figure 3

IL-17A promoter variants in PM cases with top 25% and bottom 75% concentration of serum CXCL-1. Haplotype A-C-A had a tendency of higher frequency in the top 25% concentration of CXCL-1 (top 25% vs. bottom 75%: 58.5% vs. 33.8%, OR=2.222, 95%CI: 0.982-5.028, P=0.05).

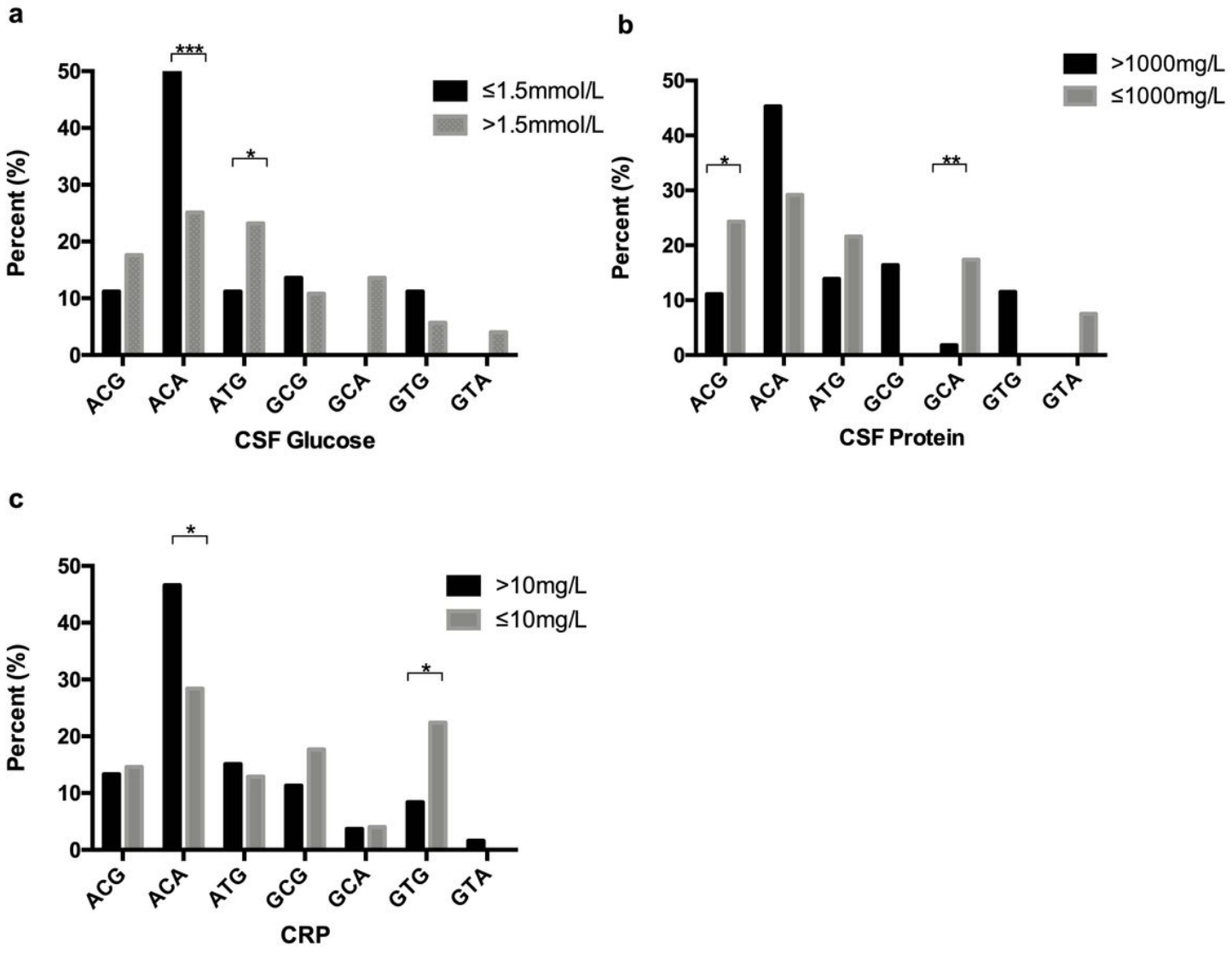


Figure 4

IL-17A promoter variants in PM cases with clinical characteristics. (a) Haplotype A-C-A frequency was significantly higher in cases with a CSF glucose concentration of less than 1.5mmol/L (52.8% vs.25.1%, OR=3.350, 95%CI: 1.787-6.280, P<0.001) while haplotype A-T-G frequency was significantly lower (11.2% vs.23.2%, OR= 0.426, 95%CI: 0.194-0.937 P=0.031). (b) Haplotype A-C-G and G-T-A frequencies were significantly lower in group with high level of CSF protein of more than 1000mg/L (11.1% vs.24.3%, OR= 0.390, 95%CI: 0.167-0.910, P=0.003; 1.8% vs.17.4%, OR= 0.001, 95%CI: 0.000-0.017, P=0.002, respectively) while Haplotype A-C-A frequency tended to be higher in this group (45.3% vs.29.2%, OR=2.010, 95%CI: 0.991-4.076, P=0.05). (c) Haplotype A-C-A frequency was significantly higher in group with a CRP concentration of more than 10mg/L (46.6% vs. 28.4%, OR=2.276, 95%CI: 1.011-5.126, P=0.043), while haplotype G-T-G frequency was significantly lower (8.4% vs.22.4%, OR= 0.321, 95%CI: 0.0119-0.864, P=0.019). (*P<0.05, **P<0.01, ***P<0.001)

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