

Relationship between interleukin-18-607C/A gene polymorphism and severity of enterovirus-71 encephalitis in Chinese children

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Abstract

Background: Interleukin-18 (IL-18), a pro-inflammatory cytokine encoded by IL-18 and known as interferon (IFN)- γ inducing factor, plays an important role in the innate immune system. Single nucleotide polymorphisms (SNPs) of IL-18-607C/A have been reported to be associated with many infectious and immune-related diseases. However, there is no evidence of its association with enterovirus 71 (EV71)-infectious encephalitis. This study aimed to explore the association between the promoter region polymorphisms (-607C/A) of IL-18 and the severity of EV71 encephalitis in Chinese children.

Methods: We analyzed the polymorphisms of IL-18-607C/A in 185 EV71-infected patients and 214 controls for genetic association research. Clinical features and auxiliary examination results were collected and compared in the cases. The serum concentration of IFN- γ was detected using enzyme-linked immunosorbent assays (ELISA).

Results: The frequency of IL-18-607 AA genotype and allele in EV71-infected patients was significantly higher than that in the controls (32.4% vs 21.5%, $P=0.042$, 55.1% vs 46.0%, $P=0.001$). Furthermore, a difference was found in severe EV71 and mild encephalitis cases (53.0% vs 20.5%, $P=0.003$, 67.3% vs 50%, $P=0.016$). The serum concentration of IFN- γ in patients with encephalitis was much lower in AA genotypes (106.0 ± 8.9 pg/mL) than in CA and CC genotypes (120.4 ± 8.9 and 128.1 ± 7.5 pg/mL, respectively, $P<0.001$). The duration of fever and blood glucose level was obviously higher in AA than in CA and CC genotypes.

Conclusions: Children with IL-18-607AA genotype were more susceptible to developing severe EV71-associated encephalitis in this study in China.

Background

Enterovirus 71 (EV71) is a small non-enveloped, single-positive-sense-strand neurovirulent RNA virus belonging to the Picornaviridae family and is an Enterovirus A species belonging to the genus *Enterovirus* [1]. It caused outbreaks of several diseases including hand-foot-mouth disease (HFMD), herpangina, encephalitis, meningitis, acute flaccid paralysis, complicated pulmonary edema (PE), hind-limb paralysis, and even shock in children in the late 1990s [2–4]. Nearly 1.5 million people (most of them are children >5 years old) were infected while 405 children developed severe neurological complications in Taiwan [2,5,6]. People with EV71 infection showed different clinical manifestations, which not only depended on the virulence of EV71, but also were inextricably linked to the host's immune system. As an important component of the immune system, interleukin (IL)–18 plays a vital role in the immune defense process [6–9,10–12].

IL–18, an 18.3-kDa pro-inflammatory cytokine, is regarded as a member of IL–1 family of cytokines [13], which is crucial to both innate and acquired immune responses. It is considered to be a partial inducing factor of IFN- γ [14]. High serum levels of IFN- γ activate macrophages and antiviral effects and promote the performance of the major histocompatibility complex (MHC) in dendritic Cells (DC) and antigen

presentation, which boosts immune regulation [13, 15, 16]. In addition, the maturation of IL-18 is controlled by activated NLRP3 inflammasome, which may protect host from fatal EV71 infection [17]. The human *IL-18* is located on chromosome 11q22.2–22.3 while IL-18-607C/A is located in the 5'-untranslated region of *IL-18* [15]. Many diseases have been reported to be associated with single nucleotide polymorphisms (SNPs) of the promoter region (-607C/A), such as asthma, rheumatoid arthritis (RA), Crohn's disease, multiple sclerosis (MS), recurrent spontaneous miscarriage (RSM), and type 1 diabetes mellitus (T1DM) [15, 16, 18–20]. This study aimed to explore the relationship between the polymorphisms of IL-18-607C/A and the severity of EV71 encephalitis in a population of Chinese children.

Methods

2.1 Case selection

In this study, we firstly obtained informed consent from 399 Chinese children in the Affiliated Hospital of Qingdao University and grouped them according to different clinical diagnostic criteria. EV71 infection was diagnosed by both clinical features and reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted from the cerebrospinal fluid (CSF), throat swabs, stool specimens, and any other tissues obtained from the patients being studied the day after admission. EV71 encephalitis was confirmed by brain parenchymal damages, CSF examination (white blood cell count $>5/\text{mm}^3$), and parenchymal lesions as identified by brain computed tomography (CT) or magnetic resonance imaging (MRI).

EV71 encephalitis patients with the following characteristics were regarded as severe cases: (1) focal or diffuse parenchymal lesions of the brain parenchyma, (2) abnormal MRI showing edema in the adjacent brain parenchyma and destruction of the brain parenchyma, (3) prolonged coma and confusion, and (4) other clinical characteristics (including oral ulcers and skin eruption on the hands and feet) [21]. All the subjects in this study were analyzed by clinical interview, physical routine examination, serological test, MRI, CSF analysis, and other auxiliary checks. Moreover, corresponding records were made.

2.2 Data collection

Clinical and laboratory data were collected from June 2011 to December 2012. In this study, we recorded demographic data such as sex and age and the results of laboratory examinations such as white blood cell (WBC) count, C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase-myocardial isozyme (CK-MB), electroencephalogram (EEG), electrocardiogram (ECG), CT of the brain, and myelencephalon MRI of the brain or spine.

A commercial kit (Qiagen) was used to extract the genomic DNA from WBCs. To detect the -607C/A polymorphism in *IL-18*, the improved multiplex ligation detection reaction (iMLDR) technique developed

by Genesky Biotechnologies Inc. (Shanghai, China) was used for genotyping at position -607 of *IL-18* on chromosome 11q22.2-22.3. The product size was 261 bp. The primers designed for the PCR are shown in Table 1. The PCR conditions were as follows: 95 °C for 2 min; 11 cycles at 94 °C for 20 s, 65 °C-0.5 °C/cycle for 40 s, and 72 °C for 1 min 30 s; and 24 cycles at 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 1 min 30 s, followed by 72 °C for 2 min and holding at 4°C. The PCR products were purified by digestion with 1 U of shrimp alkaline phosphatase at 37 °C for 1 h and at 75 °C for 15 min.

The ligation reaction mixture contained 2 µL 10 × ligase buffer, 0.2 µL Taq DNA ligase, 1 µL probe mixture, and 3 µL purified PCR product mixture. In a double connection reaction, each site contained two 5' ends of allele-specific probes and following a 3'end of an allele-specific probe. Each allele-specific connection product was distinguished by its fluorescence, while different loci were distinguished by the different lengths added to the 3'end of the allele-specific probe. The probes were TAM-TTTGGTATCCCTCTCC-MGB and TET-TTTGGTAGCCCTCTV-MGB. The ligation cycling program consisted of 35 cycles at 94 °C for 1 min and 56 °C for 4 min, and then holding at 4 °C. The SNPs were genotyped using a 3130xl Genetic Analyzer (ABI), and the raw data was validated using the Gene Mapper 4.0 (Applied Biosystems, USA).

2.3 Estimation of IFN-γ levels

Plasma concentrations of IFN-γ were detected using ELISA kits (R&D Systems) according to the manufacturer's instructions. The sensitivity of detection is 8 pg/mL as indicated by the manufacturer. Each sample was repeated, and these values were in the linear part of the standard curve.

2.4 Statistical analysis

The Chi-square test was used to compare frequencies of the three genotypes (CC, CA, and AA) and two alleles (C and A) in different groups as well as demographics (such as sex) and clinical findings and symptoms (such as abnormal ECG or MRI, temperature >39 °C, vomiting, and mental change after convulsions) among different genotype groups, respectively. The differences in demographics and clinical manifestation such as age, duration of fever, WBC count, CRP, ALT, AST, CK-MB, and BG of the different genotype groups are presented as means ± standard deviation (SD) or median values (25th-75th percentage values) and analyzed using a one-way analysis of variance (ANOVA).

The concentration of IFN-γ is presented as means ± SD and was analyzed using the *t*-test. The relationship of *IL-18-607C/A* polymorphism to the susceptibility and severity of EV71-related encephalitis was evaluated by calculating the odds ratio (OR) and 95% confidence intervals (95%CI) using logistic regression. Pearson's collection statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 21.0 IBM SPSS software, USA) and significance was set at $P < 0.05$.

Results

3.1 Study population

Three hundred and ninety-nine children were divided into EV71-infected and control groups. Subsequently, the 185 EV71-infected Chinese children (99 males, 6.20 ± 3.69 years old) were examined, and divided into the EV71 encephalitis (93 cases, 4.32 ± 2.33 years old) group, consisting of 49 severe and 44 mild (3.21 ± 3.48 and 5.50 ± 3.09 years old) cases, respectively, and the non-encephalitis group. No child died or was not followed up in this study.

3.2 Distribution of genotypes and alleles of IL-18

The distribution of genotypes and alleles of IL-18-607C/A is shown in Table 2. The genotype distributions were in accordance with the Hardy Weinberg equilibrium ($P > 0.05$). The IL-18-607 AA genotype distribution in EV71-infected children was significantly higher than that in the control group was ($P < 0.05$). A marked difference was also found in IL-18-607C/A allele frequencies between the EV71-infected and control groups ($P = 0.010$, OR = 1.441, 95%CI = 1.090–1.906).

The results in Table 3 indicate that the EV71 encephalitis group showed no difference from the non-encephalitis group, which was set as the reference ($P_1 = 0.292$, $P_2 = 0.119$, OR = 1.386, 95%CI = 0.919–2.090). Moreover, Table 4 shows that the frequency of AA genotype was significantly higher in the severe encephalitis group than in the mild encephalitis ($P_1 = 0.003$). Furthermore, a similar difference was found in alleles ($P_2 = 0.016$, OR = 2.063, 95%CI = 1.139–3.736). Compared with the C allele, carrying the A allele increased the risk of severe EV71 encephalitis by 2.063 times and, therefore, is a high-risk factor for the progression of EV71 infection to severe encephalitis.

Table 5 describes the characteristics of EV71-infected patients with different genotypes. There were significant differences in CRP level, WBC count, duration of fever, and BG level. No obvious differences were found in sex, age, ALT, AST, and CK-MB levels. In addition, some clinical manifestations, such as the distribution of vomiting, seizures, spirit change, as well as high body temperature, brain MRI, and ECG were evaluated.

3.3 Effects of IL-18 –607C/A polymorphism on serum IFN- γ level

The results shown in Figure 1 indicate that there were significant differences in serum IFN- γ among the control and mild and severe EV71 encephalitis groups (52.02 ± 15.85 , 114.60 ± 9.08 , and 128.95 ± 7.62 pg/mL, respectively). While marked differences were found in the CC and CA genotypes (75.40 ± 37.71 and 119.00 ± 21.13 pg/mL, respectively) in all cases, the CC and AA (118.84 ± 21.96 pg/mL) genotypes where

similar, whereas no obvious differences were observed between the CA and AA genotypes. In EV71-infected patients, the IFN- γ levels showed no distinct differences among the three genotypes. The IFN- γ levels were also compared among encephalitis patients and the result showed that the IFN- γ level of the AA genotype patients (106.00 ± 8.93 pg/mL) was clearly lower than that of CA (120.45 ± 8.88 pg/mL) and CC (128.14 ± 7.49 pg/mL) genotype patients in the encephalitis groups. In this study, the serum IFN- γ concentrations were compared using the Pearson's correlation statistical analysis.

Discussion

EV71 plays an important role in the etiology of epidemics of severe neurological infectious diseases in children because of its neurotropic ability. In Asia, especially China, several EV71 encephalitis incidences have caused long-term neurological sequelae, with reduced cognitive function and high mortality rate [3, 25]. Numerous hereditary factors such as the *MDA5*, *IL-10*, *IL-17F*, and *CCL2-2510* have been suggested to play a crucial role in the infectivity of EV71 [17,18,26]. A previous study demonstrated that as an NLRP3-dependent effector protein, the pleiotropic IL-18 is one of the protective factors against EV71 infection in mice [26]. Lethal EV71 challenge studies showed that the NLRP3 inflammasome was activated by EV71 3D protein, and formed an EV71-3D-NLRP3-ASC ring-like structure. The NLRP3 inflammasome controls the maturation and secretion of IL-18. The study also showed that recombinant IL-18 considerably suppressed EV71 infection in IL-18 deficient mice [17, 22].

Tavares [16] reported that the alternation from C to A at promoter region -607 position feasibly may cause *IL-18* downregulation and reduce IL-18 production. *IL-18*-deficient mice were more susceptible to EV71 infection, which caused severe EV71-associated encephalitis, similar to the experimental results of this study. Our study in humans showed an apparent association between IL-18-607A SNPs and the occurrence rate of EV71 infection. The analysis of the experimental results showed significant differences between severe and mild EV71 encephalitis depending on the distribution of the IL-18-607 genotypes and alleles, whereas no differences were observed between the encephalitis and the non-encephalitis groups. Therefore, in our study, the significantly increased risk for EV71 infection with variant genotypes of IL-18 could lead to abnormal properties and low immune responses in patients with severe EV71 encephalitis. The failure of the immune system to eliminate invasive EV71 may contribute to the development of severe EV71 encephalitis. Patients with severe EV71 encephalitis have impaired TH1-mediated immune response and stimulate NK cells to exert its cytolytic effect in patients with EV71-infected encephalitis [23, 24].

Moreover, the results indicate that patients with the -607AA genotype had higher CRP levels, WBC counts, BG degrees, and duration of fever than those with other genotypes did, which was related to the severity of EV71 infection [25, 26]. Among CC, CA, and AA genotypes, the differences in duration of fever were meaningful and, especially, important in evaluating the severity of EV71 infection [25]. However, there was no remarkable difference in sex, age, ALT, AST, and CK-MB. Significant differences were also not found in the ECG, MRI, vomiting, seizure, spirit change, and temperature over 39 °C. The inflammatory process of severe EV71 infection likely involved IL-18-607A, but the mechanism is still not clear.

IL-18-mediated viral infection likely involves several mechanisms including one that facilitates the production of IFN- γ , activated by IL-12 or IL-15 due to the activation of caspase 1. Another possible mechanism involves the direct activation of macrophages to induce chemokine and IFN- γ secretion, and stimulate FasL-mediated cytotoxicity [26]. In this study, we found an obvious difference in serum IFN- γ among controls, mild cases, and severe cases. The evaluation of all patients showed that IFN- γ levels were higher in those with AA genotype than in those with the other two genotypes. IFN- γ is one of the severity indices for EV71 encephalitis with a specific critical range [1, 24–26]. Following EV71 viral attacks, the human body can initiate the production of IFN- γ by enhanced expression of *IL-18*. A reasonable assumption could be made that IL-18-607A increases susceptibility to EV71 viral infection. The comparison of IFN- γ levels among EV71-infected patients with AA, CA, and CC genotypes did not show a clear distinction. Furthermore, patients with encephalitis in the AA genotype groups showed lower serum IFN- γ levels than those of the CA and CC groups. This difference may be associated with the involvement of the immune system in EV71-induced immunoreactions. IL-18-607A SNPs may reduce the production of serum IFN- γ in patients with EV71 encephalitis by negatively regulating *IL-18* expression at the posttranscriptional level. However, the body cannot produce adequate levels of IFN- γ to fight against EV71 similar to the previous involvement of an uncontrolled systemic inflammatory cascade induced by infectious factors.

This study has some limitations. Firstly, the sample digital object collected was not sufficient to represent the whole population, and therefore, the characteristics of the study sample only reflected a portion of the entire population. Secondly, the concentration of IL-18 and other associated molecules of the immune system in the serum was not detected and analyzed. Moreover, the mechanism of IL-18-607A SNPs affecting the IL-18 production in EV71 infection progress should be lucubrated.

Conclusion

In conclusion, the IL-18-607AA genotype, which is involved in the inflammatory process induced susceptibility to EV71 infection, enhancing the development of severe EV71-associated encephalitis in Chinese children.

Abbreviations

EV71 ☐ Enterovirus 71

IL-18 ☐ Interleukin-18

IFN- γ ☐ interferon- γ

SNPs ☐ Single nucleotide polymorphisms

ELISA ☐ enzyme-linked immunosorbent assays

HFMD☐hand-foot-mouth disease

PE☐pulmonary edema

MHC☐the major histocompatibility complex

DC☐dendritic Cells

RA☐rheumatoid arthritis

MS☐Crohn's disease, multiple sclerosis

RSM☐recurrent spontaneous miscarriage

T1DM☐type 1 diabetes mellitus

RT-PCR☐reverse transcription polymerase chain reaction

CSF☐cerebrospinal fluid

CT☐computed tomography

MRI☐magnetic resonance imaging

WBC☐white blood cell

CRP☐C-reactive protein

ALT☐alanine aminotransferase

AST☐aspartate aminotransferase

CK-MB☐creatin kinase-myocardial isozyme

EEG☐electroencephalogram

ECG☐electrocardiogram

Declarations

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Availability of data and materials:

Genotype/allele data will be available on request in this paper.

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Declarations

Ethics approval and consent to participate

All the procedures in this study were in accordance with the standards of the Ethics Review Committee of the Affiliated Hospital of Qingdao University. We obtained informed consent from 399 Chinese children's parents in the Affiliated Hospital of Qingdao University and Qingdao Women & Children's Hospital.

Consent for publication

Not applicable

Competing interest

None to disclose.

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Tables

Analysis of genotypes of IL-18

Table 1 The sequence-specific primers for C/A allele and their PCR product sizes for position -607 in the promoter of the IL-18 gene

sequence-specific	Primers	Primer sequence	Product size(bp)
-607C/A	C allele primer	5'-GTTGCAGAAAGTGTA AAAAATTATTAC-3'	261
	A allele primer	5'-GTTGCAAAGTGTA AAAAATTATTAA-3'	
	Common reverse primer	5'-TAACCTCATT CAGGACTTCC-3'	

Table 2 Genotype and allele frequencies of the IL-18-607C/A polymorphism in EV71-infected patients and controls

IL-18-607C/A SNP	EV71-infected patients (n=185)	Controls (n=214)	P-value	OR (95%CI)
Genotype				
C/C	41 (22.2%)	63(29.4%)	0.042 ^a	
C/A	84(45.4%)	105(49.1%)		
A/A	60(32.4%)	46 (21.5%)		
Allele				
A	204(55.1%)	197(46.0%)	0.010 ^b	1.441 (1.090-1.906)
C	166(44.9%)	231(54.0%)		

^a Controls vs. EV71-infected patients using the chi square-test with a 3×2 contingency table

^b Controls vs. EV71-infected patients using the chi square-test with a 2×2 contingency table

Table 3 Genotype and allele frequencies of the IL-18-607C/A polymorphism in EV71-encephalitis and Non-EV71 encephalitis

IL-18-607C/A SNP	EV71 encephalitis(n=93)	Non-EV71 encephalitis(n=92)	P-value	OR (95%CI)
Genotype				
C/C	18(19.4%)	23(25%)	P ₁ =0.292 ^a	
C/A	40(43.0%)	44(47.8%)		
A/A	35(37.6%)	25(27.2%)		
Allele				
A	110(59.1%)	94(51.1%)	P ₂ =0.119 ^b	1.386 (0.919-2.090)
C	76(40.9%)	90(48.9%)		

^a EV71 encephalitis cases vs. Non-EV71 encephalitis cases using the chi square-test with a 3×2 contingency table

^b EV71 encephalitis cases vs. Non-EV71 encephalitis cases using the chi square-test with a 2×2 contingency table

Table 4 Genotype and allele frequencies of the IL-18-607C/A polymorphism in Severe EV71 encephalitis and Mild EV71 encephalitis

IL-18-607C/A SNP	Severe EV71 encephalitis(n=49)	Mild EV71 encephalitis (n=44)	P-value	OR (95%CI)
Genotype				
C/C	9 (18.4%)	9 (20.5%)	P ₁ =0.003 ^a	
C/A	14 (28.6%)	26 (59.0%)		
A/A	26 (53.0%)	9 (20.5%)		
Allele				
A	66 (67.3%)	44 (50%)	P ₂ =0.016 ^b	2.063 (1.139-3.736)
C	32 (32.7%)	44 (50%)		

^a Severe EV71 encephalitis cases vs. Mild EV71 encephalitis cases using the chi square-test with a 3×2 contingency table

^b Severe EV71 encephalitis cases vs. Mild EV71 encephalitis cases using the chi square-test with a 2×2 contingency table

Table 5 Characteristic of EV71-infected group according to different genotypes

Parameters	CC(n=41)	CA(n=84)	AA(n=60)	P-value
Male (n=111)	29	43	39	
Female (n=74)	12	41	21	0.070 ^a
Age(years)	6.6±3.7	5.4±3.0	5.0±3.3	0.054 ^b
Duration of fever(days)	1.6±1.5	3.2±2.8	3.8±3.6	0.001 ^b
WBC(×10 ⁹ /L)	9.1±4.8	11.5±5.0	12.8±6.1	0.013 ^b
CRP(mg/L)	5.6±8.0	10.5±7.6	12.9±11.1	0.001 ^b
ALT(U/L)	18.0(14.0-23.5)	20.0(16.3-22.0)	17.0(14.0-23.0)	0.341 ^c
AST(U/L)	22.0(19.0-29.0)	25.0(22.0-31.0)	24.0(20.0-34.0)	0.526 ^c
CK-MB(U/L)	13.0(10.0-19.5)	12.0(10.0-18.75)	13.5(11.0-19.0)	0.348 ^c
BG(mmol/L)	5.4±2.2	6.3±1.9	9.23±2.7	0.015 ^b
Vomiting	3	10	11	0.250 ^a
Seizure	4	12	7	0.753 ^a
Spirit change	8	10	14	0.532 ^a
The highest temperature ≥39°C	18	34	29	0.645 ^a
Brain MRI Abnormal	3	10	7	0.716 ^a
EEG Abnormal	16	22	22	0.247 ^a

WBC, white blood cell count; CRP,C-reactive protein; BG, blood glucose; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK-MB, cardiac creatine kinase-MB fraction; MRI, Magnetic Resonance Imaging; EEG, electroencephalography

^a Groups compared using chi-square test

^b Values expressed as mean ± SD, groups compared using ANOVA

^c Values expressed as median(25th-75th percentile values)

Figures

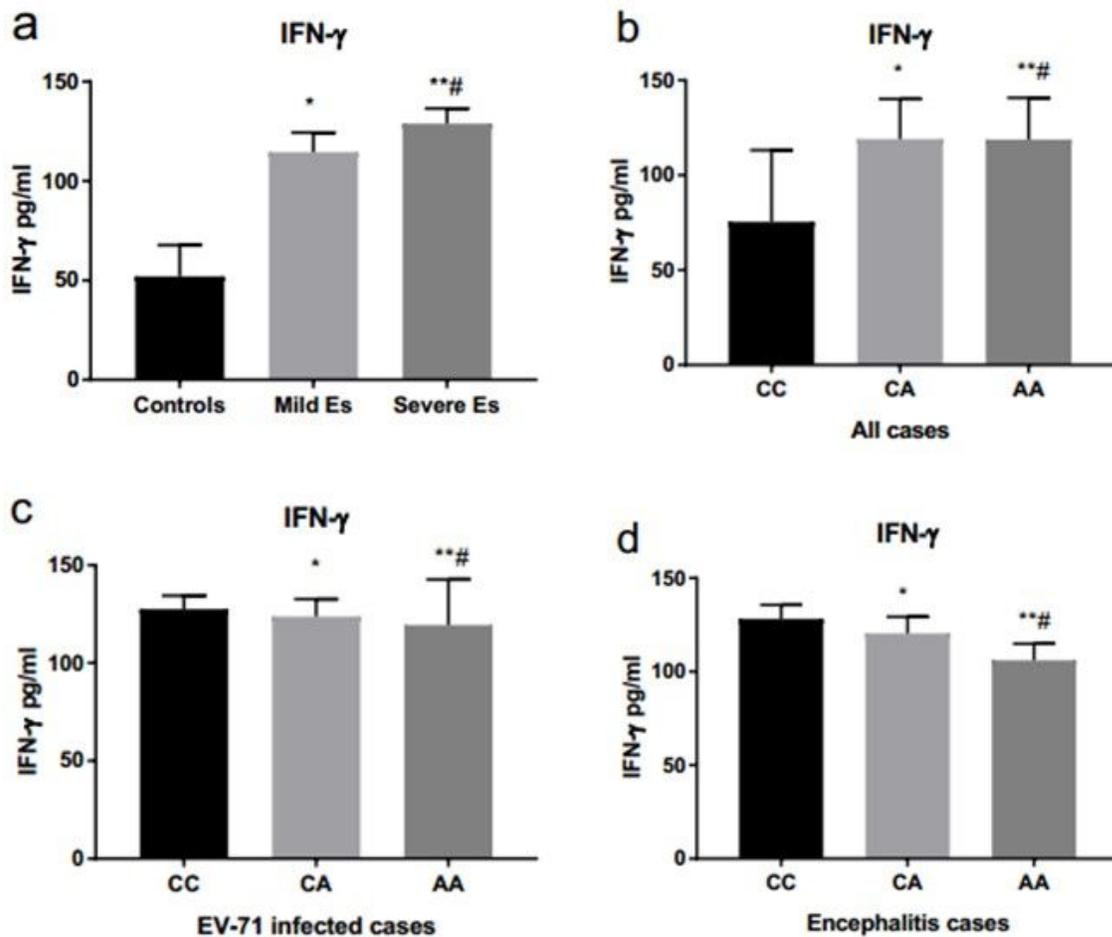


Figure 1

Serum IFN- γ levels were measured in 60 controls, 25 non-encephalitis children, 21 mild EV71 encephalitis and 17 severe EV71 encephalitis patients. Values expressed as mean \pm SD. a The serum IFN- γ level in Severe EV71 encephalitis (Severe Es) children (128.95 \pm 7.62pg/ml) were elevated significantly compared to mild encephalitis (mild Es) patients(114.60 \pm 9.08pg/ml) and controls(52.02 \pm 15.85pg/ml) (*P1 \leq 0.001,mild encephalitis vs controls; **P2 \leq 0.001,severe encephalitis vs. controls; #P3 \leq 0.001,severe encephalitis vs. mild encephalitis). b In all cases, IFN- γ concentration in CC (75.45 \pm 37.71 pg/ml) was much lower compared to CA (119.00 \pm 21.13 pg/ml) and AA (118.84 \pm 21.96 pg/ml) genotypes (*P1 \leq 0.001,genotype CC vs.CA; **P2=0.001,genotype CC vs. AA),but no significant differences in CA and AA genotype (#P3=0.99, genotype CA vs. AA). c In EV71 infected patients, no distinct differences among three genotypes in the concentration level of IFN- γ were found (*P1=0.09,genotype CC vs. CA; **P2=0.12, genotype CC vs. AA; #P3=0.41, genotype CA vs. AA). d In encephalitis cases, the level of IFN- γ in AA genotype (106.00 \pm 8.93pg/ml) patients were clearly lower than CA (120.45 \pm 8.88pg/ml) and CC (128.14 \pm 7.49pg/ml) in encephalitis groups (*P1=0.014, genotype CC vs. CA; **P2 \leq 0.001,genotype CC vs. AA; #P3=0.002, genotype CA vs. AA).