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Original Article

Correlation between miR-122 and IFN-γ cytokines in infant cytomegalovirus hepatitis

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Abstract: Infant cytomegalovirus hepatitis is one disease caused by cytomegalovirus (CMV) infection. CMV can be transmitted by mother-infant pathway. Most CMV infection had insidious onset, with jaundice, liver hypertrophy and dysfunction, with liver sclerosis and failure in severe cases. The mechanism of infant CMB hepatitis is still unclear yet. Recent studies showed the involvement of microRNA-122 (miR-122) in liver development, genetic regulation and functional metabolism. This study thus investigated the expression of miR-122 and CMV-DNA in patient blood to investigate the role of miR-122 in disease onset. 55 CMV hepatitis patients and 27 healthy control individuals were collected for blood samples, from which enzyme linked immunosorbent assay (ELISA) was employed to quantify serum IFN-γ and TNF-α levels. MiR-122 content was tested by real-time fluorescent quantitative PCR. CMV hepatitis patients had elevated levels of miR-122, IFN-γ and TNF-α compared to healthy controls ($P < 0.05$). After 1-month treatment, curing and improving infants had significantly lowered miR-122, IFN-γ and TNF-α levels ($P < 0.05$) while incurring group had no significant differences ($P > 0.05$). Their expression levels were also positively correlated with CMV-DNA contents. Cytokines including IFN-γ and TNF-α play an important role during the onset of CMV hepatitis, as is positively correlated with body's CMV-DNA contents.

Keywords: MiR-122, IFN-γ, TNF-α, CMV-DNA, cytomegalovirus hepatitis

Introduction

Human cytomegalovirus (HCMV), also named as human herpes virus 5 (HHV-5), is one linear double stranded DNA virus belonging to β-herpesvirinae, herpesviridae [1]. CMV is widely distributed in natural environment. People are susceptible for CMV. Epidemiology survey showed the frequency of CMV in China, as 83.2% of children between 1 and 3 years were positive for CMV-IgG. Such positive rates were 83.7% and 87.3% for age groups between 3 to 7 years, and between 7 and 14 years old. Women at pregnant stage had the positive rate as high as 95%, suggesting the possible occurrence of primary CMV infection at infant or children period [2, 3]. CMV can be transmitted by maternal-fetal pathway and horizontal ways, the former of which is the most important pathway for fetal and infant CMV infection [4]. CMV infection can be divided into insidious infection (without symptoms) and typical infection with symptoms, while most cases belong to the former category. In all infant CMV infection with symptoms, CMV hepatitis is the most popular disease and is one of reasons causing infant hepatitis syndrome [5], which is manifested as mild to moderate jaundice, liver expansion and dysfunction. In severe cases, liver cirrhosis, liver failure or even death may occur [6]. After invading into the body, CMV also activate Th1-induced cell immunity, during which cytokines including IFN-γ and TNF-α secreted by Th1 cells play an important roles [7]. Infant CMV hepatitis is one complicated biological process and is the hot topic in medical research. Recent study has found the critical role of microRNA (miRNA) in physiology and pathology process of liver [8]. As one kind of non-coding small molecule RNA with conserved sequences, miRNA can bind with 3' UTR of target gene mRNA and to inhibit the translation of target gene to exert biological functions [9]. It has been shown that miRNAs, which consist of only 1% of total RNA pools, can

regulate more than 30% gene regulation, and participate in various biological processes including cell proliferation, differentiation, apoptosis and activation [10]. Study has found the specific expression of miR-122 in liver tissues, and its involvement in liver cell growth, proliferation, differentiation and functional metabolism [11]. Other study also indicated the correlation between miR-122 and liver viral infection [12]. This study thus investigated the expression of miR-122 in infant CMV hepatitis infection patients, and analyzed the correlation between miR-122 and cytokine levels, in an attempt to investigate the function of miR-122 in CMV infection induced liver injury and pathogenesis mechanism.

Methods and materials

Reagents and instrument

Serum RNA extraction kit (Invitrogen, USA); Reverse-transcription PCR kit (Toyobo Life Science, Japan); SYBR fluorescent dye (Toyobo Life Science, Japan); IFN-γ ELISA kit (Xinbosheng Bio, China); TNF-α ELISA kit (Xinbosheng Bio, China); DNA extraction kit (Applied Biosystems, USA); Microplate reader (Bio-tek, USA); Waterbath (Changyuan Instrument, China); UV spectrometer (Eppendorf, Germany); Real-time fluorescent quantitative PCR (ABI, USA).

Research objects

A total of 55 CMV hepatitis infants (1 to 6 month old) were recruited in this study from August 2014 to July 2015 in the Second Affiliated Hospital. All patients received confirmed diagnosis complied with diagnostic criteria stipulated by Infectious digestive committee of Pediatric sub-group, Chinese Medical Association. There were 31 males and 24 females in the experimental group, in parallel with 27 healthy control individuals (16 males and 11 females). Inclusive criteria of control infants were: IgM negative for HCMV; Normal birth history; No infectious, metabolic or genetic disease; Normal development infant aging between 1 and 6 months. After 1-month of CMV hepatitis treatment, a total of 42 infants had improvement or cure, while 13 cases were still uncured. 3 mL peripheral blood samples were collected 1 month before and after treatment. Blood samples were centrifuged to collect serum, which were kept at -80°C fridge. This study has been pre-approved by the ethical committee of the Second Affiliated Hospital and has obtained written consents from all participants.

Serum RNA extraction

Serum was collected by centrifugation at low temperature. 0.5 mL serum was mixed with equal volume of 2 X Solution Buffer for vortex. After incubation on ice for 5 min, 1 mL phenol-chloroform mixture was added for vortex following 10 min incubation. The mixture was then centrifuged at 12000 g for 10 min at 4°C. The upper phase was saved and mixed with 1 mL absolution ethanol. The mixture was then transferred to the column for centrifugation at 12000 g for 30 s 4°C. After discarding the liquid, 0.5 mL buffer I was added for 30 s centrifugation, followed by 0.7 mL buffer II for 30 s centrifugation. The column was then transferred to a new tube and was added with 0.1 mL RNAse-free water, followed by 1 min centrifugation to elute RNA solution, which was kept at -80°C for further use.

Real-time fluorescent quantitative PCR

RNA reverse transcription was performed using test kit following manual instruction. 500 ng RNA was incubated at 65°C for 5 min, followed by iced cooling for 2 min. PCR primers, reverse transcription buffer were prepared in 20 μl system, which was incubated at 37°C for 15 min, followed by 98°C denature for 5min to synthesize cDNA. cDNA was then mixed with PCR primers, SYBR Green solution and sterilized water in 20 μl system. Real-time fluorescent quantitative PCR cycler (model VIIA 7) was used to perform PCR under the following conditions: 95°C for 5 min, followed by 40 cycles each containing 95°C denature for 15 s, 60°C annealing for 45 s and 72°C elongation for 15 s. Primer sequences were as follows: U6-F, 5'-GCTTC GGCAG CACAT ATACT AAAAT-3'; U6-R, 5'-CGCTT CACGA ATTTG CGTGT CAT-3'; miR-122-F, 5'-ACACT CCAGC TGGGT GGAGT GTGAC AATCC-3'; miR-122-R, 5'-TGGTG TCGTG GAGTC G-3'.

DNA extraction and CMV DNA assay

Peripheral venous blood sample (1 mL) was mixed with 1.5 mL EDTA buffer for vortex. After adding proteinase K, the mixture was incubated at 50°C for 90 min using intermitted vortex.

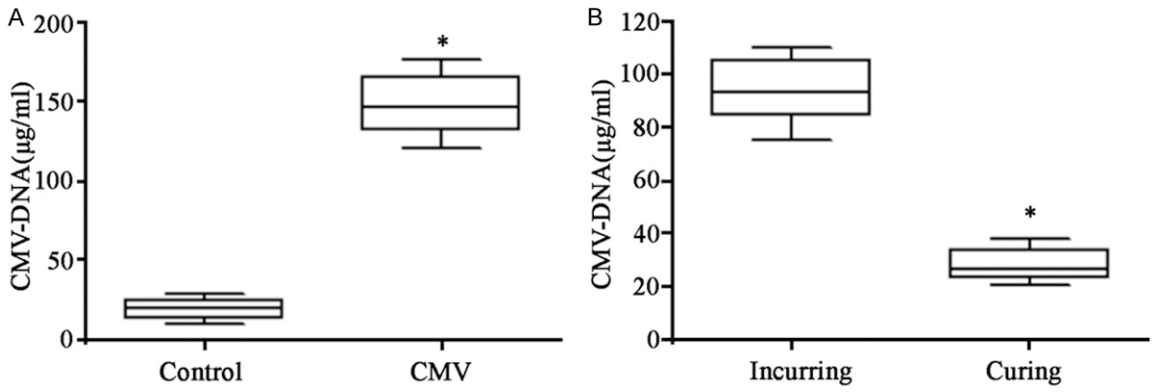

Figure 1. CMV-DNA expression. A: CMV-DNA expression in control and CMV hepatitis group; B: CMV-DNA expression in curing and incurring group. *P < 0.05 compared to control (or incurring) group.

Equal volume of balanced phenol solution was added and were centrifuged at 5000 g for 15 min. The supernatant was transferred to a new tube containing 1 mL phenol-chloroform buffer, followed by 10 min vortex and 5000 g centrifugation for 15 min. The supernatant was saved and mixed with equal volume of phenol-chloroform mixture for 5000 g centrifugation for 15 min. The supernatant was then mixed with 1/10 volume of sodium acetate solution, followed by 2.5-fold volume of absolute ethanol. The white precipitation was saved and washed by 70% ethanol twice. The mixture was centrifuged at 7500 g for 15 min. TE buffer was used to re-suspend the precipitation for obtaining DNA solution, which was quantified by UV spectrometer. DNA samples were diluted in 2% TE buffer. DNA concentration was measured at 260 nm wave length using TE buffer as the blank control.

ELISA

Peripheral blood samples collected from CMV hepatitis patients were centrifuged at 12000 g for 5 min at 4°C. Supernatants were saved and diluted. Based on manual instructions of ELISA kits for cytokines (IFN-γ and TNF-α), 50 μl standard samples were firstly added into 96-well plate, followed by test samples and blank controls in triplicates. The plate was firstly incubated at 37°C for 30 min. Then the supernatant was discarded and added with 150 μl washing buffer for 30 s. After 3 times of washing, 50 μl enzyme labeled reagent was added to each well for 37°C incubation for 30 min. The supernatant was then removed, followed by the addition of chromogenic substrate A and B (50 μl each) and 37°C dark incubation for 30 min. 50 μl stopping buffer was then added. Absorbance values at 450 nm wavelength were quantified by a microplate reader. Standard curve was plotted based on standard samples, for calculating IFN-γ and TNF-α concentration.

Statistical analysis

SPSS18.0 software package was used to process all collected data. All experiments were performed in at least triplicates. Measurement data were expressed as mean ± standard deviation. Student t-test or analysis variance (ANOVA) was used to perform statistical analysis. Category data were presented in percentage and analyzed by chi-square analysis. A statistical significance was defined when $P < 0.05$.

Results

Expression of CMV-DNA in hepatitis infants

We checked the content of CMV-DNA of infants when admitting. CMV-DNA contents were 19.25 ± 9.14 μg/ml and 154.74 ± 25.91 μg/ml for control and CMV hepatitis patients (**Figure 1A**). Compared to healthy control group, CMV hepatitis patients had significantly higher CMV-DNA contents ($t = 34.639$, $P < 0.05$). After treatment, curing patients had serum CMV-DNA contents at 26.71 ± 10.18 μg/ml while incurring group had 92.66 ± 21.54 μg/ml CMV-DNA (**Figure 1B**), which was significantly higher than curing group ($t = 10.676$, $P < 0.05$).

Serum miR-122 expression in CMV hepatitis patients

Using real-time fluorescent quantitative PCR, we found that serum miR-122 level was increased to about 2.84-fold of healthy control

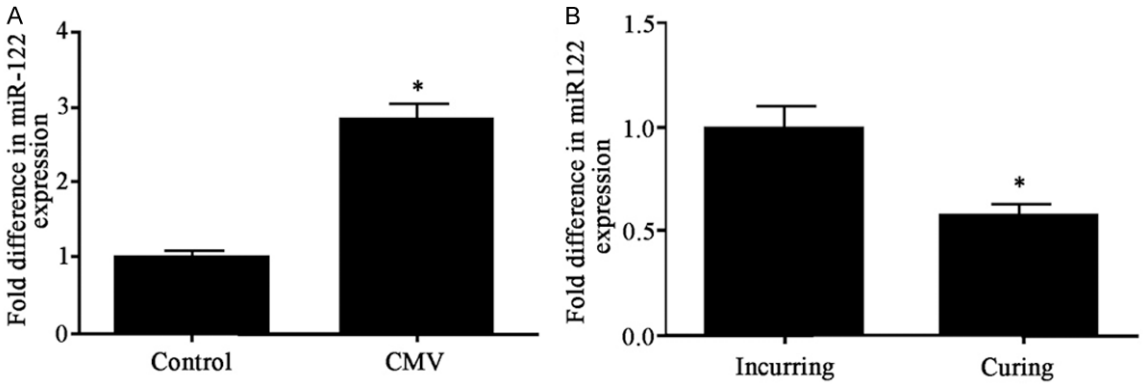

Figure 2. Serum miR-122 expression. A: miR-122 expression between healthy control and CMV hepatitis patients; B: miR-122 expression between curing and incurring patients. *P < 0.05.

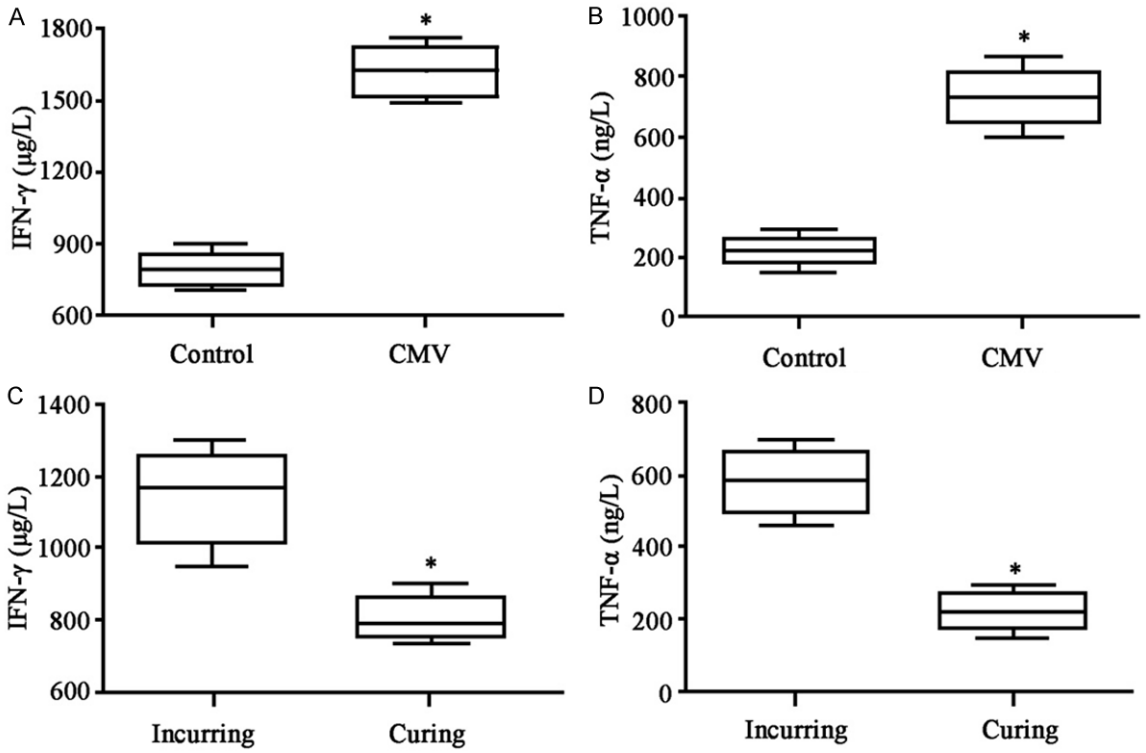

Figure 3. IFN-γ and TNF-α expression in infant blood. A and C: IFN-γ expression; B and D: TNF-α expression. *P < 0.05.

group in CMV hepatitis infants (P < 0.05, **Figure 2A**). After treatment, curing infants had serum miR-122 levels decreased by 57% compared to incurring group (P < 0.05, **Figure 2B**).

IFN-γ and TNF-α expression in serum

We further used ELISA to compared blood contents of IFN-γ and TNF-α in blood samples. When admitting, healthy control infants had IFN-γ and TNF-α levels at 786.28 ± 58.43 μg/L and 219.82 ± 38.27 ng/L, respectively. Those figures, however, were 1614.12 ± 102.84 μg/L and 732.58 ± 81.46 ng/mL. As shown in **Figure 3A** and **3B**, compared to control group, the expression level of IFN-γ and TNF-α in blood was significantly elevated in CMV hepatitis group (t = 46.370 and 53.896, P < 0.05 in both cases). After treatment, curing patients had blood IFN-γ and TNF-α levels and 826.14 ±

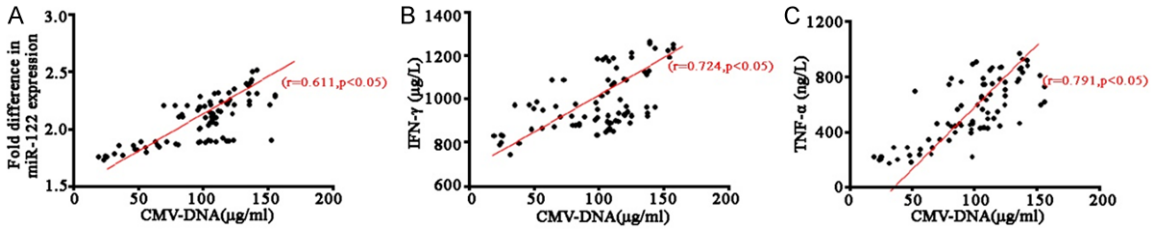

Figure 4. Correlation between miR-122, IFN-γ and TNF-α expression, and CMV-DNA. A: miR-122 and CMV-DNA; B: IFN-γ and CMV-DNA expression; C: TNF-α and CMV-DNA expression.

49.57 μg/L and 231.15 ± 36.70 ng/L, respectively. Whilst such figures were 1165.07 ± 125.73 μg/L an 585.16 ± 105.64 ng/L in incurring patients (**Figure 3C** and **3D**). Compared to incurring infants, curing patients had significantly depressed blood levels of IFN-γ and TNF-α ($t = 10.272$ and 15.214, $P < 0.05$ in both cases).

Correlation between miR-122, IFN-γ and TNF-α expression, and CMV-DNA

To better investigate the role of IFN-γ and TNF-α expression along with miR-122 in CMV hepatitis pathogenesis, we further analyzed their correlation (**Figure 4**). Results showed positive correlation between miR-122 and CMV-DNA expression ($r = 0.611$, $P < 0.05$, **Figure 4A**), and between IFN-γ and CMV-DNA expression ($r = 0.724$, $P < 0.05$, **Figure 4B**). TNF-α expression was also positively correlated with CMV-DNA expression ($r = 0.791$, $P < 0.05$, **Figure 4C**).

Discussion

Due to its relatively weak pathogenicity, CMV infection generally had no obvious clinical symptoms, as the viral replication is in latent status [13]. The infection of pregnant women may cause congenital infection of infants via placenta transmission [14]. Study has found the rapid proliferation of CMV after invading human body to cause tissue damage [15]. Due to the incomplete immune function, infants frequently cannot eliminate CMV, making it to be replicated continuously for causing CMV hepatitis [16]. After invading into the body, the activation of Th1 cells leads to immune response for suppressing *in vivo* activity of CMV [7]. IFN-γ and TNF-α are cytokines secreted by Th1 cells and can participate in Th1 cell-induced cell immunity. IFN-γ can activate NK cells and CTL cells for endowing then with anti-viral, immune modulation and anti-tumor property [17]. TNF-α is one important chemokines for leukocytes, an can enhance the toxicity for microbes via stimulating cell degranulation and secreting hydrogen peroxidase [18]. CMV hepatitis is one complicated biological process. Some studies have found the value of CMV-DNA for evaluating patient's disease condition [19], although its pathogenesis mechanism still remains unknown. Recent studies have indicated the specific expression of miR-122 in liver tissues, and their correlation with pathogenicity of CMV [20]. We thus analyzed the correlation between miR-122 and cytokines in CMV hepatitis patients, in an attempt to investigate the pathogenesis mechanism of miR-122 in CMV infection-induced live injury.

Study has found significantly elevated CMV-DNA contents in CMV hepatitis infants when admitting compared to control group, suggesting active replication of CMV inside body. Meanwhile test results showed significantly higher expression of cytokines including IFN-γ and TNF-α in blood along with higher miR-122 expression, suggesting the facilitation of IFN-γ and TNF-α secretion and miR-122 expression by CMV infection. After 1-month treatment, 42 patients had cure or improvement while 13 of them were not cured. Repeated assay showed lower CMV-DNA load in cured patients, suggesting suppression of viral replication. Those cured infants also had significantly depressed IFN-γ and TNF-α cytokine levels along with lower miR-122 expression, suggesting the down-regulation of cytokines after infection suppression. Further study also showed the positive correlation between IFN-γ and TNF-α, along with miR-122 and the level of CMV-DNA, suggesting the facilitation of cytokine and miR-122 expression by higher CMV replicative activity. When CMV infection or its replicative

activity was inhibited, IFN-γ and TNF-α and miR-122 expressions were all depressed.

In summary, CMV-DNA load in infants is closely correlated with the disease condition of CMV hepatitis patients, which had elevated expression of cytokines including IFN-γ and TNF-α, and miR-122, all of which are positively correlated with CMV-DNA load. Our results suggest the important role of IFN-γ and TNF-α and miR-122 in the pathology of CMV hepatitis.

Disclosure of conflict of interest

None.

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