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Evaluation of IL-17A and IL-17F Gene Expression in Peripheral Blood Mononuclear Cells in Different Clinical Stages of Chronic Hepatitis B Infection in an Iranian Population

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Abstract

Hepatitis B virus (HBV) infection is one of the main causes of liver damage, which can also lead to chronic hepatitis B (CHB) infection. More than 240 million individuals worldwide are chronic carriers of HBV. Among individuals with CHB who are untreated, approximately 15% – 40% will progress to liver cirrhosis or cancer. The interactions between HBV and host immune response play significant roles in the progression of CHB. CHB can be generally divided into four different clinical phases: immune tolerance (IT), immune clearance, inactive carrier, and Hepatitis B surface antigen (HBsAg)-negative reactivation phase (ENEG). Many studies showed that interleukins play important roles in anti-viral immunity and pathogenesis of chronic hepatitis. However, the relations between clinical phases of CHB and host immune transcriptome remain unclear. In this study, we aimed to investigate the expression of interleukin-17A (IL-17A) and IL-17F genes in the peripheral blood mononuclear cells (PBMCs) of patients with CHB through different clinical stages. Results were compared with the control group, which comprised individuals with no history of pre-existing medical conditions. This case-control study was carried out on 32 patients with CHB as the case group and 32 healthy individuals as the control group. According to clinical data, CHB cases were divided into two groups: active (n = 22) and inactive (n = 11). PBMC samples were obtained from all groups. After total RNA extraction and cDNA synthesis, real-time PCR was used to determine IL-17A and IL-17F expression levels. The results were analyzed by REST software, SPSS, and GraphPad Prism. The IL-17A and IL-17F gene expression levels were observed to be significantly higher in the CHB group than in the control group (*IL-17A: P = 0.0013; IL-17F: P = 0.0103*). The active phase group (including IT, clearance, and reactivation samples) significantly increased in comparison with the inactive phase (*IL-17A: P = 0.000; IL-17F: P = 0.000*). The study suggests that IL-17A and IL-17F do not only activate inflammation but are also involved in HBV-related disease progression and chronicity. Thus, mRNA levels of IL-17A and IL-17F could be used as a biomarker to diagnose CHB infection and distinguish between the active CHB phase from the inactive phase.

Abstrak

Evaluasi Ekspresi Gen IL-17A dan IL-17F di dalam Sel-Sel Inti Tunggal Darah Tepi pada Tahapan-tahapan Klinis yang Berbeda dari Infeksi Hepatitis B Kronis pada Suatu Populasi Orang Iran. Infeksi virus hepatitis B (HBV) merupakan salah satu dari penyebab utama kerusakan hati, yang dapat juga menyebabkan infeksi hepatitis B kronis (CHB). Lebih dari 240 juta individu di seluruh dunia merupakan pembawa kronis HBV. Di antara individu-individu dengan CHB yang tidak diobati, hampir 15%–40% akan berkembang menjadi sirosis hati atau kanker. Interaksi antara HBV dan respons imun inang memegang peran yang signifikan dalam perkembangan CHB. CHB pada umumnya dapat dibagi menjadi empat fase klinik yang berbeda: toleransi imun (IT), pembersihan imun, pembawa tak aktif, dan antigen permukaan hepatitis B (HBsAg)-fase reaktivasi negatif (ENEG). Banyak kajian menunjukkan bahwa interleukin memegang peranan penting dalam imunitas anti-virus dan patogenesis hepatitis kronis. Namun demikian, hubungan antara fase-fase klinis CHB dan transkriptoma imun inang tetap masih belum jelas. Di dalam kajian ini, kami bertujuan untuk menginvestigasi ekspresi interleukin-17A (IL-17A) dan gen-gen IL-17F di dalam sel-sel inti tunggal darah tepi (PBMCs) pasien penderita CHB melalui tahapan-tahapan klinis yang berbeda. Hasil-hasilnya dibandingkan dengan kelompok kontrol, yang mencakup individu-individu tanpa riwayat kondisi medis yang ada sebelumnya. Kajian kasus-kontrol ini

dilaksanakan pada 32 pasien penderita CHB sebagai kelompok kasus dan 32 individu yang sehat sebagai kelompok kontrol. Menurut data klinis, kasus-kasus CHB dibagi menjadi dua kelompok: aktif ($n = 22$) dan non aktif ($n = 11$). Sampel-sampel PBMC diperoleh dari semua kelompok. Setelah keseluruhan ekstraksi RNA dan sintesis cDNA, PCR waktu nyata digunakan untuk menentukan level-level ekspresi IL-17A dan IL-17F. Hasil-hasilnya dianalisis dengan perangkat lunak REST, SPSS, dan GraphPad Prism. Level-level ekspresi gen IL-17A dan IL-17F teramat jauh lebih tinggi di dalam kelompok CHB dibandingkan dengan di dalam kelompok kontrol (*IL-17A*: $P = 0,0013$; *IL-17F*: $P = 0,0103$). Kelompok fase aktif (termasuk sampel-sampel IT, pembersihan, dan reaktivasi) meningkat secara signifikan dibandingkan dengan fase non aktif (*IL-17A*: $P = 0,000$; *IL-17F*: $P = 0,000$). Kajian ini menyarankan bahwa IL-17A dan IL-17F tidak hanya merupakan peradangan aktif namun juga terlibat dalam perkembangan dan kronisitas penyakit yang berkaitan dengan HBV. Dengan demikian, level-level IL-17A dan IL-17F dapat digunakan sebagai suatu penanda hayati untuk mendiagnosis infeksi CHB dan membedakan antara fase CHB aktif dari fase non aktif.

Keywords: *bandpass filter, microstrip, optimization, resonator, ultra wideband*

1. Introduction

Hepatitis B virus (HBV) infection is one of the main causes of liver inflammation and diseases. Around two billion individuals have been infected worldwide, and about 240 million people are estimated to have chronic hepatitis B virus (CHB) infection [1]–[4]. The progression of CHB infection contributes to liver cirrhosis and hepatocellular carcinoma in 15% – 40% cases [5]–[7]. CHB can be classified into four clinical phases: immune tolerance (IT), immune clearance, inactive carrier (IC), and Hepatitis B surface antigen (HBsAg)-negative reactivation phase (ENEG) [7]–[32]. Serological markers are needed to determine disease stage [7]. In this regard, biomarkers are serological tests that can detect hepatitis B antigens, such as HBsAg and HBeAg [15], [26], [28]. Measuring the amount of aminotransferase enzymes, especially alanine aminotransferase (ALT) levels in the blood, can also help indicate the patient's condition with hepatitis B virus [9], [24]. During the IT phase, serum levels of ALT are normal but serum HBV DNA levels are high. In the clearance phase, serum ALT levels are high and the test result for HBeAg is positive. During the IC phase, serum ALT levels are normal, but serum levels of HBV DNA are untraceable and the test result for HBeAg is negative. In the HBsAg-negative (ENEG) reactivation stage, serum levels of ALT and HBV DNA are both high [28]. Many studies showed that the interactions between HBV and host immune response play significant roles in the progression of CHB [8], [33]–[36]. Cytokines such as IL-1, IL-2, IL-4, IL-6, IL-17, IL-18, and TNF have important roles in inflammation and HBV infections [23], [31], [37]–[39]. Recently, cytokines have been considered a hotspot in knowing the pathogenesis of chronic hepatitis and liver cirrhosis [10]. Although recent studies have focused on the association between clinical phases of CHB and host immune transcriptome, the relationship remains unclear [16], [40], [41].

The interleukin-17 (IL-17) family comprises cytokines that play significant roles in response to chronic

inflammation and control of infections [23], [27], [30], [42]. These cytokines are produced by special T helper cells known as T helper cell 17 (TH17) [43]. Previous studies proved that IL-17 signaling pathways are connected to the immunity response caused by liver laceration. IL-17 is composed of six subfamilies (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F). Of these, IL-17A and IL-17F are highly similar and commonly produced by the same cell types [42]. IL-17A and IL-17F are pro-inflammatory cytokines that can activate and recruit neutrophils. These changes may activate inflammation of big tissues (e.g., liver), metabolic disorders, and progression of autoimmune diseases [13], [17], [18], [27], [29]. Recent findings also suggested that IL-17 participates in the pathogenesis of HBV infection and anti-viral immunity [22]. Some studies have declared that IL-17 has roles in inflammatory liver damage after HBV infection [10], [44], [45]. Moreover, IL-17 has been found to play an important part in the different immune phases of CHB infection [42].

Accordingly, the present study aimed to evaluate IL-17A and IL-17F gene expression in the peripheral blood mononuclear cells (PBMCs) during the two phases of CHB infection (active and inactive) in comparison with healthy controls.

2. Material and Methods

Patient selection and sampling. This case-control study was approved by the National Research Ethics Committee, and written consent was obtained from the volunteers.

The study involved 32 healthy individuals and 32 patients suffering from CHB infection and was conducted at Taleghani Educational Hospital, Tehran, Iran, from 2016 to 2017. First, 5 mL of peripheral blood was collected from both healthy and infected individuals.

Patients with the positive ELISA test results for HBsAg and anti-HBcAb were considered the case group, and those with negative test results comprised the control group. The patients with other viral infections and who received anti-viral therapy were excluded. On the basis of ALT levels and viral load, CHB cases were divided into two groups: active ($n = 22$) and inactive ($n = 11$).

PBMC preparation. About 20 mL of Ficoll separating solution was added to a 50 mL centrifuge tube, and the blood sample was mixed with PBS at a ratio of 1:1. The diluted blood sample was carefully layered above the same volume of Ficoll separating solution to avoid mixing and then centrifuged for 30 min at $1200 \times g$ and room temperature. The lymphocyte-containing band was transferred into a new centrifuge tube. The total lymphocyte population was washed with RPMI 1640 containing 5% FBS for three times. Between washes, the cells were centrifuged for 10 min at $1200 \times g$ and room temperature to pellet down the cells. The lymphocyte pellet was diluted in RPMI 1640 to 107 cells/mL and briefly spun for 5 min at $200 \times g$ and room temperature [46].

Whole blood of the subjects containing the anticoagulant EDTA was delivered to the Virology Laboratory Research Institute of Gastroenterology and Liver Diseases of Shahid Beheshti University of Medical Sciences, Tehran, Iran, under standard conditions of temperature.

Total RNA extraction. Total RNA of fresh PBMC samples was extracted using the RNeasy mini kit (Qiagen Company) in accordance with the given instructions.

cDNA synthesis and real-time PCR. cDNA was synthesized by using a RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instruction.

The mRNA levels of IL-17A and IL-17F were then evaluated by real-time PCR using the SYBR Premix Ex Taq II kit (Takara Company).

Designed primer sequences of IL-17A, namely, 5'-TCCCACGAAATCCAGGATGC-3' (forward) and 5'-GGATGTTTCAGGTTGACCATCAC-3' (reverse), and IL-17F, namely, 5'-GCTGTCGATATTGGGGCTTG-3' (forward) and 5'-GGAAACGCGCTGGTTTTCAT-3' (reverse), were used for this purpose. GAPDH was set as the reference gene.

Statistical analysis. All the data sets in this research were analyzed by SPSS-16 using the independent samples t-test, REST software (2009), and GraphPad Prism (8.3.0). The level of significance was set at 0.05 ($P < 0.05$).

3. Results

The average age of 32 patients with CHB infection was 33.16 ± 11.11 years. The average age of the control group of 32 healthy subjects was 30.94 ± 10.30 years (Table 1).

Blood samples (10 mL) collected from each patient were tested for HBsAg and HBeAg by ELISA. All patients did not have any history of HBV vaccination or disease infection and/or other type of hepatitis.

The normal limits considered for ALT and AST were 40 and 35 IU/L, respectively. Patients with ≥ 2000 HBV DNA copies/mL and continual elevated ALT levels were considered active chronic carriers.

Specific duplication of the intended gene parts, deficiency of primer pairing, and nonspecific duplication for each gene were determined by melting curve analysis. The cycle threshold (Ct) of the reference gene (GAPDH), IL-17A gene, and IL-17F gene was used to evaluate gene expression by REST software (2009). Statistical analysis of the independent samples t-test revealed that the IL-17A and IL-17F gene expression levels in the infected group were significantly higher than those in the control group (*IL-17A*: $P = 0.0013$; *IL-17F*: $P = 0.0103$; Figures 1 and 2).

Table 1. Background Variable Information with Respect to the Healthy and Infected Individuals

Groups	Sample count	Average age	ALT	AST
Hepatitis B patients	32	21.81 ± 11.61	18.81 ± 5.600	33.16 ± 11.11
Control	31	43.71 ± 32.50	42.28 ± 24.22	30.94 ± 10.30

Table 2. Background Variable Information of the Infected Individuals Based on the Two Groups of Hepatitis B

Hepatitis B patients	Sample count	Average age	ALT	AST
Active phase (IT, Clearance, ENeg)	22	31.71 ± 11.61	21.81 ± 11.61	18.81 ± 5.600
Inactive phase	11	1135.91 ± 10.02	43.71 ± 32.50	42.28 ± 24.22

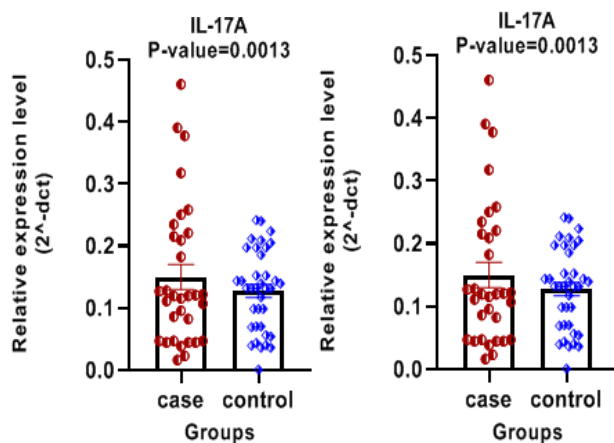


Figure 1. Relative Expression Level of IL-17A and IL-17F Among Cases and Controls

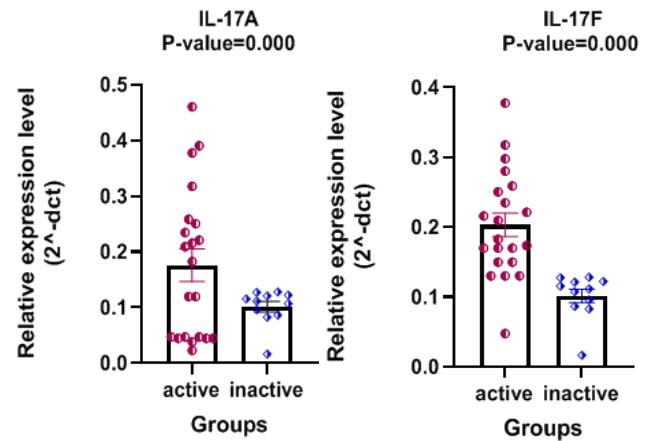


Figure 2. Relative Expression Level of IL-17A and IL-17F Among Active and Inactive Groups

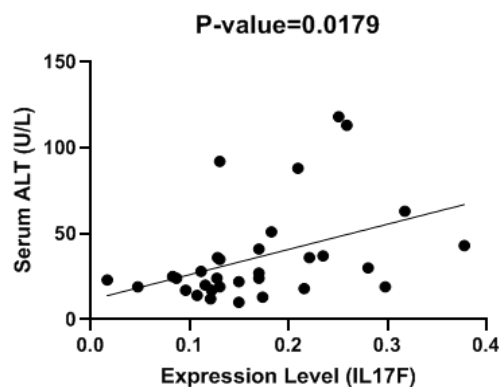


Figure 3. Analysis Between Active Group and Serum ALT

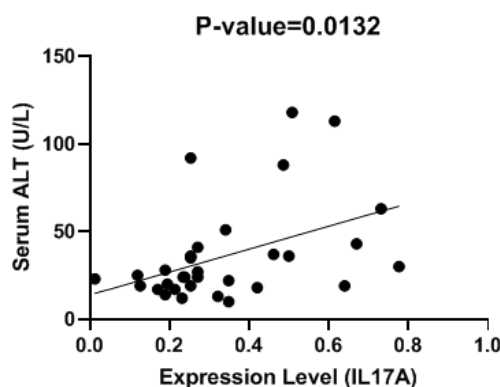


Figure 4. The Analysis between the Active Group and Serum ALT Showed a Significant Increase ($P = 0.0390$).

The expression levels of IL-17A and IL-17F also significantly increased in three stages of the active phase (IT, clear, and reactivation) in comparison with those in the inactive phase (IL-17A: $P = 0.000$; IL-17F: $P = 0.000$; Figures 3 and 4).

4. Conclusion

To our knowledge, the relations between clinical phases of CHB and host immune transcriptome are unclear. The purpose of this study was to determine the IL-17A and IL-17F gene expression levels of patients with CHB infection during different clinical phases of the disease in comparison with healthy individuals.

Despite HBV vaccination, HBV infection continues to be a global health problem [3]. HBV is an important cause of acute and chronic liver diseases [47]. About 240 million people worldwide have CHB; if left untreated, it may lead to liver cirrhosis and cancer [48]. Recent studies revealed that more than 35% of Iran's population has been exposed to HBV [19, [49]–[51]. On the basis of serological markers, CHB can be divided into four different clinical phases [14], [15], [23]–[27]. Host genetic background and immune response have significant roles in HBV outcome [52], [53]. Many studies have shown the relationship between progression of CHB infection and host immune responses [34], [54]. Cytokines, among other factors, are the most favored [10].

Previous studies determined that the IL-17 family has an important role in the different immune phases of CHB infection [10], [55]. This cytokine family consists of six members (IL-17A to IL-17F). IL-17A, which is a prototypical member, and IL-17F, which is the most similar to it, are commonly produced by the same cell types [42], [56]. Th17 is a subset of CD4+ T helper cells, which produce and secrete IL-17 cytokine in response to IL-21 and IL-6 pro-inflammatory cytokines that will consequently lead to chronic liver infection [32]. However, IL-17 is also produced by neutrophils and NK cells [14]. IL-17 cytokine plays a substantial role in the delayed type of inflammatory reactions. These cytokines will summon neutrophils, monocytes,

macrophages, and fibroblasts by an increase in chemokine secretion [21]. In 2015, Zhang *et al.* showed that the cell signaling pathways concerning the IL-17 cytokine serve as a convenient approach for the treatment of hepatic diseases. Therefore, IL-17 can be used as a pharmaceutical target [8].

Many studies showed the relation between genetic variants of IL-17 and HBV progression [12], [57], [58]. In 2016, Wang, Jian *et al.* studied the association of IL-17A and IL-17F gene polymorphisms with CHB and HBV-related liver cirrhosis in a Chinese population. They revealed that the IL-17A rs4711998 genetic variant contributes to HBV–liver cirrhosis susceptibility [12]. In another study, Xi *et al.* exposed the polymorphism of IL-17A and IL-17F genes, which can cause HBV-related hepatocellular carcinoma [11]. Studies have reported an associated between IL17 and HBV infection. In 2018, Yang *et al.* showed that IL-17 gene expression in PBMCs and IL-17 protein levels in all four phases of CHB were significantly higher compared with those in controls [10]. Our results were consistent with the previous findings. Thus, the increase in IL-17 gene expression levels may actively participate in CHB infection, just as in other inflammatory diseases, although further investigations are necessary.

We also investigated the relation between serum ALT level and the expression levels of IL-17A and IL-17F. Our findings suggested an increased level of IL-17 in the peripheral blood samples of patients with CHB compared with the healthy individuals. Therefore, IL-17 can be used as a biomarker to diagnose CHB infection and distinguish between the active CHB phase from the inactive phase. If the presence and effect of TH17 cells in inflammation caused by hepatitis is proved, then the interference in their activity or their products can lead to novel effective aspects of treatment for this disease.

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