International Journal of Medical Laboratory 2017;4(1):15-24.

International Journal of Medical Laboratory
Shahid Sadoughi University of Medical Sciences

Original Article

Decreased Expression Levels of S100A12 and RAGE May Be Associated with Chronic HBV Infection

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Article history
Received 4 Oct 2016
Accepted 15 Dec 2016
Available online 7 Mar 2017

Key words
Chronic hepatitis B
RAGE
S100A12

A B S T R A C T

Background and Aims: Engagement of the receptor for advanced glycation end products (RAGE) and its ligand "S100A12 protein" induce a cascade of reactions that eventually might lead to develop an inflammatory response dependent on NF-κB. Although involvement of S100A12 and RAGE in some autoimmune disease have proved, in chronic hepatitis B (CHB) infection functions of the proteins are not clear thus far. Determining of expression of S100A12 and RAGE in peripheral blood cells of the CHB patients was the aim of the present study.

Materials and Methods: In the case-control study the mRNA levels of S100A12 and RAGE genes of the sixty CHB patients and sixty healthy donors were measured by real-time polymerase chain reaction method. The patients and healthy donors were sex and age-matched.

Results: The findings demonstrated expression of S100A12 and RAGE in the CHB patients significantly decreased compared to the healthy donor group ($p=0.001$). Expression levels of the genes were not altered among HBeAg-positive and HBeAg-negative CHB patients. The HBV-DNA copy number/ml did not affect the expression of S100A12 and RAGE in the patients ($p>0.05$).

Conclusions: The results of the study suggested that down regulation of RAGE and S100A12, because of their role in inducing inflammation, might have a considerable role in chronicity of hepatitis B.

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Introduction

Despite efficient vaccine and potent antiviral treatment, which have developed in the recent years, chronic hepatitis B virus (HBV) infection and liver-related diseases are still threatening public health in many countries [1]. Chronic hepatitis is characterized by persistence of HBV virus particles in the body fluidics for more than six months [2, 3]. In the chronic hepatitis B (CHB) patients, chance of spontaneous HBsAg clearance is only 1-2% per year [4]. This devastating infection has affected about 3.6 % of worldwide population. Persistent infection increases the risk of developing cirrhosis and hepatocellular carcinoma. With increasing of age, the risk of developing the chronic infection will be declined. Whereas 20-60% of infected children under 6 years develop chronic infection, in the adult the risk of chronicity is less than 5%. According to the new investigations, prevalence of chronic HBV infection is decreasing in many countries. However, higher mortality and morbidity of infected patients comparing to the past is a big concern of health centers [3, 5]. Innate and adaptive immune responses are recruited by HBV. Although adaptive immune responses against the virus are well known, many aspects of innate immunity behaviors in the infection remain to be clear [6, 7].

In human, the S100 protein family consists of 21 subtypes that share structural similarities, but they have divers functions [8]. The S100 subtypes, S100A8 (calgranulin A), S100A9 (calgranulin B) and S100A12 (calgranulin C) are phagocyte specific. S100A12 is considered as neutrophils activation marker [9, 10]. Once secreted from activated phagocytes, S100A8/A9 and S100A12 act as proinflammatory ligands of Toll-like receptor-4 or receptor for advanced glycation end products (RAGE). The mechanisms of chemotactic activities related to inflammation (S100A8/A9 and A12), neurotrophic activities (S100B), and angiogenic effects (S100A4 and S100A13) as well as the nature of high affinity surface receptors remain largely unknown [8]. Based on other studies, S100A12 gene is regulated by other inflammatory cytokines, including interleukin-16. Although elevated levels of the inflammatory protein are reported in some bacterial infectious diseases, including acute otitis media in young children, juvenile idiopathic arthritis, importance of the protein in viral infection has not clear yet.

RAGE belongs to immunoglobulin superfamily and express on wide variety of stressed cells. This receptor is activated upon binding of S100A12, S100A13, S100P, and S100B [9, 11-13]. Activation of RAGE by binding S100 isoforms play crucial role in regulating cell metabolism, inflammation responses, apoptosis, proliferation and autophagy [14]. RAGE expression was investigated by other studies in thoracic aortic aneurysm, THP-1 macrophages and other inflammatory situations [15, 16]. According to these finding RAGE plays an inflammatory role. Blocking RAGE as effective therapeutic potentials is under extensive investigation especially in cardiovascular diseases. Several studies have shown RAGE induces inflammatory pathways by involving the NF-κB and the Mitogen-activated protein kinase

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(MAPK) pathways. NF-κB and MAPK signaling lead to the production a large number of pro-inflammatory cytokines and chemokines [17-19].

Because of the important role of S100A12 and RAGE in inducing inflammation as well as the very limited information in the case of the molecules in CHB infection [11, 20, 21], it may be hypothesized that alteration in expression of the proteins can affect the quality of the immune response and play a role in chronic HBV infection.

Materials and methods

Human subjects

This study was approved by the Ethics Committee of the Shahid Sadoughi university of medical sciences in Yazd. All subjects provided prior written informed consents. In the case-control study, 60 chronic HBV patients and 60 healthy donors were selected from Rafsanjan Blood Transfusion Organization, Iran. Chronic infection was confirmed by enzyme-linked immunosorbent assay (ELISA) test for hepatitis B surface antigen (HBsAg), Hepatitis B e antigen (HBeAg) and HBV-DNA polymerase chain reaction (PCR). All the patients were examined by a gastroenterologist, using the Scheuer scoring system; nobody showed fibrosis. The patients

did not show apparent auto-immune hepatitis, primary biliary cirrhosis, alcoholic liver disease, sclerosing cholangitis, Wilson's disease, cirrhosis, α1-antitrypsin deficiency, current or past history of alcohol abuse, and previous liver transplantation or evidence of hepatocellular carcinoma. None of the patients had received antiviral therapy before taking part in the study in order to avoid the possible effect of therapy on the expression of S100A12, RAGE and NF-κB. The positive sera for human immunodeficiency virus type 1 (HIV-1), hepatitis C virus (HCV), Syphilis were excluded from our study. Anti-HIV and anti-HCV antibodies were detected using commercial ELISA kits (Diapro, Diaplus, Italy). Syphilis disease was detected by serologic tests. No patients with metabolic disease such as diabetes were included. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in HCV patients were measured by the Prestige auto-analyzer, (Tokyo, Japan) and the biosystem kit (Spain). Total bilirubin and HBV-DNA copy (Cinnaclone, Iran) number in the patients were determined before they took part in the study.

Table 1. Illustration of the primer sequences, which were used for evaluation of mRNA Levels of S100A12, RAGE and NF-kB by Real-Time PCR

Gene		Primers
S100A12	Forward	GGAGGGAATTGTCAATATC
	Reverse	ATCTTGATTAGCATCCAGG
RAGE	Forward	GACCCTGGAAGGAAGGAAGCAG
	Reverse	CCCCTTACACTTCAGCACC
Beta-actin	Forward	GGCACCCAGCACAATGAAG
	Reverse	AAATGAGCCCCAGCCTTC

RAGE; Receptor for advanced glycation end products

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Sixty sex and age-matched healthy subjects also were selected from the Rafsanjan Blood Transfusion Organization, Iran. They were sero-negative for HBV, HIV-1, HCV and Syphilis. The healthy donors did not receive any immunosuppressant drugs at least one month before taking blood samples. Five milliliters of each peripheral blood sample were taken in sterile vacutainer tubes containing ethylenediaminetetraacetic acid. Obtaining blood samples from subjects were transferred immediately to the laboratory with special conditions in terms of temperature, safety and absence of microbial contamination and mRNA extracted from the samples.

RNA extraction and reverse transcription

collected blood cells were centrifuged, lysed and homogenized in presence of Trizol (RNX TM-PLUS, Cinnaclon, Iran) at the ratio of 1 ml TRI/10^7 cells. Total cellular RNA was extracted using the RNA Extraction kit (Parstus, Iran). The quantity and purity of the RNA were assessed by measuring the absorbance at 260 nm and the ratio A260/A280 in a UV-spectrophotometer (Photo Biometer, Eppendorf, Germany). To make linear and preventing of forming secondary structures of RNA, the samples were kept for 10 min. in 60ºC in Bio-Rad thermocycler (Bio-Rad, USA, C1000). A total of 5 µg of RNA was reverse transcribed into single-stranded complementary DNA (cDNA) using a commercial kit (cDNA Synthesis kit, Paretus, Iran). Reverse transcribed according to kit instructions. Briefly, the reaction mixture containing 5 µg of extracted RNA, 0.5 µg of oligo (dT), 50 ng/µl random hexamer and cDNA mastermix (Parstus, Iran) in 20 ml of total volume was incubated at 42ºC for 60 min. The products were analyzed on 1% agarose gel stained with a green viewer. The cDNA was kept at -20ºC.

Real-time PCR

Real-time PCR of S100A12 and RAGE was performed using the real Q-PCR master mix kit (Ampliqon III, Denmark), according to the kit protocol, and the mRNA expression levels of S100A12 and RAGE were quantified using real time PCR system (Bio-Rad, CFX96, USA). Beta-actin was used as a housekeeping gene for normalization of the amount of mRNA expression of the genes of interest. Primers were designed according to published sequences in the Genome database (Genbank) using the PRIMER3 software. The sequences of the primers are shown in table 1. Briefly, 3 ng of cDNA was used for each PCR with 2pM of forward and reverse primers in a total volume of 20 µL. The thermal cycling conditions comprised of 5 min. at 94°C, followed by 45 cycles at 95°C for 1 min., 58°C for 30 seconds, and 72°C for 30 seconds. The relative quantity of the target mRNA was normalized to the level of the internal control beta-actin mRNA level. Gene expression was expressed in relative units ($RQ=2^{-\Delta\Delta CT}$) where ΔCT is the difference between the gene of the target and the housekeeping gene, and ΔΔCT is the change between the ΔCT for each sample and the control group. Each S100A12 and RAGE gene was then described as the fold change from

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the control group. Real-time PCR data were analyzed by BIORAD CFX manager software.

Statistical analysis

Statistical analyses were performed using SPSS, version 18 (SPSS Inc., Chicago, IL, USA). Continuous variables (AST, Bilirubin, age, and ALT) are presented as mean (±SD). Paired sample t-test was used for comparing of cycle threshold (CT) mean between CHB patients and the healthy donors. A P value less than 0.05 was considered as a significant difference.

Results

Patient's Characteristics

Characteristics of the patients and healthy subjects are shown in table 2.

Table 2. Characteristics of CHB patients and Healthy donors

Characteristics	CHB patients	Healthy donors	Pv
Number	60	60	
Age	37 (12.1)	38(9.2)	0.11
Gender (male/female)	33/27	35/25	0.32
HBV DNA (log copies/ml)	Male: 10.2 (9.3-11.5) Female: 9.32 (8.1-10.4)	-	0.10
ALT (IU/L)	45 (20-78)	28(15-48)	0.087
Total bilirubin (mg/dL)	0.8 (0.5-1.6)	0.7(0.4-0.9)	0.15
AST (IU/L)	48 (32-84)	36(24-65)	0.098

CHB; Chronic Hepatitis B, HBV; hepatitis B virus, HBeAg; hepatitis B e antigen, ALT; alanine aminotransferase, AST; aspartate aminotransferase

Matching of age and gender between the patients and healthy donors was done. The CHB group's age was followed for an average of 37±12.1 years, whereas the control group was followed for an average of 38±9.2 years. In the CHB group, male patients (55%) had HBV-DNA (10.2 log copies/ml) and female (45%) had 9.3 log copy/ml. HBV infection duration in the patients was not cleared, but at the time of sampling none of the CHB patients showed developed liver cirrhosis symptoms. Although in the most of CHB patients ALT and AST enzymes showed slightly rising, statistical analysis did not confirm a significant difference between the patients and healthy donors.

RT-PCR of S100A12 and RAGE

The confirmed CHB patients and healthy donors evaluated for mRNA expression in peripheral blood cells according to the materials and methods. The results demonstrated the average log S100A12 gene expression in the CHB patients (0.034±0.017) have decreased significantly compared to the control group (0.242±0.059) (Fig. 1) (p=0.001).

RAGE as the main receptor of S100A12 was investigated on the level of mRNA in peripheral blood cells of the CHB patients by RT-PCR. Expression of RAGE gene in the CHB patients (0.005±0.002) also showed substantially decreasing compared to the healthy donors (0.095±0.031) (Fig. 2) (p=0.001).

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HBV-DNA copy number per ml was determined by PCR in all patients, and 28, 14, and 18 patients were carrying <20,000, 20,000–100,000, and >100,000 HBV copy number/mL, respectively. Our results in the case of HBV-DNA copy/ml did not show significant correlation between loads of the virus and expression levels of the S100A12 and RAGE genes (Fig. 3) (p>0.05).

Measuring HBsAg and HBeAg by ELISA method also were carried out in all of the patients. All the CHB patients were HBsAg positive, while only 16 (26.7%) of the patients showed HBeAg in their serum. Expression of S100A12 and RAGE between HBeAg negative and HBeAg positive did not show a significant difference (p>0.05) (Fig. 4).

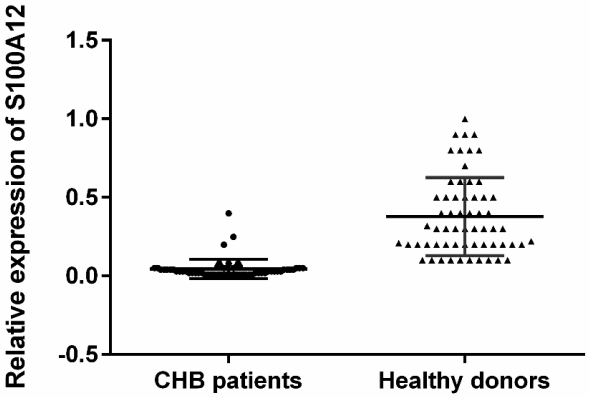

Fig 1. The S100A12 gene expression in the CHB group and the healthy donors. As the figure shows the expression levels ofS100A12in the patients with chronic hepatitis B significantly reduced compared to the control group (p=0.001).

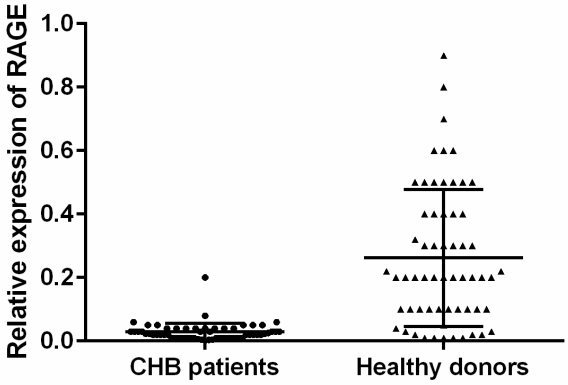

Fig 2. Relative expression of RAGE in the CHB patients. The results reveal that the expression levels of RAGE in patients with chronic hepatitis B significantly reduced compared to the control group (p=0.001).

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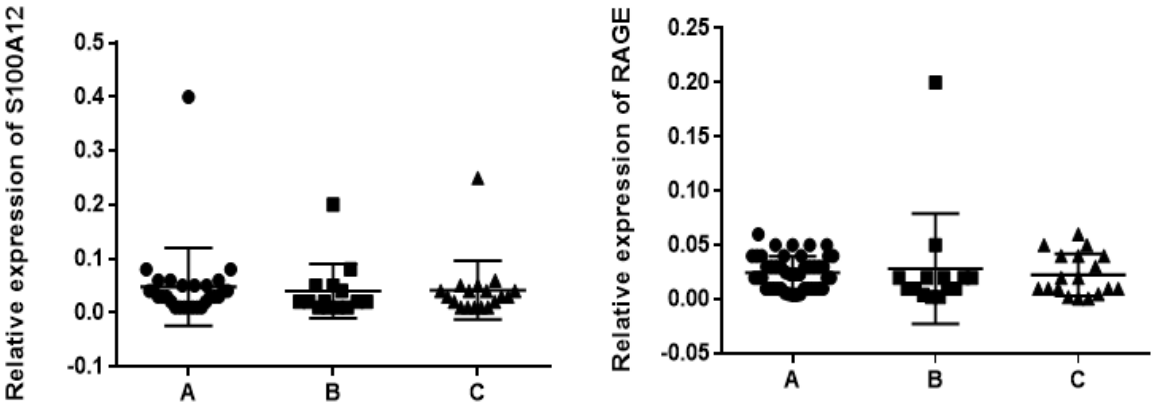

Fig 3. Relationship between HBV-DNA viral load, S100A12 and RAGE expression levels. The CHB patients were divided to 3 groups base on HBV-DNA copy number/ml: group A <20000, group B between 20000-100000 and group C>100000. Statistical analysis did not show significant differences between expression levels S100A12 and RAGE and viral load (p>0.05).

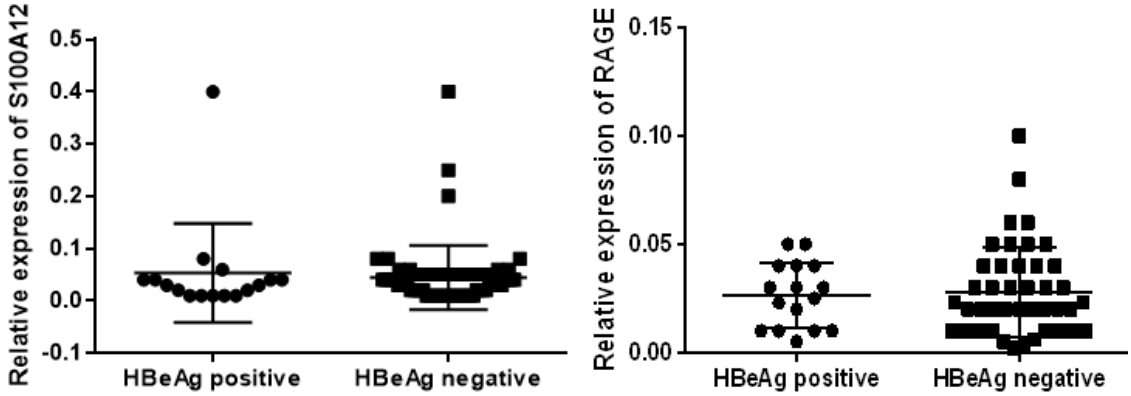

Fig 4. Relative expression of S100A12 and RAGE in HBeAg-positive and HBeAg-negative CHB patients. The results showed mRNA expression levels of S100A12 and RAGE were not altered among HBeAg-positive and HBeAg-negative CHB patients (p>0.05).

Discussion

Plenty of studies have demonstrated that S100 proteins are able to direct proliferation, migration, tissue repair, antimicrobial activity and inflammation via different receptors on surface of cells [22]. S100 proteins, including S100A8/A9 and S100A12 efficiently induce inflammatory responses and are considered as systemic inflammatory markers [23]. According to the recent reports TNF production after engaging S100A12 with its receptor on macrophages substantially augmented [24-26]. Additionally, in the bowel disease related to bacterial infection, there is a cooperation between S100 proteins and neutrophils and

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TH17 cells [27]. However, our knowledge of S100 family proteins function in viral infection disease and especially HBV infection remain obviously to be elucidated.

Results of the study confirmed that S100A12 and RAGE genes expression were significantly decreased in the CHB patients when compared to the healthy subjects. The compromised ability of peripheral blood cells in CHB patients for expressing S100A12 did not show significant correlation with HBV viral load and HBeAg concentration. The concentrations of ALT and AST enzymes in the patients were also roughly similar to the healthy subjects. Defects in producing of the proinflammatory agents may lead to inappropriate immune responses resulting in developing chronic infection. In consistent with our results, Cai and colleagues also showed, S100A12, HMGB1 and sRAGE levels in serum of hepatitis B virus related acute-on-chronic liver failure (HBV-related ACLF) patients, a serious condition with a high mortality, showed significant elevation when compared with CHB and healthy controls. Based on the results, increase of S100A12 and HMGB1 concentrations are related to the poor prognosis in the HVB-related ACLF [28]. In the liver cirrhosis patients, S100A12 concentrations were lower than those in HBV-related ACLF patients. However, the patients who were selected in our study according to clinical examination and liver enzymes measurements and other confirming criteria had not shown cirrhosis and fibrosis. On the other hand, our survey was carried out on peripheral blood cells and on mRNA expression. Although the CHB patients did not show severe involvement and liver injury, but declined S100A12 comparing to healthy subjects which showed the CHB patients may have some defect to produce S100A12 appropriately. Nevertheless, possible mechanisms, which may lead to increase S100A12 in severe liver tissue damage such as HVB-related ACLF are not clear so far, but it seems that S100A12 and its receptor RAGE may serve as biomarkers that show a correlation with deteriorating liver tissue damage in the advanced liver injury caused by HBV and could be considered as predicting factor in prognosis of HBV infection. However, our study results confirmed that in the CHB patients who have no acute inflammation or intensive liver tissue damage, S100A12 and RAGE were decreased. Although this down regulation may show non-inflammatory situation in the CHB patients, but declined ability of peripheral blood cells for production of the inflammatory proteins may play a role in inappropriate immune responses resulting in chronic infection disease.

Conclusion

Taken together, impaired expression of S100A12 and its receptor might direct a role in developing chronic CHB infection. To address this hypothesis in more detail it needs to investigate expression of S100A12 in the liver tissue of the CHB patients and in blood cells of cleared patients of HBV infection. As effective acquired immune responses to some extent are dependent to innate immunity agents such as S100A12, more studies need to address the exact role of the proteins in CHB patients.

Conflict of Interests

The authors have no conflicting financial interests.

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Acknowledgments

This study was supported by a grant of International Campus, Shahid Sadoughi of Medical Sciences.

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