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Are serum GRP78 levels significant in chronic hepatitis C patients? A case-control study

Kronik hepatit C hastalarında serum GRP78 düzeyleri anlamlı mı? Vaka-kontrol çalışması

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Abstract

Aims: Glucose-regulated protein 78 (GRP-78) is one of the basic markers of endoplasmic reticulum (ER) stress in tissues. It is known that ER stress develops in the livers of patients infected with hepatitis C. In this study, the aim was to assess serum GRP78 levels which have not previously been investigated as a stress marker in chronic hepatitis C patients (CHC).

Methods: This case control study includes patients with chronic hepatitis C (CHC) infection in our Infectious Diseases clinic (n=60) and a healthy control group without any additional chronic disease (n=60). Serum GRP78 levels were measured with enzyme-linked immunosorbent assay (ELISA), then correlation analysis was performed for serum GRP78 levels with alanine aminotransferase (ALT), aspartate aminotransferase (AST) and HCV-RNA levels.

Results: A significant positive correlation was observed between HCV-RNA, ALT and AST levels in CHC patients (P<0.001 and P=0.008, respectively). Serum GRP78 was identified at similar levels in both the control and HCV subgroups. While a significant positive correlation was identified between serum GRP78 and AST levels (P=0.046), no significant correlation was detected for serum ALT levels.

Conclusion: Though liver injury induced by HCV is shown to cause ER stress, our results showed there was no significant increase in serum GRP78 levels during chronic HCV infection.

Keywords: Glucose-regulated protein 78, Hepatitis C infection, Endoplasmic reticulum stress

Öz

Amaç: Glikozla düzenlenen protein 78 (GRP-78) dokudaki endoplazmik retikulum (ER) stresinin temel göstergelerinden birisidir. Hepatit C ile enfekte hastalarda karaciğerde ER stresinin geliştiği bilinmektedir. Bu çalışmada kronik hepatit C (KHC) hastalarında bir stres belirteci olarak daha önce incelenmemiş olan serum GRP78 düzeylerinin değerlendirilmesi amaçlanmıştır.

Yöntemler: Çalışmamız, Enfeksiyon Hastalıkları polikliniğimize başvuran Kronik Hepatit C (KHC) enfeksiyonu tanısı almış hasta grubu (n=60) ve ek kronik hastalığı olmayan sağlıklı kontrol (n=60) grubundan oluşan bir vaka kontrol çalışmasıdır. Serum GRP78 seviyesi Enzyme-Linked Immuno Sorbent Assay (ELISA) ile ölçülmüş, ardından GRP78 ile alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), HCV-RNA düzeyleri arasında korelasyon analizi yapılmıştır.

Bulgular: KHC hastalarında HCV-RNA düzeyleri ile ALT ve AST düzeyleri arasında anlamlı bir ilişki izlenmiştir (sırasıyla P<0,001 ve P=0,008). Serum GRP78 hem kontrol hem de HCV alt gruplarında benzer seviyelerde saptanmıştır. Serum GRP78 seviyesi ile AST düzeyleri arasında anlamlı bir pozitif korelasyon saptanırken (P=0,046), serum ALT düzeyleri ile anlamlı bir ilişki saptanamamıştır.

Sonuç: HCV ile indüklenen karaciğer hasarında ER stresinin geliştiği gösterilmiş olmasına rağmen, sonuçlarımız kronik HCV enfeksiyonu sırasında serum GRP 78 düzeyinde anlamlı bir artış olmadığını göstermektedir.

Anahtar kelimeler: Glikozla düzenlenen protein78, Hepatit C enfeksiyonu, Endoplazmik retikulum stres

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Introduction

Hepatitis C virus (HCV) infection is a widespread and significant public health problem throughout the world. In the last decade, the global seroprevalence in general reached 2.8% and it is estimated there are more than 185 million infected individuals [1]. Due to complications associated with chronic hepatitis C (CHC), nearly 350,000 people die annually [2]. Together with a reduction in the incidence of new cases through the years, it is predicted that secondary mortality from CHC will continue to increase for 20 years [3]. Most people infected with HCV cannot naturally clear the infection and a chronic infection process begins to develop, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC). HCV infection is associated with 15 to 20 times increased risk of HCC development [4,5]. However, the molecular mechanisms related to how HCV infection causes this disease are still not fully understood.

Endoplasmic reticulum (ER) is a quality control center for protein synthesis. Newly synthesized proteins that will be released by cells into the extracellular area or will participate in membrane structures are folded, modified, and combined correctly in ER [6,7]. A variety of physiological, pharmacological, and pathological conditions disrupt ER homeostasis, reducing the capacity to fold proteins. If the work of folding and releasing proteins is not completed effectively in cells, which is defined as "ER stress", it triggers a cell-protective response called unfolded protein response (UPR). The aim of UPR is to increase protein folding capacity, ensuring widening and reorganization of the ER membrane to reduce the stress response. If stress is not solved by severe and adaptive precautions, it may cause programmed cell death (apoptosis) [8-12]. ER stress was shown to play a role in a variety of liver diseases such as non-alcoholic steatohepatitis (NASH), alcoholic liver disease, ischemia reperfusion injury, toxic liver injury and viral hepatitis. HCV and hepatitis B virus (HBV) head the list of viruses inducing ER stress in the liver [13-15].

Replication of the HCV genome within hepatocytes causes accumulation of copious amounts of viral protein and RNA replication intermediate material in ER [16], which induces a significant stress response. The link between development of this stress response and the chronic infection process is not fully known. In mammalian cells, UPR is activated by three ER-stress sensors: RNA-dependent protein kinase-like ER-resident kinase (PERK), activating transcription factor (ATF6) and inositol-requiring enzyme 1 (IRE1). In ER stress, activation of these sensors is completed by separation of GRP78 (glucose-regulated protein), an ER chaperone protein found linked to luminal domains [13]. When ER stress develops, GRP78 expression clearly increases. When sufficient HCV protein accumulates in the ER, it binds to misfolded proteins, is titrated and activates the three pathways to begin UPR. It was shown that both structural and non-structural proteins in HCV trigger ER stress [17].

GRP78, one of the basic markers of ER stress in tissue, is shown to pass into circulation in studies completed recently [19,20]. It was previously shown that an ER stress response develops in hepatitis C patients; however, there was no marker studies that could show ER stress in circulation. This study evaluated the variations in GRP78 levels in circulation of patients with chronic HCV infection.

Materials and methods

Patients

We used serum samples from patients referred to Ordu University School of Medicine Hospital, Infectious Diseases clinic with diagnosis of hepatitis C infection. The hepatitis C patient group (n=60) included anti-HCV positive and HCV RNA positive, HBsAg negative and anti-HIV negative patients. Patients with HCV infections were separated into subgroups according to HCV RNA values as low (20 - $1x10^2$ IU/ml, n=20), moderate ($1x10^3$ - $1x10^5$ IU/ml, n=20), and high HCV-RNA ($1x10^6$ - $1.7x10^8$ IU/ml, n=20). The control group (n=60) included patients with no other chronic disease, and no chronic liver disease, anti-HCV negative, HBsAg negative anti-HIV negative patients. All serum samples were stored at -40 °C until evaluation. All patients and control serum samples had serum GRP78 levels investigated with the ELISA method. The study was conducted in accordance with the Declaration of Helsinki. It was planned retrospectively, and permission was obtained from the local ethics committee (Ordu University Clinical Research Ethics Committee, 2017-157).

Real-Time PCR

The COBAS AmpliPrep automatic extractor system was used for viral nucleic acid extraction. The COBAS AmpliPrep/COBAS Taqman 48 (Roche, Branchburg, NJ, USA) system was utilized to perform real-time PCR analysis for quantitative HCV-RNA. All procedures were performed according to manufacturer's recommendations. The measurement interval for HCV-RNA was 20-$1.7x10^8$ IU/ml.

Detection of GRP78

Analysis of GRP78 in serum used a commercial kit for human-GRP78 protein identification (Elabscience; E-EL-H5586). Using the sandwich-ELISA principle, the measurement interval for this kit was 0.63-40 ng/ml and sensitivity is reported as 0.38 ng/ml. Serum samples and standards were loaded in the appropriate wells of the micro ELISA plate and kit instructions were followed. After spectrophotometric reading at optical density of 450 nm (BioTek, ELx800 brand REF ELX508 SN1310149), serum GRP78 levels were calculated based on a standard graph. Results are presented as ng/ml.

Statistical analysis

Results are given as mean (standard deviation). The one-way ANOVA and Bonferroni post-hoc test were used for statistical assessment. Pearson correlation analysis was used for correlation assessments. $P<0.05$ was considered statistically significant.

Results

This study included a total of 60 chronic HCV patients (34 female and 26 male), with a mean age of 63.03 (14.2) years, and a total of 60 controls (24 female and 36 male), with a mean age 52.97 (19.7) years. Chronic HCV patients were divided into 3 different subgroups according to HCV-RNA levels as low (20 - $1x10^2$ IU/ml, n=20), moderate ($1x10^3$ - $1x10^5$ IU/ml, n=20) and high ($1x10^6$ - $1.7x10^8$ IU/ml, n=20). The mean ages in these subgroups were: 55.40 (16.5) years in the low HCV-RNA group,

66.65 (10.0) years in the moderate HCV-RNA group and 67.05 (12.6) years in the high HCV-RNA group.

ELISA measurements showed serum GRP78 concentration in the control group was 15.09 (10.30) ng/ml. Serum GRP78 was identified as 10.84 (9.6), 12.12 (8.4) and 14.41 (12.0) ng/ml in low, moderate and high HCV-RNA groups, respectively. There was no significant difference when compared with the control group. The value of serum GRP78 was similar in both the control and study subgroups.

There were statistically significant positive correlations between HCV-RNA, ALT and AST levels (P<0.0001 and P=0.008, respectively). However, no significant correlation was detected between HCV-RNA and serum GRP78 levels (P=0.238) (Table 1). A significant positive correlation was established between serum GRP78 and AST (P=0.046), while none was identified with serum ALT levels (Table 2).

Table 1: Correlation between HCV-RNA level and serum ALT, AST, GRP78 levels (n=60)

Correlation	r	R square	*P*-value
HCV-RNA vs. ALT	0.530	0.281	<0.001
HCV-RNA vs. AST	0.352	0.124	0.008
HCV-RNA vs. GRP78	0.161	0.026	0.238

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GRP78: Glucose-regulated protein78

Table 2: Correlation between serum GRP78 level and ALT, AST (n=60)

Correlation	r	R square	*P*-value
GRP78 vs. ALT	0.033	0.001	0.805
GRP78 vs. AST	0.270	0.072	0.046

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GRP78: Glucose-regulated protein78

Discussion

The liver's capacity for protein synthesis is very high and ER stress appears to be one of the important mechanisms in pathophysiology of liver diseases. To date, ER stress was shown to play a role in many liver diseases like alcoholic liver disease, diabetes related steatotic liver disease, toxic liver injury and viral hepatitis. Chronic hepatitis C disease causes cirrhosis and hepatocellular carcinoma, and is characterized by a microenvironment where oxidative stress, inflammation and regeneration processes are dominant in the liver [21]. The oxidative microenvironment and increased protein synthesis are important factors triggering UPR, with hepatitis C virus being one of the leading viruses inducing ER stress in the liver [15].

The HCV genome, coding nearly 3000 amino acids, ensures synthesis of many polypeptides including structural and non-structural proteins. As the host transcription system is used for the virus to replicate, rapid viral replication and accumulation of viral proteins in ER triggers the ER stress response [20]. In HCV, the three ER stress sensors may be activated, and it was found that GRP78 amounts increased significantly in the HCV subgenomic replicon system [7,22]. Studies of biopsy samples obtained from hepatitis C patients showed changes to the ER structure (widening and disorganization) indicating stress with electron microscopy. Additionally, clear increases were identified in proximal sensors (ATF6α, ATF6β, sXBP1, phosphorylated PERK) of the ER stress response and the levels of downstream effectors (GRP78, fosfo-elF2alpha, ATF4) of these sensors [7]. GRP78 shows protective effects against cell death due to cytotoxic T lymphocytes; however, it increases tumoral changes and resistance to antitumor medication [23]. As a result, the increase in GRP78 expression may play a role in HCV infection becoming chronic and carcinogenic.

Another study including HCC patients used immunohistochemistry and the Western Blot method on liver biopsy samples to show increases in ER stress markers of sXBP1, GRP78 and ATF6 [24].

Contrary to these results, there are findings showing that HCV does not cause ER stress. McPherson et al. [25] compared GRP78, GRP94, sXBP1 and EDEM mRNA levels in HCV positive and negative liver biopsies using RT-PCR and observed no significant difference between the two groups. This result may be due to random sampling. They stated they could not identify clear variations in mRNA/protein levels as mixed infected and non-infected hepatocytes were studied. This study simultaneously assessed viral load and UPR correlation, as in our study, and similarly stated there was no correlation.

The ER stress response varies according to whether hepatocytes are infected with HCV or not; as a result, there are other publications stating that random biopsy samples may have different results [7,22].

The association between HCV RNA viral load and liver histology has been researched in many studies. Gretch et al. [26] assessed 121 cases and proposed that hepatic activity index and fibrosis scores were higher among patients with high HCV RNA levels. Magrin et al. [27] concluded that liver histology was worse in patients with low HCV RNA in a 100-case series. Karakuş [28] stated that HCV RNA elevation was a parameter affecting liver histopathology; however, they interpreted different results in studies as indicating the liver injury may not always be shown due to proliferation of HCV in extrahepatic regions. This may explain the lack of connection between GRP78 and HCV RNA in our study.

Currently it is known that transaminase values and liver histopathology may not always comply. In our study, there was a positive correlation identified between AST and GRP78 elevation. AST is known to increase earlier and by higher amounts in ischemic and toxic liver injury [29,30]. However, there is a need for advanced studies to state the correlation with ER stress.

Limitations

Limitations of our study may be listed as the limited number of patients studied and the lack of accompanying histopathologic assessment.

Conclusion

Though ER stress is documented as an important mechanism in chronic liver injury associated with HCV, our results show serum GRP78 concentration does not change during chronic HCV infection, and that there is no significant correlation between parameters associated with HCV and GRP78, other than AST. Considering that random biopsy samples may have different results, identification of a serum/plasma marker for use to assess hepatic ER stress is clinically very important. There is a need for studies about a variety of markers in larger patient groups to explain the molecular mechanisms associated with ER stress in the process extending to chronic disease and cancer in patients infected with HCV and to identify appropriate markers in circulation.

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