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Impact of interplay between autophagy and interferon alpha in HCV and HCV/HIV infection

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Summary. – Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are among the most dangerous pathogens globally. Infection with HCV has been reported in a high percentage of HIV patients. Viruses are obligate intracellular pathogens and their survival is associated with their capability to subvert antiviral defenses of cells and to improve cellular processes required for their replication. The aim of this study was to compare the expression rate of the key gene for autophagy process, Beclin-1, as a cellular response to viral infections, and its effect on interferon alpha (IFN-α) expression in both HCV and HCV/HIV patient groups. In this study, a total number of 40 samples of peripheral blood mononuclear cells (PBMCs) including 20 HCV and 20 HCV/HIV patients before treatment were evaluated. The HCV viral load in both groups was evaluated by semi quantitative real-time PCR. The level of Beclin-1 and IFN-α gene expression was examined in all samples by semi quantitative real-time PCR assay. The median viral load was 8.3×10^5 copies/ml in HCV group and 2.1×10^6 copies/ml in HCV/HIV patients. While the expression level of Beclin-1 gene in HCV group was significantly higher, the level of IFN-α expression was lower compared to the HCV/HIV group (P <0.03). Furthermore, an inverse correlation was observed between Beclin-1 and IFN-α level of gene expression in co-infected patients (P <0.05). According to the results of this study, the positive or negative correlation between IFN-α and Beclin-1 gene expression along with other genetic and physiological host factors can be considered as important agents involved in HCV and HIV pathogenesis, and a probable reason for progression toward to cirrhosis in co-infected individuals.

Keywords: autophagy; Beclin gene; HCV; HIV; IFN gene

Introduction

Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are among the most serious pathogens globally (Torriani *et al*., 2003). HCV has infected more than 110 million people all around the world and also offers a huge burden to universal health, with the very high prevalence rates in developing countries (Shrivastava *et al*., 2011). Generally, HCV is asymptomatic, and in 70% of infected people the virus infection develops to chronic liver disease, which may progress toward steatosis, cirrhosis, fibrosis and hepatocellular carcinoma (Poynard *et al*., 2003; Hajarizadeh *et al*., 2013). On the other hand, as of December 2014, an estimated 36.9 millions of individuals were living with HIV, 2 millions were newly infected, and 1.6 million died (Boulle *et al*., 2014; Hoseinpour *et al*., 2015). Infection with HCV has been reported in one quarter of HIV-positive individuals in the United States and more than of 9% of drug abuser HIV patients in Iran (Olea *et al*., 2018; Amiri *et al.,* 2016). Infection with HIV influences the outcome of HCV therapy and worsens HCV progression to cirrhosis. Viruses usually affect autophagy as one of the early protecting mechanisms of cellular defense against invading pathogens (Lee *et al*.,

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Abbreviations: HCV = hepatitis C virus; HIV = human immunodeficiency virus; IFN-α = interferon alpha; PBMCs = peripheral blood mononuclear cells

2007; Ke and Chen, 2011). Of the more than 35 human autophagy-associated genes involved in autophagy, ten are now considered to be important for HIV replication (Campbell and Spector, 2013; Lee and Iwasaki, 2008). Beclin-1 protein is an important compartment in the generation of autophagosomes, starting and developing autophagy. This protein reacts with other proteins such as kinase complex called Vps34 and also other proteins such as Vps15 and Atg14L. In one evaluation performed for expression rate of Beclin-1 in human immortal hepatocytes IHH, it was defined that its expression rate was increased in the primary stages of infection with HCV until the 6th day after infection. In this study, also the effect of various HCV proteins on the promoter of Beclin-1 gene was evaluated and it was found that NS5A protein has an important role in increasing the expression rate of Beclin-1. HCV induces autophagy to increase its replication through using autophagy-induced intracellular membranes (Mizushima *et al*., 2008; Funderburk *et al*., 2010; Shrivastava *et al*., 2012). HIV decreases autophagy in dendritic cells (DCs) (Sagnier *et al*., 2015), while in macrophages, it initially increases autophagy in order to enhance virus proliferation and generation of viral proteins, and then it prevents the final stages of autophagy in order to arrest cell destruction (Borel *et al*., 2012). It was shown that the reaction of viral Nef protein with Beclin-1 blocks Beclin-1 activity and prevents maturation of autophagosomes (Campbell *et al*., 2015). Although there are some reports on the effect of viral gp41 in inducing autophagy in cells, the effect of virus on T-cell autophagy mechanism is not well recognized. Despite reducing the number of circulating plasmoid cells, increase of IFN-α expression is observed in acute and chronic forms of HIV infection. Blocking of IFN-α signaling may decrease the levels of interferon stimulatory genes and leads to the disease development. With regards to above notes and interaction between autophagy and IFN-α signaling (Levine *et al.*, 2011), the aim of the present study was to evaluate the difference in Beclin-1 protein expression and its effect on the expression of IFN-α in patients suffering from simultaneous infection of HCV and HIV compared with those infected with HCV only.

Materials and Methods

Patients. In this cross-sectional study, 40 samples (including 20 HCV patients and 20 HCV/HIV co-infected patients) of peripheral blood mononuclear cells (PBMCs) were evaluated. These samples have been collected from naïve HCV 1a patients referred to Alborzi infectious research center of the Shiraz Namazi Hospital before starting the treatment. The samples were divided into two groups balanced with respect to demographics and virologic characters. Ten samples from healthy people who had no obvious infectious disease during 4 weeks before sampling were considered as reference samples.

Table 1. The sequence of forward and reverse primers for interferon, Beclin-1 and GAPDH

Length	Sequence	Primer	Gene
bp 171	CTCAAGCCATCTGTGTCCTCC	Forward	IFN
bp 171	CTACCACCCCCACCTCCTGT	Reverse	
163 bp	CGCTGAGGGATGGAAGGGTCTAAG	Forward	Beclin
163 bp	CCTGGGCTGTGGTAAGTAATGGAG	Reverse	
68 bp	ACCTGACCTGCCGTCTAGAAA	Forward	GAPDH
68 bp	CCTGCTTCACCACCTTCTTGAT	Reverse	

PBMCs isolation with Ficoll. Five milliliters of total blood were diluted with 5 ml PBS and gently mixed with 3 ml Ficoll. Specimens were centrifuged at 400 g for 30 minutes at 18 to 24°C. Four layers containing serum, PBMCs, Ficoll and red blood cells were observed after centrifuging. The PBMCs layer was separated and washed with equal volume of PBS.

RNA extraction. RNA extraction was done according to guidelines of manufacturer of High pure RNA isolation kit (Roche company, Germany) on 10^6 PBMCs in a volume of 200 µl of PBS.

Primer design. The primers used in this survey were designed by lasergene7 software. The related sequences of interferon genes, Beclin-1 and GAPDH were obtained from nucleotide database. The sequences were aligned for each gene by MegAlign and the conserved areas were characterized for designing the primer. The primers were designed for separate exons or connection areas of exons (Table 1). The specificity of primers was evaluated by NCBI Primer Blast.

Synthesis of cDNA for real-time PCR. A reaction mix containing 2.5 µg of RNA was incubated with the 250 ng random hexamers (Sigma) and pre-mix cDNA synthesis buffer (QuantiTect Reverse Transcription kit, Qiagen, Germany), including Quantiscript Reverse Transcriptase, 5x Quantiscript RT Buffer, RT Primer Mix, dNTP, dithiothritol (DDT) and RNase inhibitor, gDNA Wipeout Buffer, RNase-Free Water. Finally, DEPC water was added and the reaction was kept in thermocycler for 15 min at 42°C and immediately was incubated at 95°C for 3 min in order to inactivate the RT enzyme.

Real-time qPCR reaction. A Rotor Gene RT-PCR machine (ABI, USA; Model RG3000) was used for the duplicated PCR reactions with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Cat. No. 204243). After 12 min activation of the modified Taq polymerase at 95°C for 15 min, 40 cycles of 95°C for 15 s, 30 s at each gene's annealing temperature and 30 s at 72°C were performed. Then, the ΔCT values were used for data analysis (Livak and Schmittgen, 2001).

Detection of viral load. Viral load in two groups of patients was determined by quantitative Real-Time PCR using Quantification of Hepatitis C Virus Advanced kit (PrimerDesign Ltd., Millbrook Technology Campus, Southampton, UK).

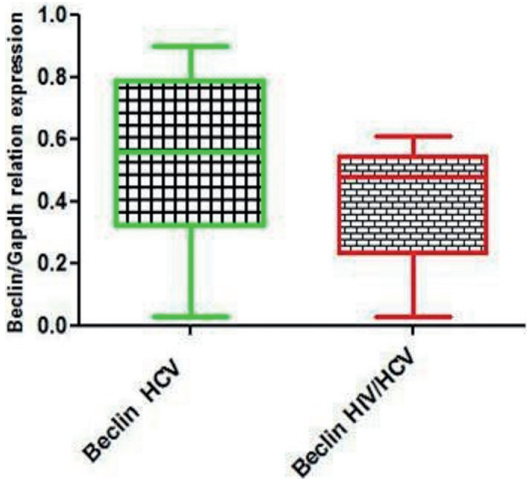

Fig. 1

Comparison of the expression rate of Beclin gene in HCV and HCV/HIV patients

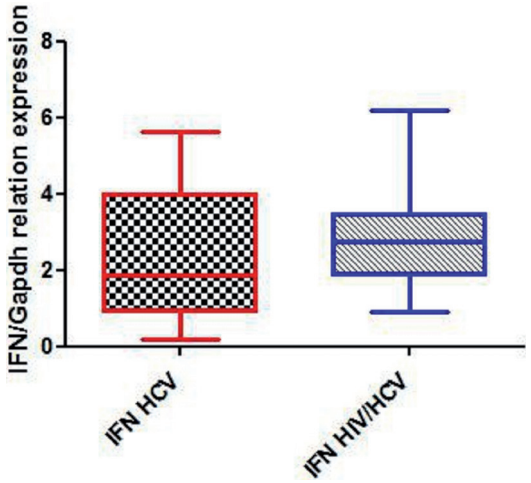

Fig. 2

Comparison of the expression rate of IFN-α gene in HCV and HCV/HIV patients

Data analysis. The data analysis was performed by SPSS and/or GraphPad software. The relationship of Beclin-1 and interferon mRNA expression in each group was analyzed by Pearson's correlation coefficient.

Results

Forty patients were included in the present study, 20 HCV and 20 HCV/HIV patients, with averages age of 47.85±13.99 and 39±6.76 years, respectively. There was no significant difference in this aspect between the two groups (P >0.05).

The median viral load was 8.3×10^5 copies/ml in HCV group and 2.1×10^6 copies/ml in HCV/HIV group according to the results of the semi-quantitative real-time PCR assay.

The results of semi-quantitative real-time PCR showed that the gene expression level of Beclin-1 in HCV group was significantly higher than in HCV/HIV group (P <0. 03), (Fig. 1), while the gene expression level of IFN-α in the HCV group was significantly lower compared with HCV/HIV group (P ≤0.3) (Fig. 2). An inverse correlation was observed between Beclin-1 and IFN-α only in co-infected patients (r = −0.54, P <0.05, Pearson's correlation), while in HCV group the correlation was 0.367 (P ≥0.05), which was not significant.

Discussion

Viruses can escape from the effect of the inherent immune system by manipulating the autophagy pathway and thus reinforce their proliferation. Various genes are involved in the initiation and regulation of autophagy process during viral infection. Studies have shown that some viruses stimulate autophagy and others inhibit it (Choi *et al.*, 2018). Various studies have evaluated the correlation between HCV and/or HIV and genes involved in autophagy modulation, with paradoxical results. As yet, no study has been conducted about the correlation between co-infection with HCV and HIV and autophagy. In the present study, we evaluated the association between autophagy and co-infection with HCV and HIV and its comparison with HCV mono-infection.

The results of our study showed that the expression rate of Beclin-1 in HCV group was significantly higher compared with HCV/HIV group, in spite the fact that the expression of IFN-α was significantly higher in the HCV/HIV group compared with HCV patients. Novel studies showed that there is a complex correlation between HIV infection and autophagy. HIV infection influences the autophagy process by several ways and has different effects on autophagy in different tissues and also different at stages of infection. In some cells and at certain stages of infection HIV inhibits autophagy, while other cases the virus stimulates autophagy (Tallóczy *et al.*, 2002; Dreux *et al.*, 2009; Shrivastava *et al.*, 2012; Dinkins *et al.*, 2014; Sagnier *et al.*, 2015).

Various studies in cell culture and in liver samples of patients have shown that the number of autophagosomes is increased in HCV infection. HCV infection increases the stress in hepatocytes, especially in endoplasmic reticulum and activates unfolded protein response (UPR), which plays a key role in stimulation of autophagy (Borel *et al.*, 2012).

Beclin-1 protein is an early important component of autophagosomes and in initiation of autophagy. This protein reacts with other proteins such as a kinase complex called Vps34 and also proteins Vps15 and Atg14L. In a study conducted to determine the expression rate of Beclin-1 in hepatocytes, it was found that in the early stages of HCV infection, the expression rate of Beclin-1 is increased. Studies have shown that various cytokines can affect autophagy in cell in different ways. Zhao *et al*. showed that IFN-α stimulates autophagy by stimulation of Beclin-1 signaling pathway. The use of IFN-α as a stimulator of autophagy has been reported as a treatment in some cancers (Zhao *et al*., 2014).

Despite the fact that IFN-α stimulates autophagy in cancer, the activation of autophagy pathway following HCV infection represses IFN activation mediated by the virus-induced PAMP (pathogen-associated molecular pattern)(Ke and Chen, 2011). It was also shown that Beclin-1 knockdown in HCV-infected cells resulted an increased expression of IFN-α and some other mRNAs mediated by the interferon signaling pathway (Shrivastava *et al*., 2011). This reverse relation between autophagy and IFN expression together with the results of the present study showing reduction of autophagy in HCV/HIV infected patients, may be accounted as a factor for enhancing IFN expression in co-infected patients compared to HCV infected individuals.

Concurrent infection of HIV/HCV leads to activation of HCV and acceleration of the disease into cirrhosis mediated by the increase of profibrogenic cytokines and oxidative stress and also increase of hepatocellular carcinoma incidence (Mastroianni *et al*., 2014). Additionally, the pathologic effect of HIV virus on liver has also important roles in HCV disease acceleration (Guadalupe *et al*., 2003; Kaspar and Sterling, 2017). HIV patients with concurrent HCV infection have higher morbidity and mortality because of higher viral load of HCV and increased risk of cirrhosis (Puoti *et al*., 2003).

Kottilil *et al*. showed that concurrent HCV and HIV infection lead to alteration in expression of genes in PBMCs, and HCV viremia due to the influence on cellular immunity, especially IFN-α. Chronic immune activation and elevated production of inflammatory cytokines in co-infected patients lead to the progression of liver disease into fibrosis due to the effect on immunoregulatory and pro-inflammatory pathways (Kottilil *et al*., 2009).

The decreased level of autophagy, which leads to an increase in IFN-α production in co-infected patients compared to HCV infected individuals, suggests a possible role in cirrhosis progression in concurrent HIV/HCV infection.

In conclusion, the reduced rate of Beclin-1 in co-infected patients, which increases the expression rate of IFN-α as an immune activation cytokine, can be considered as a factor that promotes immune system toward destruction of cells and cirrhosis progression.

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