TRANSCRIPTOME ANALYSIS OF APOPTOTIC MARKERS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF HCV GENOTYPE-3A INFECTED PATIENTS

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ABSTRACT

Objective: To examine the HCV genotype 3a specific effects on caspases involved in apoptosis, expression levels of caspases-3, 8, 9, and 10 in peripheral blood mononuclear cells (PBMCs) of HCV genotype 3a infected patients.

Methodology: This hospital based case controlled study was carried out between May 2014 to March 2015 on 72 subjects (36 HCV cases and 36 healthy controls). Blood samples of already diagnosed HCV patients of genotype 3a who were not on any antiviral therapy and HCV negative controls were collected. After extracting Ribonucleic acid (RNA) from samples it was reverse transcribed into complementary deoxyribonucleic acid (cDNA). Relative gene expression was performed for caspases-3, 8, 9 & 10.

Results: Overall alanine transaminase and aspartate transaminase (ALT and AST) were raised, i.e. 85.20 ±40.64 and 97.43 ±2.77 in HCV infected subjects, as compared to controls 54 ±29.5 and 33 ±18.3 respectively ($p < 0.001$). Gene expression analysis exhibited a statistically significant increase of 3.77, 3.74, 4.36 and 2.86 folds of caspases-3, 8, 9 & 10 respectively ($p < 0.001$) in HCV patients.

Conclusion: HCV genotype 3a has the tendency to induce both intrinsic and extrinsic pathways of apoptosis in peripheral blood mononuclear cells, facilitating HCV infection of hepatocytes by inhibiting the cells related to immune system.

Key Words: Hepatitis C virus, Apoptosis, Caspases

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INTRODUCTION

Hepatitis C virus (HCV) affects almost 170 million individuals around the globe[1]. In Pakistan, about 4.7% people are infected with hepatitis C virus[2]. Out of these infected individuals, around 50% to 80% proceed towards the chronic stage of the disease. Chronic HCV infected patients have the tendency to develop life-threatening complications and about 20% of the cases enter cirrhosis and out of them about 4% develop hepatocellular carcinoma (HCC)[3].

HCV is a small virus of Flaviviradae family having a positive-strand RNA genome of 9.6 kb enclosed in protein capsid and further surrounded by a lipid bilayer of cellular origin. The genome of HCV encodes an approximately 3000 a.a. (amino acids) long precursor polyprotein which is further divided into three structural proteins and seven non-structural proteins[3]. HCV has been categorized into seven genotypes with 50 or more sub-types on ground of its different nucleotide arrangement. However, different but related viral genomes are present in the infected individual which are termed as quasispecies which occur due to the fast viral life cycle and viral RNA polymerase which acts without proof reading[4].

Persistence of HCV infection and consequent HCC is greatly attributed to the inhibition of apoptosis and studies indicate that HCV proteins have pro- and anti-apoptotic properties[5,6]. Two different pathways generally mediate Apoptosis: intrinsic pathway and extrinsic cell death pathway. In extrinsic pathway, cell death occurs when activated caspase-8 binds with Fas-associated protein with death domain (FADD), resulting in activation of caspases-6, -9, and ultimately caspase-3[7]. Mitochondrion activates its intrinsic pathway as a result of DNA damage, oxidative stress and viral protein action. It involves the activation of caspase-9 which then triggers a cascade of events involving caspase-3 and caspase-7 activation[8]. Regulation of apoptosis is through caspases in which the initiator caspases

are caspases-2, 8, 9 & 10, effecter caspases include caspases-3, 6 & 7[9]. HCV causes apoptosis by extrinsic and intrinsic pathways[10,11], and their expression is raised in HCV infection resulting in increased apoptosis of liver cells.

PBMCs being the alternative site for HCV replication are affected by the viral proteins, which alter the expression of caspases in order to modulate the apoptotic pathway favoring HCV in infecting the target hepatocytes[10,12]. Very few studies have been undertaken to fully elucidate the intricate interplay between various HCV genotypes and the expression of caspases in PBMCs as to reveal the dubiety of different induction responses in different genotypes. In this preliminary study, expression levels of caspases-3, 8, 9 & 10 in PBMCs of HCV genotype 3a infected patients were analyzed. Caspases in HCV patients can provide information regarding various "in vivo" findings without venturing for any invasive procedures e.g. liver biopsy or histological examination. There is a strong need of non- invasive biomarkers and their relationship with disease and knowledge regarding caspases levels can be a milestone for this.

METHODOLOGY

This hospital based case controlled study was carried out between May 2014 to March 2015. The sample size was calculated using the following parameters in the formula: anticipated proportions =70% & 100% with effect size of 30%[5]; power of study =90%; and desired level of significance = 5%. A total of 72 subjects (36 HCV cases and 36 healthy controls) were included. Convenient sampling was done after taking written consent from participants. The diagnosed untreated patients of HCV genotype 3a were selected.

Blood samples of already diagnosed HCV patients (ELISA and PCR positives) of genotype 3a who were not on any antiviral therapy were collected from the Jinnah Hospital, Lahore. Patients with hepatitis B virus (HBV), recent history of any other viral infection and bacterial diseases were excluded. Patients on interferon therapy and pregnant women were also excluded. Controls for the study included those who were anti-HCV negative. All the subjects responded to the questionnaire of socio-demographic and basic clinical data. Medical records showed clinical variables like jaundice, edema, scratch marks, e.t.c. University of Health Sciences (U.H.S) provided funds for the study. The study was performed after the approval from Ethical review board of U.H.S, Lahore, Pakistan. Moreover, norms of the Helsinki Declaration of 1975, as revised in 2000 and 2008 concerning human and animal rights were observed by authors.

RNA was obtained from collected blood samples by TriZOL reagent (Ribo LS kit, Germany). For the assessment of quality and quantity of RNA, NanoDrop ND-1000 spectrophotometer was used. Reverse transcription of RNA into cDNA was carried out by RevertAid First Strand cDNA Synthesis Kit (Life technologies, USA). The primers used for gene specific amplification of caspases-3, 8, 9 & 10 were same (Table 1) as described previously[5]. In order to evaluate effects of HCV genotype 3a on expression of cellular genes caspases-3, 8, 9 & 10, real-time PCR was conducted, gene specific-primers & SYBR Green mix (Fermentas) were used. Real time PCR monitors and quantifies PCR reaction and PCR product (DNA, cDNA or RNA). For internal normalization of data GAPDH was used with similar PCR profile as caspases-3, 8, 9 & 10. Primers of caspases-3, 8, 9 & 10 were optimized at annealing temperature of 55°C while annealing temperature of GAPDH was 58°C. The software "iQ5 2.1" (Bio-Rad, USA) was used to analyse gene expression. Each of the experiment was performed in triplicate.

All statistical analyses were performed using SPSS software (version 20.0, SPSS Inc). Qualitative data were presented as frequency while quantitative data were shown with mean ±SD. Student's t-test was used to analyze numerical data. P value ≤ 0.05 was considered statistically significant.

RESULTS

Results showed that every patient was having the history of fatigue and majority were experiencing loss of appetite, intolerance and itching etc. as shown in the Table 2. As for as the clinical examination was concerned more than 90% patients had jaundice, scratch marks and liver/spleen palpations. Overall ALT and AST were raised, i.e. 85.20 ±40.64 and 97.43 ±52.77 respectively (Table 3).

Results of our study, indicated a statistically significant enhanced gene expression of caspases-3, 8, 9 & 10, in patients infected with HCV genotype 3a in comparison with healthy controls. Target genes expression among healthy controls were considered as 1.0. A statistically significant ($p < 0.001$) relative gene expression increase of 3.77 ±1.08, 3.74 ±1.43, 4.36 ±1.30 and 2.86 ±0.92 folds in caspases-3, 8, 9 & 10 respectively was observed (Figure 1).

DISCUSSION

HCV infection could induce apoptosis at early phase of infection and modulates apoptosis causing both hepatocyte damage and repair, which is a characteristic of HCV infection, ultimately leading to HCC. Due to involvement of caspases in apoptosis, it is proposed that the detection of caspase activity in serum can serve as a reliable and sensitive marker for determining levels of HCV infection as compared to the traditional markers such as ALT and AST. In addition, detection of caspase

Table 1: Sequences of primers used for this study

Pair	Gene	Primer	Optimized Annealing Temp	Product (BP)
01	Caspase-3 F	5′ ATGGAAGCGAATCAATGGAC 3′	55°C	484
	Caspase-3 R	5′ GCCATGTCATCATCAACACC 3′		
02	Caspase-8 F	5′ TATGGCACTGATGGACAGGA 3′	55°C	232
	Caspase-8 R	5′ GCAGAAAGTCAGCCTCATCC 3′		
03	Caspase-9 F	5′ ATGTCGTCCAGGGTCTCAAC 3′	55°C	400
	Caspase-9 R	5′ GGAAACTGTGAACGGCTCAT 3′		
04	Caspase-10 F	5′ AGTGACAGGTATGGGCGTTC 3′	55°C	280
	Caspase-10 R	5′ GCAGCACCTCAACTGTACCA 3′		
05	GAPDH-F	5′ ACCACAGTCCATGCCATCA 3′	58°C	453
	GAPDH-R	5′ TCCACCACCCTGTTGCTGTA 3′		

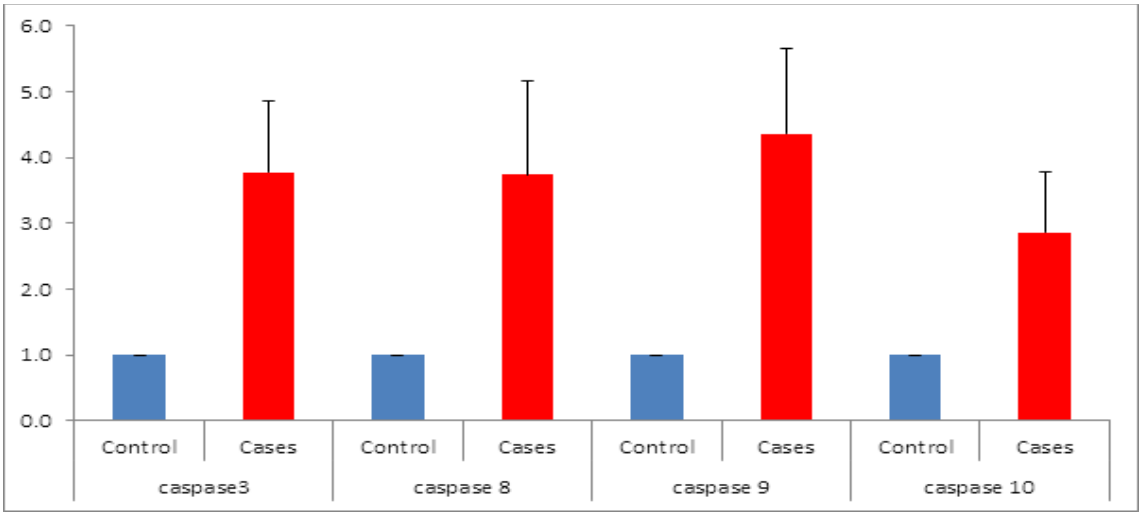
Table 2: Summary of clinical examination and symptoms reported by patients

Parameters	Present n (%)	Absent n (%)
Clinical History		
Fatigue	36 (100%)	00 (0%)
Weariness	31 (86.1%)	05 (13.9%)
Flatulence	23 (63.9%)	13 (36.1%)
Loss of Appetite	26 (72.2%)	10 (27.8%)
Nausea	22 (61.1%)	14 (38.9%)
Intolerance	27 (75.0%)	09 (25.0%)
Itching	33 (91.7%)	03 (8.3%)
Impotence	20 (55.6%)	16 (44.4%)
Upper Abdominal Pressure	20 (55.6%)	16 (44.4%)
Clinical Examination		
Jaundice	35 (97.2%)	01 (2.8%)
Spider Naevi	23 (63.9%)	13 (36.1%)
Palmer Erythema	20 (55.6%)	16 (44.4%)
Smooth Red Tongue	20 (55.6%)	16 (44.4%)
Gynecomastia	13 (36.1%)	23 (63.9%)
Paper Money skin	20 (55.6%)	16 (44.4%)
White Nails	27 (75.0%)	09 (25.0%)
Chvostek's Habitus	14 (38.9%)	22 (61.1%)
Dupuytren's Contracture	20 (55.6%)	16 (44.4%)
Scratch Marks	34 (94.4%)	02 (5.6%)
Hemorrhages	30 (83.3%)	06 (16.7%)
Palpable Liver/Spleen	34 (94.4%)	02 (5.6%)
Dark Urine	17 (47.2%)	19 (52.8%)
Edema/ Ascites	31 (86.1%)	05 (13.9%)
Hepatic foetor	16 (44.4%)	20 (55.6%)
Alcholic stools	17 (47.3%)	19 (52.8%)

Table 3: General demographics and transaminases levels of patients and controls

Parameters	Controls Values n (%) / Mean ± SD	Cases Values n (%) / Mean ± SD	P value
Gender			
Male	20 (55.6%)	11 (30.6%)	0.224
Female	16 (44.4%)	25 (69.4%)	
Age	45.78 ± 11.87%	47. 08 ± 11.87	0.637
Enzyme SGPT (ALT)	54 ± 29.5	85.20 ± 40.64	<0.001
Enzyme SGOT (AST)	33 ± 18.3	97.43 ± 52.77	<0.001

Figure 1: Cumulative relative gene expression of caspases-3, 8 , 9 & 10



activity in serum might be a more sensitive method of detecting early liver injury. Measurement of caspases activity alone or with conventional parameters might provide a novel diagnostic tool, especially for patients with normal aminotransferase levels having histologically active hepatitis[13]. HCV also leads to carcinoma so determination of caspases earlier in the disease might help as a sensitive bio-marker for early detection of hepatocellular carcinoma.

Our results show a significant higher mRNA level of caspase-3, -8, -9 and 10 in PBMC's of HCV patients of genotype 3a. In Brazil, Albertoni et al[10] investigated caspase-3, -8 and -9 expression in PBMC's of HCV patients and found high levels of caspase-3 and caspase-8 which lead to activation of caspase-9 ($p < 0.05$). Interestingly, we observed that caspase-9 gene expression had increased 4.35 folds suggesting that the intrinsic pathway of apoptosis is more prominent compared with the activity of extrinsic pathway as shown by the expression of caspase-8 which was around 3.73 folds compared with the normal control group. However, the expression of Caspase-10 was found to be only 2.86 folds as compared with the normal control group. Studies indicate that caspase-10 is activated by caspase-8[8]. However, it can also act as an independent caspase and both (caspase -8 and 10) can function independently without depending on each other. Based on the results of the study, Wang et al[14] emphasized on the fact that caspase-8 and caspase-10 might have different substrates for apoptosis, and might be playing an individual role in the apoptotic process. We also found increased levels of caspase-10 (2.862 ±0.92) but they were not as enhanced as levels of caspase-8 (3.74 ±1.43) and other caspases. Therefore, we can speculate that caspase-10 might be working independently of caspase-8. Importance of activation levels of caspase-10 can be quite expressive in the process of apoptosis as it has been shown that its decrease amounts can make the body prone towards malignancy; causing resistance to apoptosis[14]. Caspase-3, which is the main downstream effector and also known as executioner caspase, was raised 3.77 folds in cases as compared with controls. Since all caspases were statistically higher than controls ($p < 0.05$), these results confirm that enhanced genes expression are involved in apoptosis in HCV patients, as

reported earlier[10]. Many earlier studies, such as of Deng et al[7] on human hepatoma cell line passage (Huh) 7.5 and of Bantel et al[15] in vivo study on 20 patients of HCV, support our data of increased gene expression of caspase-3, 8, 9, & 10 in HCV patients. *In vitro* studies of Jahan et al[5] and Berg et al[16], however, are in contrary to our data. These studies suggest that decreased apoptosis lead to progression of HCV. The difference in results could be because our study was carried out purely on PBMCs and not in hepatocytes and also it was an *in vivo* study in which numerous biological factors like immunity and other responses of the body towards disease may have played an important role in increasing the apoptotic machinery. It is, therefore, suggested, that a detailed study with higher number of patients be commissioned, so that the exact role of apoptotic factors and caspases be delineated clearly.

CONCLUSION

Hepatitis C virus genotype 3a has the tendency to induce both intrinsic and extrinsic pathways of apoptosis in PBMCs, which may facilitate HCV infection of hepatocytes by inhibiting the cells related to immune system. Moreover, compared with conventional surrogate markers such as ALT and AST, detection of caspase activity in serum can serve as better tool for detecting early liver injury. Studies correlating the degree of liver damage with the transcriptome and proteome levels of caspases may provide further insight into the disease process and progression and its control.

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CONTRIBUTORS

KPL conceived the idea, designed the study, analysed the data and drafted the manuscript. AZ helped acquisition of data, performed the experiment and did data analysis. SK performed the experiment, did data analysis and drafted the manuscript. SJ performed the experiment and did data analysis. All authors contributed significantly to the submitted manuscript.