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Quantitative evaluation of insulin resistance markers in Pakistani patients suffering from HCV-associated type 2 diabetes mellitus

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Summary. – The current study was designed to determine the role of the host genes involved in the development of chronic hepatitis C-associated type 2 diabetes mellitus. This study was carried out in patients in four different stages of chronic hepatitis C virus (HCV) infection, including treatment-naïve HCV patients, HCV-positive patients with type 2 diabetes mellitus (T2DM), non-responders and responders. The mRNA expression level of host genes, such as glucose-6-phosphatase (G6Pase), tumor necrosis factor α (TNF-α) and different adipokines including adiponectin, leptin and resistin, was quantified and compared to healthy controls. HCV infection was found to be associated with insulin resistance, a step towards type 2 diabetes mellitus (T2DM). The results also suggest the potential role of adipokines in chronic HCV (CHC)-associated T2DM. The upregulation of gluconeogenic genes, such as G6Pase and resistin, and a decreased mRNA expression level of adiponectin suggest the potential role of selected markers in the CHC-associated T2DM in Pakistani population. Based on these results, it is concluded that upregulation of TNF-α, G6Pase and resistin in chronic HCV patients leads to gluconeogenesis, eventually favoring T2DM. Collectively, these findings suggest that CHC patients are more prone to T2DM.

Keywords: HCV associated T2DM; role of adipokines in HCV infection; TNF-α and HCV

Introduction

Hepatitis C virus (HCV) is a lethal blood-bore pathogen that shows hepatotropism. Once infection is established, it can progress from acute to chronic, eventually advancing in multiple hepatological disorders including fibrosis, cirrhosis, steatosis, insulin resistance (IR), type 2 diabetes mellitus (T2DM) and hepatocellular carcinoma (HCC) (Lau *et al.,* 1998; Bièche *et al*., 2005; Fartoux *et al*., 2005; Pekow *et al*., 2007). The morbidity rate of patients infected with HCV is more than 170 million, making about 3% of the world's population (Simmonds, 2004).

Insulin is an anabolic hormone that aids in selective absorption of glucose and maintains normal blood glucose level. This involves a complex array of metabolic signaling, where insulin receptor substrate (IRS) acts as an adaptor protein and couples insulin to the cytosolic domain of insulin receptor. Any artifact in this signaling pathway is likely to disrupt the glucose metabolism and favors insulin resistance, T2DM as well as lipogenesis (Parvaiz *et al.,* 2014).

HCV core and NS5A proteins are considered to be the potential candidates towards IR development (Banerjee *et al*., 2008; Parvaiz *et al*., 2014). Under normal insulin signaling, activation of IRS involves phosphorylation at tyrosine residues, while serine phosphorylation is favored during infection or disrupted metabolic signaling (Withers, 2001;

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Abbreviations: CHC = chronic hepatitis C; G6Pase = glucose-6-phosphatase; HCV = hepatitis C virus; IR = insulin resistance; IRS = insulin receptor substrate; SVR = sustained virological response; TNF-α = tumor necrosis factor α; T2DM = type 2 diabetes mellitus

White, 2003). Previous studies have shown that upregulation of TNF-α triggers serine phosphorylation of the IRS, thereby modulating entire downstream insulin signaling pathway. IL-6 and TNF-α are important proinflammatory cytokines that are upregulated in CHC patients (AlDosary *et al.*, 2002). Previous studies have also indicated a plausible role of TNF-α in T2DM (Saliba *et al.*, 2007).

Contrary to the inflammation, naturally various anti-inflammatory mediators, such as adiponectin, IL-10, IL-6, etc. are activated in HCV infection (Tilg and Moschen, 2006). Previous studies have also linked the resistin-mediated upregulation of TNF-α with inflammation (Silswal *et al.*, 2005). Some studies have also linked the upregulation of TNF-α with increased gluconeogenic gene expression, such as G6Pase and phosphoenolpyruvate carboxykinase (PEPCK). PEPCK is a rate-limiting step for gluconeogenesis that converts pyruvate back to glucose, eventually favoring increased blood glucose level. (Deng *et al.*, 2010).

The molecular mechanism behind HCV-induced IR is still a great mystery. Pakistan is an under-developed country with great challenges in morbidity and mortality due to HCV infection. This study was designed to unveil plausible markers involved in CHC-induced IR in Pakistani population.

Table 1. Primer sequences

Genes	Primer sequence (5'-3')	Product size
TNFα-F	TCCTTCAGACACCCTCAACC	206bp
TNFα-R	CAGGGATCAAAGCTGTAGGC	
G6P-F	CATTGACACCACACCCTTTGC	89bp
G6P-R	CCCTGTACATGCTGGAGTTGAG	
ADIPOQ-F	GTGTATGGGGAAGGAGAGCG	296bp
ADIPOQ-R	TTTGTGAAGCTCCCCAGGAC	
LEP-F	ACGTGCTGGCCTTCTCTAAG	241bp
LEP-R	ACCTGGAAGCCAGAGTTCCT	
RETN-F	CGCCGGCTCCCTAATATT TA	218bp
RETN-R	GCTGCACACGACAGCAGC	

F: Forward, R: Reverse.

Materials and Methods

Study design. In this study, 36 patients chronically infected with HCV were screened and analyzed. The patients were categorized into four different groups depending upon their response to the standard therapy for HCV. First group included 10 treatment-naïve (TN) chronic HCV patients (Table 2), second group 10 treatment non-responders without T2DM (NR) (Table 3), third group

Table 2. Chronic hepatitis C (treatment naïve) patients

Sr No.	Age	Gender	BMI	ALT (0–42 U/l)	Viral titer (copies/ml)	Bilirubin (0–17 umol/l)	Serum alkaline phosphatase (40–130 IU/l)	HCV genotype
1	29	F	19.2	70	36586	15	54	3a
2	35	M	22.7	24	475695	23	65	3a
3	54	F	21.6	11	22440	11	42	3a
4	65	M	22.8	25	134357	23	65	3a
5	35	M	22.8	32	6265	6	63	3a
6	22	M	19.8	87	4923	13	96	3a
7	32	M	20.6	56	112988	25	76	3a
8	34	F	21.7	23	502670	15	53	3a
9	35	M	27.5	37	359400	12	87	3a
10	52	M	21.4	50	3540	23	95	3a

Table 3. Chronic hepatitis C (non responder without T2DM) patients

Sr No.	Age	Gender	BMI	ALT (4–42 U/l)	Viral titer (IU/ml)	Bilirubin (0-17 umol/l)	Serum alkaline phosphatase (40–130 IU/l)	HCV genotype
1	29	F	22.5	70	327645	32	43	3a
2	35	M	19.9	240	436758	54	65	3a
3	34	M	23.8	46	1100323	03	112	3a
4	37	M	21.6	45	1053106	34	45	3a
5	34	M	19.9	28	92539	19	32	3a
6	50	M	25.3	42	25220	37	65	3a
7	32	M	21.5	56	84365	25	87	3a
8	34	F	22.7	23	54687	15	45	3a
9	36	M	19.5	37	32540	10	88	3a
10	31	M	23.5	37	526553	07	78	3a

Table 4. Chronic hepatitis C (non responder with T2DM) patients

Age	Gender	BMI	ALT (0–42 U/l)	Bilirubin (0–17 umol/l)	Viral titer IU/ml	Serum alkaline phosphatase (40–130 IU/l)	Total cholestrol (<5.2 mmol/l)	HbA1c	HDL (>1.04 mmol/l)	VDL (<3.2 mmol/l)	TG (0.4–1.6 mmol/l)
32	M	30.4	110	14	23546	54	143	6.3	0.9	2.1	2.4
46	F	32.3	34	23	45667	57	152	5.4	1.5	1.4	2.3
52	M	21.2	69	65	52220	76	4.1	7.1	1.7	1.6	1.2
40	M	21.6	47	17	134357	65	130	5.8	0.4	2.4	1.2
37	F	22.7	91	23	323910	86	4.6	6.1	1.1	2.3	2.5
48	F	25.3	240	12	130044	43	154	7.8	1.2	2.3	1.5

included 6 treatment non-responders with T2DM (NR-T2DM) (Table 4) and the final group of 10 patients, who achieved sustained virological response (SVR) after the treatment (Table 5). The control group included 15 healthy individuals (HCV negative). The treatment offered to patients was in accordance with the standards of care set for the HCV genotype 3 infections. Patients were given pegylated interferon along with ribavirin for 24 weeks. The study design was approved by the ethical committees of the Combined Military Hospital (CMH), Military Hospital (MH) Rawalpindi, and the Atta-ur-Rahman School of Applied Biosciences (ASAB) NUST Islamabad, Pakistan.

Blood collection. Blood samples positive for hepatitis C virus infection were collected within a period of 10 months. Patients with adequate documentation of abnormal ALT for over 6 months, anti-HCV antibody and blood sugar estimation were included in the study. Patients infected with HCV (both male and female) genotype 3a, with age ≥ 25 years, were included. The blood samples from these patients were subjected to a series of biochemical analyses, such as genotyping, complete blood count, lipid profile, liver function tests and hemoglobin A1c (HbA1c) diagnostic test for diabetes, in Armed Forces Institute of Pathology (AFIP), Rawalpindi. PCR-based confirmation of HCV RNA was performed in ASAB diagnostic laboratories. The quantification of HCV RNA was carried out by real time PCR according to the manufacturer's protocol using Applied Biosciences 7300 Real time PCR System (Imran *et al.*, 2015). The samples were run in parallel along with the quantification standards (synthetic internal controls). HCV RNA level in each sample was quantified with help of CT values for the sample and standard curve resulting from the analysis of quantification standard and the assay-specific calibration coefficient. The HCV RNA concentration is expressed in IU/ml, where one IU/mL is equal to two RNA copies/ml. The genotype of the virus was determined using genotype-specific primers reported by Ohno *et al.* (1997). Patients with co-infection of HBV, HIV or any other associated liver disease were excluded from this study. A written consent form was duly signed by the patients, healthy controls and ethical committee.

RNA extraction, quantification and reverse transcription. Total cellular RNA was extracted from blood of patients and healthy controls using TRIZOL method according to manufacturer's protocol. To avoid cellular contamination, the extracted RNA was treated with DNAase I (Fermentas, MD, USA). To determine quality and quantity, the extracted RNA was subjected to spectrophotometry (Eppendorf BioPhotometer plus™). The complementary cDNA strand was synthesized from DNase I-treated RNA using oligo (dT) 18 primer (Fermentas) and RevertAid H minus reverse transcriptase (Fermentas, MD, USA) in the presence of Ribolock RNase Inhibitor (Fermentas).

Optimization of PCR amplification and quantification. The gene expression of selected markers (TNF-α, resistin, leptin, adiponectin and G6Pase) was analyzed by PCR amplification using primers

Table 5. Hepatitis C SVR (without T2DM)

Sr No.	Age	Gender	BMI	ALT (042 U/l)	Serum Alkaline Phosphatase (40–130 IU/l)	Bilirubin (0–17 umol/l)
1	43	F	21.5	90	43	12
2	45	F	22.3	50	65	0
3	31	M	22.6	37	112	07
4	33	M	23.4	50	45	9
5	51	M	23.7	30	32	11
6	28	M	19.4	50	65	5
7	40	M	21.8	30	87	14
8	25	M	19.9	69	45	12
9	32	M	21.9	32	88	03
10	41	M	22.5	35	78	06

Table 6. Variation in expression of gene markers

Patients	TNF-α	G6P	Adiponectin	Leptin	Resistin
TN	↓	–	↓	↓	↓
NR	↑	–	–	–	–
NR-T2DM	↑	↑	↓	–	↑
SVR	↓	↓	↑	↓	↓

specific for selected markers, designed in Primer3web software Version 4.0.0 (Untergasser *et al*., 2012). Primer sequences are listed in Table 1. PCR reactions were carried out in Applied Biosystems PCR GeneAmp 9700 Thermocycler. PCR conditions used for the amplification of the selected markers comprised of initial denaturation at 95°C for 10 min, with 35 cycles of amplification [95°C (40 s), 60°C (40 s), and 72°C (40 s)] and final extension at 72°C for 7 min. Results were validated by using multiple samples to ensure the consistency, and high concentration of the template cDNA was used so that the variation in the expression of the selected markers can be detected. PCR amplification products were visualized on 2% agarose gel stained with ethidium bromide.

For the quantification of selected cellular genes in a signaling pathway, PCR for each gene was optimized using 1 μg of cDNA. Amplification plots for all genes were optimized by qRT-PCR in Applied Biosystems 7300 Real-Time PCR System using Maxima® SYBR Green/ ROX qPCR Master Mix (Fermentas, USA). Gene expression data was normalized to the expression levels of control or "housekeeping" genes. GAPDH was used as a housekeeping gene, a constitutive gene, expressed in all cells of the body. The relative gene expression analysis was carried out using the $2^{-\Delta\Delta CT}$method to calculate the fold change of difference. Each individual experiment was performed in triplicate.

Statistical analysis. All statistical tests were performed using Graph-Pad Prism version 5.0 (GraphPad Software, Inc., CA, USA). Data are presented as mean ± standard error of the mean. The expression of the selected markers in the CHC patient samples and healthy controls was compared statistically by unpaired Student's *t* test. p-values <0.05 were considered statistically significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$).

Results

To determine the regulatory genes involved in HCV-induced T2DM, selected genes were quantified using quantitative real time PCR.

Quantification of targeted genes in chronic HCV patients with and without T2DM

To understand the regulatory role of pro-inflammatory cytokines and adipokines in CHC patients, qRT-PCR was performed along with healthy controls. These CHC patients were without T2DM and categorized as treatment-naïve (received no HCV treatment) and non-responders

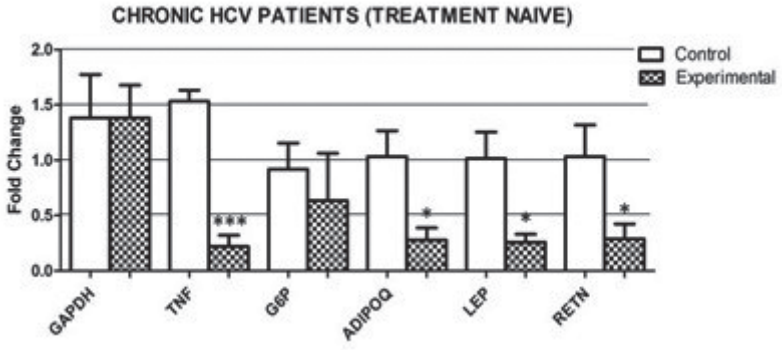

Fig. 1

Gene expression of treatment naïve chronic HCV patients compared to that of healthy controls

All values are expressed as mean ± SEM. TNF-α showed significantly decreased (0.22-fold) expression, while AdipoQ (Adiponectin), Lep (Leptin) and RETN (Resistin) levels in CHC-associated diabetes non responders as compared to the healthy controls showed significant increase (0.27- 0.26- and 0.29-fold respectively). G6P levels showed no significant change. ***shows $p < 0.001$, **shows $p < 0.01$, *shows $p < 0.05$. The expression was studied at least in 3 separate studies with similar results. All reactions were set up in duplicate.

(HCV-positive after receiving standard HCV treatment as mentioned in the Materials and Methods section). The results indicated a significantly reduced transcriptional level of TNF-α in treatment-naïve patients, while a significant increase in expression was found in non-responder group in comparison with healthy controls (Fig. 1 and 2). The data also suggest a decreased transcriptional expression of G6P in both groups, as T2DM has not developed yet (Fig. 1 and 2). Furthermore, data have also suggested a decreased transcriptional expression of adipokines in both targeted groups as compared to healthy controls (Fig. 1 and 2).

The results suggested a significantly increased mRNA expression of TNF-α (1.94-fold), G6Pase (1.92-fold) and resistin (1.95-fold), while the Adiponectin mRNA expression levels (0.27-fold) were decreased significantly in NR-T2DM patients compared to the healthy controls (Fig. 3).

Expression level of targeted genes in SVR

The analysis of the selected markers in SVR and healthy controls revealed that the expression of mRNA of TNF-α (0.11-fold), G6Pase (0.25-fold), Lep (0.41-fold) and resistin (0.06-fold) decreased significantly in SVR, while there was a significant increase in the mRNA expression levels of Adiponectin in this group (Fig. 4).

Discussion

HCV-associated T2DM is considered to be the outcome of chronic HCV infection (Hoofnagle, 2002). Several studies have reported that HCV infection induces ER stress and promotes hepatic gluconeogenesis, resulting in increased

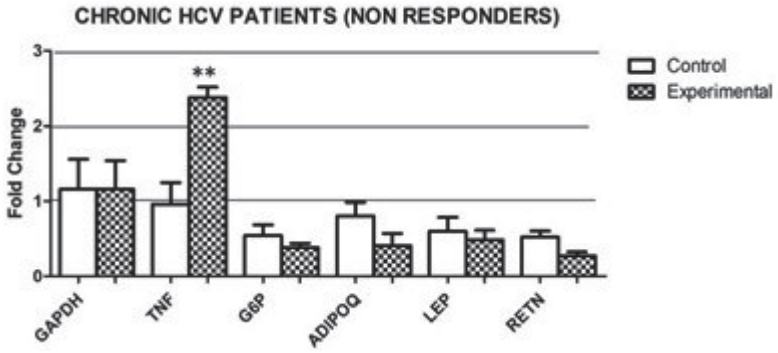

Fig. 2

Gene expression of non-responder chronic HCV patients compared to that of healthy controls

All values are expressed as mean ± SEM. TNF-α showed significantly enhanced (2.38-fold) expression, while G6P, ADIPOQ (Adiponectin), Lep (Leptin) and RETN (Resistin) levels in CHC-associated diabetes non responders as compared to the healthy controls remained unchanged. ***shows $p < 0.001$, **shows $p < 0.01$, *shows $p < 0.05$. The expression was studied at least in 3 separate studies with similar results. All reactions were set up in duplicate.

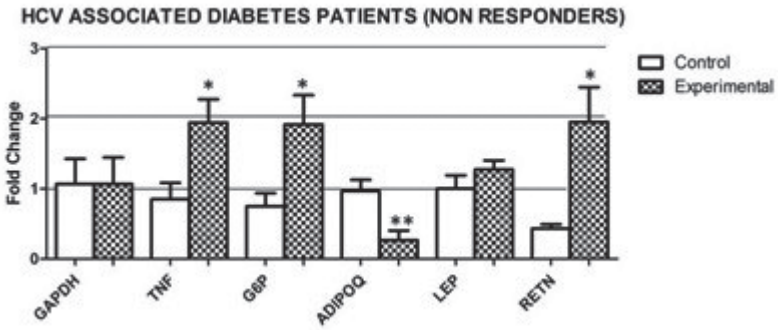

Fig. 3

Gene expression of chronic HCV-associated diabetic patients compared to that of healthy controls

All values are expressed as mean ± SEM. TNF-α, G6P and RETN (Resistin) were increased significantly (1.94-, 1.92- and 1.95-fold respectively), while Lep (Leptin) levels in CHC-associated diabetes non responders as compared to the healthy controls remained unchanged. The levels of ADIPOQ (Adiponectin) were significantly decreased (0.27-fold). ***shows $p < 0.001$, **shows $p < 0.01$, *shows $p < 0.05$. The expression was studied at least in 3 separate studies with similar results. All reactions were set up in duplicate.

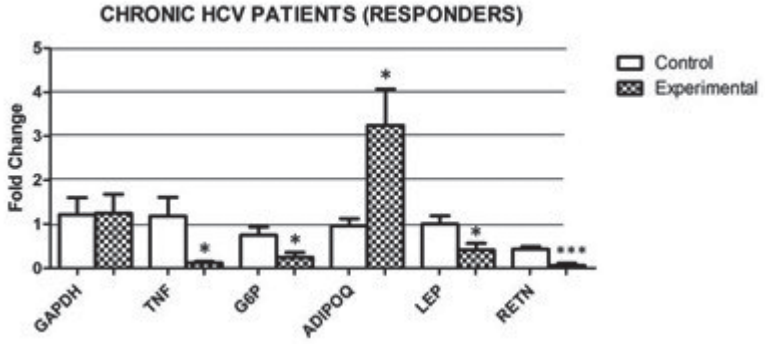

Fig. 4

Gene expression of HCV responders compared to that of healthy controls

All values are expressed as mean ± SEM. TNF-α, G6P, Lep (Leptin) and RETN (Resistin) levels in CHC responders as compared to the healthy controls were significantly decreased (0.11-, 0.25-, 0.41- and 0.06-fold, respectively). The level of ADIPOQ (Adiponectin) was enhanced significantly (3.24-fold). ***shows p <0.001, **shows p <0.01, *shows p <0.05. The expression was studied at least in 3 separate studies with similar results. All reactions were set up in duplicate.

glucose production in hepatocytes, eventually leading to T2DM (Deng *et al.,* 2011).

Current study is focused on determining the role of several host genes in HCV-associated T2DM. The results of our study suggest an increase in the mRNA expression of TNF-α in non-responders with and without T2DM association. These results are in accordance to the previous study that indicates an upregulation of oxidative stress during HCV infection (Parvaiz *et al.,* 2011). One of the possible mechanisms, through which these cytokines block normal insulin signaling, is the phosphorylation of IRS that modulates entire downstream insulin signaling pathway (Parvaiz *et al*., 2011).

G6Pase is one of the important enzymes favoring gluconeogenesis. The G6Pase elevation in non-responder patients with T2DM correlated with the decreased glucose utilization and favors hyperglycemia (Shlomai *et al.*, 2012). Our results also suggested increased G6P expression.

Adiponectin and resistin are important adipokines that play pivotal role during inflammation. (Durazzo *et al*., 2013). Our data suggest somewhat varied expression of adipokines in different categories of chronic HCV patients. TNF-α was found to be upregulated in NR and NR-T2DM. Our results indicated that there is no change in the expression of G6Pase in TN and NR groups, while an increased expression was observed in HCV-associated T2DM patients, revealing that there is strong role of G6Pase in the glucose impairment. Adiponectin is thought to have a hepatoprotective role and is found to be downregulated during hepatocyte damage (Kukla *et al.,* 2011). We also observed an increased expression of adiponectin in responders group, while a decreased expression of adiponectin was observed in patients with CHC-associated T2DM. Conversely, resistin was shown to have increased expression in HCV-associated T2DM patients, while decreased expression was observed in SVR. The expression levels of each of the four categories of patients have been compared and shown in Table 6.

Present study demonstrated the molecular mechanism involving adipokines in HCV pathogenesis, leading towards HCV-associated T2DM. Based on these findings, it is suggested that HCV induces insulin resistance, which down-regulates the expression of adipokines. This study also suggests the disease condition can be detected at early stages by evaluating the mRNA expression levels of the selected markers. The expression levels of certain cytokines and adipokines are affected due to the complexity of the metabolic pathways and the various transcription factors involved in them, showing the disease trend towards T2DM in chronic liver disease patients. The current study illustrates the need to investigate the roles of other adipokines in inducing HCV-associated T2DM to unveil the molecular pathways abrogated or perturbed by these events, leading to a more severe disease.

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