

Osteopontin as a marker for response to pegylated interferon Alpha-2b treatment in Chronic HCV Saudi patients.

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Abstract:

Background: Many recent studies support the idea that osteopontin (OPN) can be used to predict the success of pegylated interferon (PEG IFN) alpha-2b/ribavirin therapy in chronic HCV patients.

Objective: Our aim was to investigate the role of plasma OPN and its gene polymorphism at nt – 443 in response to PEG IFN in Saudi patients with chronic HCV.

Methods: Blood was collected from 87 patients with chronic hepatitis C before treatment, then patients received PEG IFN α 2b plus ribavirin combination therapy. Another 25 healthy subjects, matched for age and sex to patients, were enrolled as controls. Single nucleotide polymorphism (SNP) in OPN at nt – 443 and its blood level were analyzed.

Results: The frequency of patients who reached sustained virological response (SVR) was increased in patients with T/T at nt – 443 than in those with C/C or C/T. Also the frequency of T allele was increased in responders than in non-responders. However, this increase was not statistically significant. The blood level of OPN was significantly increased in non-responders (Mean \pm SD=37.21 \pm 3.9) in comparison to responders (Mean \pm SD=33.22 \pm 4.1).

Conclusion: Osteopontin blood level can be considered as a reliable predictor to PEG IFN α 2b plus ribavirin therapy in chronic HCV Saudi Patients.

Keywords: OPN, PEG IFN, HCV, SVR, SNP, RT-PCR.

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Introduction

Infection with hepatitis C virus (HCV) is a common global cause of chronic liver disease, which is also true in Saudi Arabia. HCV prevalence in Saudi Arabia varies in different provinces. The highest of which being the Western and Southern provinces¹. HCV was responsible for 35.4% of chronic liver disease among Saudis, 74.2% among Egyptians, 28.6% among Yemenis and 24% among Palestinians². Patients with post-necrotic liver cirrhosis secondary to HCV are also liable to develop hepa-

tocellular carcinoma (HCC) with an annual rate of 1 to 4%^{3,4,5}.

OPN was shown to be essential for initiation of T helper 1(Th1) immune response at the upstream of IL-18 and IL-12 in a cytokine network. Th1 immune response is involved in the development of inflammation in chronic hepatitis C, and the hepatocytes infected with HCV are eradicated by Th1 response during IFN-based therapies⁶. Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation that has been employed for the prediction of both disease progression⁷ and therapeutic response⁸. Also, SNP was reported to be associated with a virologic response to IFN-based therapy⁹.

The aim of our study was to analyze OPN protein level in the blood of chronic HCV patients under treatment with interferon (responders and non-responders) and

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their value as pre-treatment predictor of responsiveness to treatment. Also, to compare the results with non-infected patients.

Patients and methods:

Patients:

The present study was an observational case-control study. One hundred individuals were included in this study and divided into two groups; 87 naive chronic HCV-infected Saudi patients and 25 healthy Saudi patients. Patients visited out-patient clinics of Hepatology Department, King Abdul-Aziz Hospital, Taif, Saudi Arabia, during the period from September 2013 to December 2013 and were diagnosed as chronically infected HCV patients. The selection and sampling of patients was done before starting treatment with PEG-IFN, then patients received interferon based therapy at a dose of 1.5mg/kg subcutaneously every week plus ribavirin at dose of 600–1200mg/day, according to the patient's body weight, for 48 weeks. All patients and control gave written informed consent to participate in the study.

The control group enrolled in the study comprised of 25 healthy volunteer subjects with no evidence of any chronic medical condition who matched age and sex with patients. Patients that participated in the study fulfilled the following inclusion criteria: age 18–54 years, elevated alanine transaminase (ALT) and aspartate transaminase (AST); within 6 months prior to entry of the study, positive HCV antibodies, detectable HCV-RNA, HCV genotype 4, liver biopsy showing histological evidence of chronic hepatitis without cirrhosis or fibrosis, and they were never previously treated with IFN. Patients that presented with liver cirrhosis or hepatocellular carcinoma, haemoglobin (<11 g/dl), total leucocytes count (<3000/mm³), neutrophil (<1500/mm³), platelets (<100 000/mm³), the presence of anti-nuclear antibodies (titre <1/160), hepatitis B surface antigen seropositivity or auto-immune hepatitis or co-infection with HIV, and the presence of any chronic systemic illness were excluded from this study.

According to response to treatment, patients were classified into two groups: Responders (50 patients) in whom serum HCV-RNA was not detected for 6 months after the discontinuation of IFN-based therapies, they were classified as patients with SVR; and non-responders (37

patients) who received treatment for 6 months and failed to clear the virus as the viral load measurement was assessed by real time PCR. Liver biopsies were evaluated by a single expert pathologist and scored using the Ishak system in separate reports for grading and staging. All the participants in the study were F-0 by Ishak's score.

Methods

Venous blood samples were collected in ethylene diamine tetraacetic acid (EDTA) and centrifuge tubes. The part in the centrifuge tube was incubated at 37°C for 15 minutes, then centrifuged at 3000 rpm for 10 minutes at room temperature. Serum was separated and used for biochemical characterization of HCV specific antibody titers by enzyme-linked immunosorbent assay and enzymatic evaluation of ALT, AST, bilirubin, albumin and complete blood picture. These tests were performed at weeks one, two, four and monthly thereafter during treatment to detect the development of any adverse side effects to the drugs necessitating dose modification, temporary or permanent stoppage of treatment. Markers of Hepatitis virus: hepatitis B surface antigen, anti-HBc and anti-HCV were assessed by routine methods using commercially available assays.

Viral RNA was extracted using viral RNA extraction kit (Qiagen) and stored at - 80°C. HCV-RNA titer was measured before and after treatment by real-time PCR by Quanstudio 3 real time PCR system provided by ThermoFisher scientific. Thyroid function tests (T3, T4 and thyroid stimulating hormone) were performed (using Immulite) to all patients before receiving the treatment. Auto-antibodies (anti-nuclear antibodies and anti-DNA) were performed using Immunofluorescence kits.

Quantitative detection of HCV-RNA: Before treatment Quantitative detection of HCV-RNA in serum was measured routinely at weeks 24 and 48 after treatment and graded into low, moderate and high levels. HCV-RNA was quantitated in all patients' serum using real time polymerase chain reaction (RT-PCR) (Stratagene, LaJolla, CA, USA). According to quantitative PCR value, we can determine the level of viremia and the response to the treatment. Each patient received sub-cutaneous injection of PEG-IFN-a2b, at 1.5 mg/kg body weight once a week, combined with daily oral administration of ribavirin at a dosage that was determined on the basis

of the patient's body weight (600mg for <60 kg, 800mg for 60–80 kg and 1000–1200mg for >80 kg). Successful treatment was ascertained on the basis of SVR, defined as HCV RNA-negative 6 months after cessation of therapy.

Estimation of serum osteopontin protein: Human OPN in serum was measured using enzyme-linked immunosorbent assay kit provided by RayBio (RayBiotech, Inc., NY, USA).

Analysis of SNP in the promoter region of OPN at nt – 443: Genomic DNA was extracted from peripheral blood mononuclear using the QIAamp DNA minikit (Qiagen, USA) following the manufacturer's instructions. SNP in the promoter region of OPN, at nt – 443 was determined by direct sequencing of DNA fragments amplified by PCR.

Extracted genomic DNA was amplified in a 50 ul solution (using both forward and reverse primers) with the oligonucleotide primers determined from the sequence of the promoter region of human OPN as follows: Forward 50-TGTCAGTAGTGCCATTTGT-30 and reverse 50-TGTACCTTGGTCGGCGTTTG-30 . The reaction mixture was kept at 94°C for 3 minutes for the enzyme activation, followed by 35 amplification cycles. Each cycle consisted of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and primer extension at 72°C for 60 seconds. Then, the mixture was incubated at 72C for 7 minutes for final primer extension and the PCR products were checked by DNA agarose gel electrophoresis. The PCR amplification product was detected at 630 bp. Direct sequencing was performed with the use of The Big Dye Terminator mix V 3.1 system and ABI 310 sequencer (ABI, Lincoln Center Drive Foster City, USA).

Statistical analysis of data: Statistical package for social sciences (IBM-SPSS), version 19 IBMChicago, USA was used for statistical data analysis. Data expressed as mean, standard deviation (SD), number and percentage. Mean and standard deviation were used as descriptive value for quantitative data. Student t test was used to compare the means between two groups, and one-way analysis of variance (ANOVA) test was used to compare means of more than two groups. Number and percentages were used to describe qualitative data. Chi square was used to compare percentages between cases and controls. For all these tests, the level of significance (P-value) was considered significant if <0.05.

Informed consent: Written informed consent was obtained from each patient before participating in the study according to the research and ethical committee of Taif university, Taif, Saudi Arabia.

Results

Demographic and clinical features of subjects included in the study: Among 87 patients, there were 52 (59.8%) men and 37 (40.2%) women, aged 40.62 ± 8.56 years (mean \pm SD), with a range of 18 to 54 years in the twenty five subjects control there were 19 (51.35%) males and 18 (48.65%) females with an average age of 33.10 ± 8.16 (mean \pm SD). All the patients were genotype 4a and were treated with PEG-IFN alfa 2-b with a dose of 1.5 μ g/kg weekly and ribavirin (600 – 1200 mg/day) for 48 weeks. All the included patients were compensated and had no evidence of fibrosis as assessed by clinical, laboratory and imaging investigations. The patients were classified into two groups: responders who included 50 patients and non-responders who included 37 patients. All biochemical tests were significantly higher in hepatitis C patients than control group while there was no significant difference observed regarding age and sex (Table 1).

Table 1: Demographic and biochemical characteristics of subjects included in the study

Variables	Studied Groups		P value
	Hepatitis C patients (n=87)	Control (n=25)	
	Mean ± SD	Mean ± SD	
Age	40.62 ± 8.56	35.20 ± 7.35	0.05
Sex			
Males %	52 (59.8%)	15 (60%)	0.3
Females %	35 (40.2%)	10 (40%)	
ALT (U/L)	87.8±58.2	36±8.0	P=0.0001*
AST (U/L)	107±65.8	33.8±7.5	P=0.0001*
Total Bilirubin (mg/dl)	1.43±0.79	0.83±0.3	P=0.0001*
Direct Bilirubin (mg/dl)	0.46±0.28	0.19±0.08	P=0.0001*
Alkaline phosphatase (U/L)	123.5±53.4	48.7±8.6	P=0.0001*
Albumin (g/l)	3.29±0.45	3.93±0.53	P=0.0001*
Alfa fetoprotein (ng/ml)	14.2±8.3	4.7±1.8	P=0.0001*
Prothrombin time (seconds)	13.5±1.7	10.1±0.4	P=0.0001*
Osteopontin protein (ng/ml)	35.21±4.3	24.3±8.8	P=0.0001*

* Indicate a statistical significant difference

According to the response to PEG-IFN- α 2b plus ribavirin combination therapy, patients were divided into responders (n = 50) and non-responders (n = 37). Patients were considered to be responders if they showed clearance of the virus revealed by negative HCV-RNA after six months of PEG-IFN- α 2b plus ribavirin combination therapy. Interestingly, before starting the PEG-IFN- α 2b plus ribavirin combination therapy, responders

and non-responders showed no significant difference as regards sex and age. There were 46 responders and 34 non responders. Responders showed significantly lower levels of ALT, AST, ALP, PT, AFP (P < 0.05) and significantly higher plasma ALB level (P = 0.01) than non-responders. The clinical background features of responders and non-responders before starting therapy were shown in Table (2).

Table 2: Biochemical features between responders and non responders

Variables	Studied Groups		P value
	Responders (n=50)	Non responders (n=37)	
	Mean ± SD	Mean ± SD	
Age	41.4±9.4	42.5±9.8	0.24
Sex			
Male	27 (54%)	19 (51.35%)	
Female	23 (46%)	18 (48.65%)	0.12
ALT (U/L)	70.2±33.5	92.4±29.8	P=0.000*
AST (U/L)	62.7 ±28.9	88.3±37.6	P=0.000*
Total Bilirubin (mg/dl)	0.83±0.33	0.75±0.4	P=0.74
Direct Bilirubin (mg/dl)	0.24±0.21	0.22±0.24	P=0.58
Alkaline phosphatase (U/L)	93.3±9.4	96.2±7.5	P=0.02*
Albumin (g/l)	3.47±0.31	3.63±0.27	P=0.01*
Alfa fetoprotein (ng/ml)	10.5±3.8	10.9±4.2	P=0.06
Prothrombin time (seconds)	13.3±2.6	13.8±1.9	P=0.004*
Osteopontin protein (ng/ml)	34.75±6.4	37.82±4.8	P=0.0001*

* Indicate a statistical significant difference

The genotypes and allele frequencies at the promoter region of osteopontin gene at nt – 443 were significantly different between patients and controls and also between responders and non-responders. In patients who reached

sustained virological response (SVR) the frequency of T/T genotype at nt – 443 was higher than C/C or C/T. Also, the frequency of T allele was increased in responders than in non-responders. (Table 3)

Table 3: Prevalence of single nucleotide polymorphism (-443) in the promoter region of osteopontin gene and allele frequency in patients and control

SNP at nt – 443	Genotypes				Allele frequency		
	C/C	C/T	T/T	P-value	C	T	P-value
Patients	8 (9.2%)	35(40.2%)	44 (50.6%)	0.22*	51 (29.3%)	123 (70.7%)	0.2
Control	4 (16%)	13 (52%)	8 (32%)		21(42%)	29 (58%)	
Responders	4 (8.0%)	20 (40%)	26(52%)	0.65*	28 (28%)	72 (72%)	0.7
Non responders	4 (10.8%)	16 (43.25%)	17 (45.95%)		36 (48.65%)	38 (51.35%)	

* The comparison was made between TT genotype against C/T+C/C assuming a dominant model

Discussion

Hepatitis C virus belongs to the genus *Hepacivirus*, a member of the family *Flaviviridae*. Until recently it was considered to be the only member of this genus¹⁰. The majority of HCV infected patients do not clear the virus and this leads to chronic hepatitis which may progress to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC)^{4,5,11}. The goal of HCV treatment is to cure the virus, which can be done with a combination of drugs. Although the treatment of chronic HCV has now been revolutionized with new drugs, during the time period of conducting the study the treatment protocol of chronic HCV was PEG-IFN in combination with ribavirin. Being expensive, involving severe side effects and requiring long treatment duration, the therapy is creating a huge burden on the country. Predictive factors regarding treatment response are not well established for each individual and do not allow personalizing the treatment. Therefore, accurate prediction of response to the therapeutic regimen is of great interest. So, finding genomic based predictors of pre-treatment response to anti-viral therapy is an essential issue^{12,13}.

This current therapy is inefficient, mainly due to usually late diagnosis and high recurrence rate within the remaining cirrhotic liver after surgical resection. It is a multistep

process involving different genetic alterations that ultimately lead to malignant transformation of hepatocytes. HBV and HCV chronic infections account for 75% of HCCs, whereas non-viral etiologies such as alcohol and genetic or metabolic disorders represent less than 25% of cases. HCV-induced chronic liver injury can lead to progressive fibrosis, cirrhosis and eventually HCC^{4,5,11,14}. HCV-associated HCC has been reported to promote tumor growth and metastasis due to an increased recurrence after liver resection. However, the underlying mechanisms responsible for metastatic spread of HCV-induced HCC are not fully elucidated¹⁵.

Osteopontin (OPN) is a secreted phosphoprotein, originally characterized in malignant-transformed epithelial cells. OPN is associated with tumor metastasis of several tumors and over-expressed in hepatocellular carcinoma (HCC)¹⁶. Importantly, OPN is significantly up-regulated in liver injury, inflammation and HCV-associated HCC. However, the underlying mechanisms of OPN activation and its role in HCV-mediated liver disease pathogenesis are not known¹⁵. Previous studies have demonstrated that OPN is involved in tumor metastasis and has been detected not only in numerous cancers but also in plasma of HCV related HCC patients, suggesting a correlation between high levels of OPN expression and malignant invasion^{16,17,18}.

Osteopontin is shown to be essential for initiation of Th1 immune response at the upstream of IL-18 and IL-12 in a cytokine network. The Th1 immune response is involved in the development of inflammation in chronic hepatitis C, and the hepatocytes infected with HCV are eradicated by Th1 response during IFN-based therapies⁶.

In this study, we analyzed OPN blood levels in chronic HCV patients before treatment with interferon, according to their response to interferon they were either (responders or non-responders). and their value as a pretreatment predictor of responsiveness to treatment. The present study was conducted on 87 patients with chronic hepatitis C who received PEG-IFN alpha-2b plus ribavirin. There were 52 men and 35 women, aged 40.62 ± 8.56 years, with a range of 18 to 54 years. No significant difference was observed as regards to different genotype distribution between them. The results of the current study revealed that, the sustained virological response (SVR) to PEG-IFN alpha-2b plus ribavirin therapy was 60 %. This is comparable with response rate (63%) predicted by Al Ashgar et al.,¹⁹ but is higher compared to that reported by kamal et al.,²⁰ which was 48 %. And is lower compared to that reported by Al Ashgar et al.,²¹ which was (75.6%).

Results of our study concluded that the allele frequencies but not genotypes at the promoter region of osteopontin gene at nt – 443 were significantly different between patients and controls. Frequency of patients who reached sustained virological response (SVR) was increased in patients with T/T at nt – 443 than in those with C/C or C/T. Also the frequency of T allele was increased in responders than in non responders. However, the genotypes and allele distribution showed no statistically significant difference between responders and non responders. In the present study, a clear relationship between baseline of OPN protein level in blood of chronic hepatitis C patients and response to treatment was observed. Lower pre-treatment plasma OPN protein was correlated with better response. This finding is further verified by Huang et al.,²² study which found that OPN concentrations were significantly increased in the HCV individual with extensive fibrosis and inflammation. They provide evidence that the surveillance of plasma OPN concentration may be a non-invasive biomarker for the evaluation of severity of liver damage in HCV-infected subjects. Other previous results suggest that HCV infection induces OPN via altered Ca^{2+} homeostasis in the endoplasmic reticulum and elevation of reactive oxygen species in the mitochondria.

Also, Ca^{2+} mediated mitochondrial dysfunction has been suggested to play an important role in HCV-mediated liver disease pathogenesis²³. In other studies, they observed that the induction of OPN is mediated by the activation of cellular kinases²⁴.

Also, Cheng and Mahato²⁵ study showed that the OPN might be a central pathway of HSC (hepatic satellite cell) activation. HSC is largely responsible for the development of liver inflammation and cirrhosis in the liver by increasing the ECM (extra cellular matrix) deposition in the sub-endothelial space between hepatocytes and endothelial cells. Our results highlight the correlation between plasma OPN concentration in HCV-infected subjects and response to combination treatment (PEG -INF and ribavirin) of chronic hepatitis C patients.

Conclusion

The results of this study proved that osteopontin level but not its SNP at nt – 443 is a good predictor of response to interferon based therapy in Saudi patients with chronic HCV infection.

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Conflict of interest

All the authors declare that there is no conflict of interest regarding publication of this paper.

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