

ORIGINAL ARTICLE

Protein kinase expression as a predictive factor for interferon response in chronic hepatitis C patients



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ABSTRACT

Egypt has the highest prevalence of hepatitis C virus (HCV) worldwide. Currently, combined pegylated interferon and ribavirin therapy are the standard treatment. The biological activity of interferon (IFN) is mediated by the induction of intracellular antiviral proteins, such as 2'-5' oligoadenylate synthetase, and dsRNA-activated protein kinase. IFN-inducible double-stranded RNA-activated protein kinase (PKR) is thought to play a key antiviral role against HCV. Some studies observed that PKR expression was higher in sustained viral responders compared with the non-responders. The PKR is considered as antiviral toward HCV and responsible for IFN's effect against HCV while others have showed that, there were kinetic results indicate that HCV infection is not altered by reduced levels of PKR, indicating that HCV is resistant to the translational inhibitory effects of the phosphorylated forms of PKR. This study was conducted on 50 consecutive patients with chronic HCV infection (CHC) and 20 healthy controls. All the patients were subjected to clinical and laboratory assessment, abdominal ultrasound, and liver biopsy. Determination of PKR gene quantity by using a real time PCR was done at the baseline and at the end of treatment for all patients and controls. Pre-treatment levels of protein kinase gene were significantly higher in responders in comparison with non-responders ($P < 0.001$). It was found that 97.06% of patients who were responding to treatment had the expression of protein kinase gene greater than 2^6 cycle threshold.

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Introduction

Chronic liver disease and hepatocellular carcinoma are major worldwide public health problems in countries with endemically high levels of viral hepatitis (B and C) [1]. Chronic hepatitis C virus (HCV) infection affects more than 170 million persons worldwide and responsible for the development of

liver cirrhosis in many cases [2]. Hepatitis C virus (HCV) is considered the most common etiology of chronic liver disease (CLD) in Egypt, where prevalence of antibodies to HCV (anti-HCV) is approximately 10-fold greater than in the United States and Europe [3]. The prevalence of genotype 4 as the main HCV genotype with different subtypes as well as different virological, biochemical and histopathological responses to treatment in comparison to the other well-known isolated five genotypes; made it an important and interesting task for many researchers to study the interaction between the viral genome and the anti-viral preparations specially IFN which has many preparations and has both an anti-viral as well as an immune-modulator role in combating the virus [4]. As a result of the continuing research for better medications; the development of new and efficient medications remained an important concern of many research institutions in the field of hepatology. Interferon alpha (IFN alpha) has been widely used as therapy for chronic hepatitis C. The attachment of an inert Poly Ethylene Glycol (PEG) molecule to the standard IFN had resulted in the production of long acting IFN which was named Pegylated Interferon (PEG-IFN). Standard treatment with pegylated alpha IFN in combination with the nucleoside analogue ribavirin leads to a sustained virologic response in approximately half of the patients [2]. Although the efficacy of antiviral therapy in chronic hepatitis C has improved since interferon was introduced, nonresponse to this therapy remains common. Several factors have been shown to influence response [5]. The biological activity of interferon (IFN) is mediated by the induction of intracellular antiviral proteins, such as 2'-5' oligoadenylate synthetase, dsRNA-activated protein kinase and MxA protein. Interferon (IFN)-inducible double-stranded RNA-activated protein kinase (PKR) is thought to play a key antiviral role against hepatitis C virus (HCV) [6]. Double-stranded RNA-activated protein kinase (PKR) plays a role in cell defense against virus infection. Ribavirin was able to up-regulate the levels of phosphorylated PKR and phosphorylated eIF2 alpha, leading to suppression of HCV-RNA replication. The molecular mechanisms that regulate PKR function in normally dividing cells are largely unknown. PKR is implicated in controlling HCV replication and mediating interferon-induced antiviral state against HCV replication [7]. Some viruses including hepatitis viruses can evolve various devices to down-regulate PKR and overcome the host defense mechanism against virus replication [8]. In addition to PKR, PKR-independent antiviral pathways are believed to play important roles in cellular defense against HCV replication [9].

Patients and methods

This study was a prospective study. The sample size was 70 subjects divided into two groups. Group I consists of 20 healthy volunteer subjects who matched age and sex with patients. With men-to-women ratio of 11/9, their age ranged from 18 to 47 years. Group II consists of 50 naïve patients to be treated with PEG-IFN-a2b, at a dose of 1.5 µg/kg subcutaneously every week plus ribavirin at a dose of 1000–1200 mg/day, according to the patient's body weight, for 48 weeks, with men-to-women ratio of 34/16; their age ranged from 18 to 60 years. Strict inclusion criteria were set to nullify the effect of confounding variables and further minimize selection bias. The inclusion criteria are adult men or women (18–60 years

old) with proven chronic hepatitis C genotype 4, elevation of aspartate aminotransferase and alanine aminotransferase levels, positive serum HCV-RNA by quantitative PCR, naive patients (not previously treated with any antiviral drugs including IFN, ribavirin, thymosin and lamivudine). The exclusion criteria are decompensated liver disease, histological evidence of hepatic cirrhosis diagnosed by hepatic histopathology, pregnant or nursing female, concomitant hepatic schistosomal infection (excluded by rectal snip and pathologically), alcohol intake, other etiologies of chronic hepatitis (e.g. autoimmune, hepatitis B virus infection and drug-induced liver injury) and presence of any chronic systemic illness.

All the patients were subjected to clinical assessment. Height and weight were determined at baseline and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (weight in kilograms/height in meters).

Laboratory investigations

Liver enzymes including ALT (alanine aminotransferase), AST (aspartate aminotransferase), serum albumin (Alb), serum bilirubin including total bilirubin (T BIL), prothrombin time (PT), complete blood count (CBC), lipid profile and fasting blood sugar were assayed using Beckman CX4 chemistry analyzer (NY, USA, supplied by the Eastern Co. For Eng. & Trade-Giza, Egypt). Alpha fetoprotein (AFP) and viral status were measured using Abbott, Axyam (USA, Supplied by Al Kamal Company Cairo, Egypt).

Molecular tests

Quantitative detection of HCV-RNA in serum by real-time PCR and RNA extraction from lymphocytes for quantitative gene expression of PKR by real-time PCR were performed for all patients and controls.

Quantitation of hepatitis C virus-RNA in serum by real-time PCR RNA was extracted from serum and reverse transcriptase polymerase chain reaction was performed initially and repeated periodically throughout the period of the study (3, 6 and 12 months). HCV-RNA was quantitated in all patients' serum using real-time PCR (Stratagene, LaJolla, CA, USA).

According to quantitative PCR value, we can determine the level of viremia and the response to the treatment.

Quantification of PKR gene expression by real-time PCR PKR gene expression was performed at the beginning of treatment and repeated after 3 months during the study.

Preparation of PBMCs obtained from peripheral blood of all patients and controls were isolated by Ficoll density centrifugation and sedimentation. RNA was extracted from PBMCs cells using QIAamp viral RNA extraction kit (QIAGEN GmbH, Hilden, Germany). Quantification of PKR gene was performed using TaqMan Gene Expression (Applied Biosystems Inc, Foster City, CA, USA). B-actin was used as house-keeping gene (endogenous reference cDNA). Fractional threshold cycles (CT) were expressing the initial concentration of target sequence. Relative mRNA quantification was calculated using the arithmetic formula $2^{-\Delta Ct}$ where, CT was the difference between the CT of a given target cDNA and an

endogenous reference cDNA. Thus, this value yields the amount of the target normalized to an endogenous reference.

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS, USA). Data are expressed as means \pm standard error. The Mann–Whitney, Wilcoxon Signed Ranks, Chi-square–Fisher’s Exact tests were used for the comparisons of proportions. A $p < 0.05$ was considered significant.

Informed consent was obtained from all participating subjects before the study. The study protocol was approved by the ethics committee of the National Hepatology and Tropical Medicine Institute, Cairo, Egypt, and conformed to the ethical guidelines of the 1975 Helsinki Declaration. Its Serial Number was: 7-2009 in date 22-8-2009.

Results

The median value of PKR gene expression in control and group II was 2^{14} and 2^9 , respectively. There was a statistically significant difference between group II and controls ($P < 0.0001$) (Mann–Whitney test). Cases (group II) showed significantly higher level of AST, ALT, Tbil, AFP, PT, Tsh when compared to control group ($P < 0.05$) Table 1. The median level of PKR gene expression at baseline was 2^9 , and after treatment, it was 2^{15} . There was a statistically significant increase in PKR gene expression after treatment (Wilcoxon Signed Ranks test) ($P < 0.0001$) Table 2. Table 3 showed that, there was no statistically significant difference ($P > 0.05$) between male and female or obese and non-obese patients regarding PKR gene expression. Non-obese patients showed significantly higher rate of response than obese ($P < 0.05$) while regarding gender, there was no significant difference

Table 1 Comparison between HCV patients before IFN treatment (gpII) and controls (gpI).

	Control (gpI)			HCV patients Before treatment (gpII)			Z	P-value	Sig.
	P25	Median	P75	P25	Median	P75			
AST	26.00	31.00	37.50	48.50	60.00	70.00	5.57	< 0.0001	HS
ALT	26.00	29.00	35.75	43.50	60.00	68.50	6.13	< 0.0001	HS
T BIL	0.60	0.80	0.90	0.80	1.00	1.20	3.48	0.001	S
D BIL	0.10	0.10	0.20	0.20	0.20	0.30	3.76	< 0.0001	HS
Alb	3.63	3.85	4.00	3.60	3.80	4.00	0.53	0.59	NS
Glucose	90.75	99.50	111.50	90.00	100.00	103.50	1.02	0.30	NS
AFP	4.00	6.00	7.15	5.00	8.00	12.00	2.59	0.009	S
PT	11.00	11.00	12.00	90.00	90.00	100.00	5.06	< 0.0001	HS
TSH	1.93	2.75	3.78	3.10	3.60	4.00	3.21	0.001	S
Creatinine	0.83	1.00	1.10	0.90	1.00	1.00	0.61	0.544	NS
PKR gene	2^{11}	2^{14}	2^{15}	2^4	2^9	2^{11}	4.30	< 0.0001	HS
Hb	10.25	11.00	13.75	10.00	12.00	13.00	0.47	0.63	NS

P: Percentiles, ALT: alanine aminotransferase; AST: aspartate aminotransferase, TBil: total bilirubin, Hb: hemoglobin, Alb: albumin, AFP: alpha fetoprotein, TSH: thyroid stimulating hormone, PKR: Protein kinase gene.

Mann–Whitney test, is used as to compare between the two groups.

Z: It is a value on horizontal axis of standard normal distribution curve, P: probability, HS: high significant, S: significant, NS: non-significant.

Table 2 Comparison between all biochemical parameters in HCV patients before and after IFN treatment.

	HCV patients						Z	P-value	Sig.
	Before treatment (gpII)			After treatment (gpIII)					
	P25	Median	P75	P25	Median	P75			
AST	48.50	60.00	70.00	30.00	38.50	60.00	4.65	< 0.0001	HS
ALT	43.50	60.00	68.50	30.00	35.50	40.00	5.73	< 0.0001	HS
T BIL	0.80	1.00	1.20	0.70	0.80	1.00	2.13	0.03	S
D BIL	0.20	.20	0.30	0.10	0.20	.20	1.87	0.06	NS
Alb	3.60	3.80	4.00	3.40	3.90	4.00	1.66	0.10	NS
Glucose	90.00	100.0	103.5	90.00	100.0	130.00	2.46	0.01	S
AFP	5.00	8.00	12.00	7.75	9.00	10.00	.76	0.45	NS
PT	90.00	90.00	100.0	87.00	90.00	92.00	2.08	0.04	S
TSH	3.10	3.60	4.00	3.00	4.00	5.00	2.53	0.01	S
Fibrosis	3.00	3.00	4.00	2.00	2.00	4.00	1.87	0.06	NS
Creatinine	0.90	1.00	1.00	0.90	1.00	1.20	2.22	0.03	S
PKR	2^4	2^9	2^{11}	2^2	2^{15}	2^{16}	6.12	< 0.0001	HS
Hb	10.00	12.00	13.00	9.00	10.50	11.00	4.63	< 0.0001	HS

P: Percentiles, Wilcoxon Signed Ranks test is used to compare between the HCV patients before and after treatment. ALT: alanine aminotransferase; AST: aspartate aminotransferase, TBil: Total bilirubin, Hb: hemoglobin, Alb: albumin, AFP: alpha fetoprotein, TSH: thyroid stimulating hormone, PKR: Protein kinase gene. Z: it is a value on horizontal axis of standard normal distribution curve, P: probability, HS: high significant, S: significant, NS: non-significant.

Table 3 Comparison between male and female and obesity regarding PKR gene expression.

Before treatment	Male			Female			Z	P-value
	P25	Median	P75	P25	Median	P75		
PKR	2 ⁴	2 ⁸	2 ¹¹	2 ³	2 ¹⁰	2 ¹¹	0.20	0.84
PKR	Obese 2 ³	2 ⁶	2 ¹¹	Non-obese 2 ⁸	2 ¹⁰	2 ¹¹	1.72	0.09

P = Percentiles, Mann–Whitney test is used to compare between male and female before treatment and between obese and non-obese before treatment regarding PKR.

The unit of PKR gene was: CT (cycle threshold), and equation for calculation of PKR gene expression was: $2^{-\Delta ct}$ cycle threshold.

Table 4 Comparison between responders and non-responders.

		Group				P-value
		No response		Response		
		N	%	N	%	
Load of viremia	Low	3	21.4	11	78.6	> 0.05
	Moderate	7	30.4	16	69.6	
	High	6	46.2	7	53.8	
BMI	Non-obese	3	11.5	23	88.5	0.002*
	Obese	13	54.2	11	45.8	
Sex	Female	4	25.0	12	75.0	0.53
	Male	12	35.3	22	64.7	

* Chi-square test–Fisher's Exact test.

Table 5 Comparison between HCV patients who are responders and non-responders to the treatment with INF.

	No response (16)			Response (34)			Z	P-value	Sig.
	P25	Median	P75	P25	Median	P75			
Age* (mean ± SD)	± 7.87	40.50		± 8.30	40.35		0.06	0.95	NS
AST	60.00	69.00	90.00	45.50	50.00	68.50	3.15	0.002	S
Alt	47.00	57.00	67.50	40.00	60.00	68.50	0.40	0.69	NS
T BIL	0.80	1.00	1.43	0.80	1.00	1.20	0.22	0.83	NS
D BIL	0.20	.20	0.30	0.20	.20	.25	0.79	0.43	NS
Alb	3.53	3.60	4.00	3.60	3.80	4.00	0.95	0.34	NS
Glucose	81.25	90.00	103.75	90.00	100.00	103.50	0.97	0.33	NS
AFP	6.00	9.00	14.00	4.00	8.00	12.00	0.47	0.64	NS
PT	90.00	90.00	97.50	90.00	95.00	100.00	1.23	0.22	NS
TSH	3.00	3.40	4.00	3.45	3.70	4.00	1.63	0.10	NS
Fibrosis	3.00	3.00	4.00	3.00	3.00	4.00	0.65	0.51	NS
HIA	5.25	6.00	7.00	5.00	6.00	6.50	1.64	0.10	NS
Creatinine	0.90	1.00	1.15	0.90	0.90	1.00	0.99	0.32	NS
PKR	2 ⁴	2 ⁹	2 ¹¹	2 ²	2 ¹⁵	2 ¹⁶	5.38	< 0.0001	HS
Hb	12.00	13.00	13.75	10.00	12.00	13.00	1.54	0.12	NS

Mann–Whitney test, P = Percentiles. ALT: alanine aminotransferase; AST: aspartate aminotransferase, TBil: Total bilirubin, Hb: hemoglobin, Alb: albumin, AFP: alpha fetoprotein, TSH: thyroid stimulating hormone, PKR: Protein kinase gene .

Z: It is a value on horizontal axis of standard normal distribution curve, P: probability, HS: high significant, S: significant, NS: non-significant.

* Unpaired *t* test

between male and female cases in response to IFN treatment, also there was no significant difference regarding level of viremia and response to interferon treatment ($P > 0.05$) Table 4. At the end of the study, patients were subdivided into responders and non-responders to treatment. Table 5 shows that

responders had significantly higher initial PKR gene expression (2¹⁵) compared to non-responders (2⁹) ($p < 0.0001$).

Responders showed a higher percentage of cases with initial PKR > 2⁶, the number of responder cases was 34(68%) vs 16(32%) in non-responders Table 6. Receiver operating

Table 6 The relationship between the response to IFN treatment and initial PKR gene expression.

		Response		No response		Total	P-value
		N	%	N	%		
PKR	< 2 ⁶	1	6.25	15	93.75	16	< 0.001*
	≥ 2 ⁶	33	97.06	1	2.94		

Patients who had the expression of protein kinase gene greater than or equal to 2⁶ CT showed higher responding percentile to the treatment compared to those who had the expression of protein kinase gene less than 2⁶ CT. So 2⁶ CT was considered the best cut off value to detect the response.

* Fisher's Exact test.

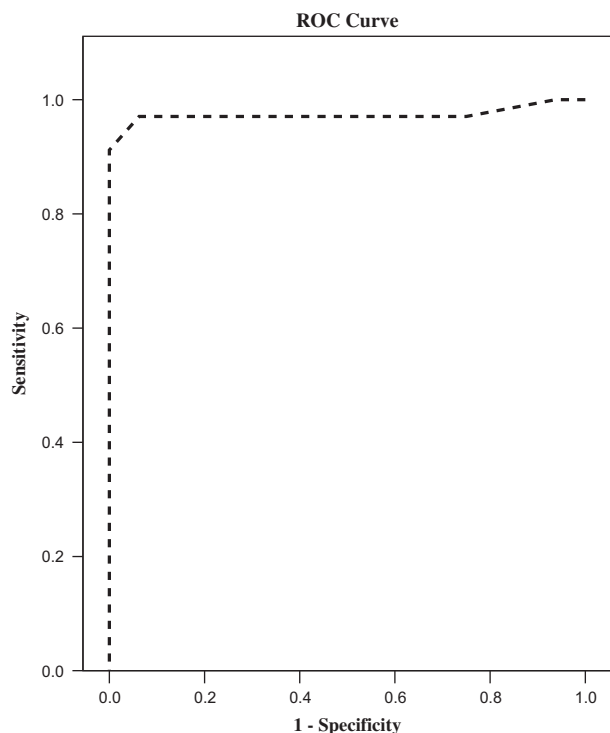


Fig. 1 Receiver operating characteristic (ROC) curve to define the best cutoff value to PKR expression to detect response.

characteristic curve was plotted Fig. 1 to identify the best cut-off point. A value of 2⁶ was the best cut-off point to predict response. So, PKR levels of 2⁶ provided a sensitivity of 97%, a specificity of 94% and a positive predictive value of 97%, negative predictive value of 94% with area under the curve 0.97 and *P* value less than 0.0001. According to these results, PKR gene had a good predictive ability.

Discussion

HCV infects 2–3% of the world population. A majority of infected people fail to clear the virus and are at risk for developing serious liver complications [10]. It was found that HCV accounts for about 20% of cases with acute hepatitis, 70% with chronic hepatitis, 40% with cirrhosis, 60% with hepatocellular carcinoma and 15–30% with liver transplantation [11]. Opportunities for prospective studies are rare because

most infections are asymptomatic [12]. The effectiveness of therapy for patients with chronic hepatitis C has greatly improved in the last few years, [13]. PKR is well-recognized as an important effector of the antiviral response through its ability to arrest protein synthesis and its importance is highlighted by the number of viral and cellular products that are able to abrogate or modulate its action [14]. In the case of HCV, some viral proteins such as NS5A and a cytosolic soluble form of E2 were reported to interact with PKR, and were proposed to be viral inhibitors of the antiviral action of PKR [15]. Gale et al. [16], had a direct proof that NS5A interacts with and inhibits the IFN induced protein kinase, PKR. Importantly, they found that the ISDR was required for NS5A interaction with PKR and repression of PKR activity. These data thus provide the first evidence for the molecular mechanisms underlying HCV resistance to IFN therapy and agree with our results which indicate the effective role of PKR gene against Hepatitis C virus infection in our responder patients who were higher comparing with non-responders regarding to PKR gene expression value.

It has been proposed that mutations in the RNA-dependent protein kinase (PKR) binding domain (PKRBD) within the HCV viral NS5A gene disrupt NS5A-PKR interactions and are important factors contributing to IFN sensitivity and repression of viral function [17]. This present study showed that, there was a statistically significant increase in PKR gene expression in group I (control) when compared with patients (group II) (CHC) at *P* < 0.0001 Table 1. This may be due to the ability of HCV virus to counteract the PKR gene response to viral infection by encoding proteins that inhibit PKR gene function. There was a statistically significant difference (*P* < 0.0001) between responders and non-responders as regards the PKR gene expression 2¹⁵, 2⁹, respectively using Mann–Whitney test. Responders showed a statistically higher value of PKR compared to non-responders Table 5. These results were in agreement with some studies which observed that PKR expression in response to PEG-IFN was higher in sustained viral responders compared with the non-responders [18]. It is considered as antiviral agent towards HCV and responsible for IFN's effect against HCV [6] The results of Chang et al. [19] suggest that PKR is inhibitory to HCV RNA replication which is in agreement with our results indicating that PKR gene high expression leads to high chance for HCV patient response to interferon treatment with ribavirin.

However, the present results disagreed with those of Gar-aigorta and Chisari [20] who have showed that, their kinetic results indicate that HCV infection is not altered by reduced

levels of PKR, indicating that HCV is resistant to the translational inhibitory effects of the phosphorylated forms of PKR and eIF2 α that it induces during infection.

In our study we found that, there was no significant difference between responders and non-responders ($P > 0.05$) regarding alanine transaminase (ALT), albumin (Alb), creatinine, hemoglobin and prothrombin time Table 5 which was in agreement with Ogawa et al. [21]. Regarding the gender, Akram et al. [22] found significantly high SVR rates at p value ($p < 0.01$) in male patients when compared with female patients, while in another study, they found that, female sex decrease the risk of disease progression [23]. On the contrary, we found no significant difference regarding gender in the different responding groups to interferon treatment Table 4. Regarding the comparison between responders and non-responders, George et al. [24] found that there was no significant difference between first and last collected samples in the mean alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), hemoglobin (Hb) which was in contrary with our study in Table 2 where we found that for ALT ($p < 0.0001$), AST ($p < 0.0001$), T BIL ($p = 0.03$), and Hb ($p < 0.0001$). However their results agreed with our results in finding that there was no significant difference in albumin ($p = 0.10$). Our results indicated that, there was significant difference in BMI among responders and non-responders (the number of responder non-obese subjects were 23 and obese subjects were 11 while the non-responder non-obese subjects were only 3 and obese subjects were 13) which was in agreement with Ascione et al. [25], who reported that, overweight and obesity were considered from the pretreatment factors causing a decrease in the sustained virological response (SVR) Table 4.

In this study, it was found that PKR gene expression is perfect and reliable to predict (at $P < 0.0001$), where the receiver operating characteristic (ROC) curve is plotted to determine the best cut-off value of PKR gene expression which is 96 with sensitivity of 96%, specificity 96%, diagnostic accuracy of 96% and area under the curve (AUC) is 99%.

Conclusion

PKR gene expression is a sensitive biological marker for viral replication. These results highlight the importance of the detection of PKR gene expression at the start of therapy as a predictable factor for assessing the likelihood of HCV genotype 4 SVR for treatment with IFN- α 2 in combination with ribavirin.

Conflict of interest

The authors have declared no conflict of interest.

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