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ROLE OF MXA AND NEUTRALIZING ANTIBODIES IN EARLY RESPONSE TO INTERFERON TREATMENT IN HEPATITIS C EGYPTIAN PATIENTS

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ABSTRACT
Pegylated interferon-alpha (PEG-IFN-α) and ribavirin (RBV) treatment does not succeed to attain a sustained virological response (SVR) in about 20-50% of patients with chronic hepatitis C virus (HCV) infection. It has been found that Myxovirus resistance protein A (MxA) gene expression is a reliable and sensitive marker for the presence of exogenous type I interferons (IFNs) during IFN treatment. In addition, considering the involvement of anti-IFN-α binding antibodies (anti-IFN-α2a IgG Abs) and anti-IFN-α neutralizing antibodies (NAbs) on the non-response to treatment with PEG-IFN-α2a/RBV, Fifty-two PEG-IFN-α2a/RBV treated patients and sixteen healthy controls (without Hepatitis C) were enrolled in this study. We examined the correlation between MxA gene expression, anti-IFN-α2a IgG Abs and NAbs and the early response to treatment. Quantification of MxA mRNA performed by real-time PCR showed a statistical significant elevation in the mean value of MxA mRNA in early virological responders (EVR) (4.10±1.54) when compared with both controls (3.19±0.89, $P \leq 0.026$) and non-early virological responders (non-EVRs) (2.56±1.09, $P \leq 0.001$). Anti-IFN-α2a IgG Abs were assessed using indirect ELISA. The percentage of positivity of anti-IFN-α2a IgG antibodies in EVRs was 2/26 (7.7%) and in non-EVRs 4/26 (15.4%). There was no statistical significant difference between the mean values of anti-IFN-α2a IgG antibodies in EVRs (0.46±0.06) and non-EVRs (0.52±0.11), ($p \leq 0.321$).). NAbs were distinguished by means of a neutralizing antibody assay that evaluated the neutralizing effects of serum samples against PEG-IFN-α2a. Serum samples were assessed for the presence of NAbs using rabbit polyclonal antihuman IFN-α2a as a positive control. In the 26 EVRs, no NAbs were detected. Of the 26 non-EVRs, two (7.7%) were positive for NAbs ($p \leq 0.149$)). This study ensured the importance of the detection of MxA expression as a factor for early assessing the probability of HCV genotype 4 patients to respond to treatment with PEG-IFN-α2a/RBV. Also, it showed that antibody production against IFN-α2a in patients getting treatment for chronic HCV infection is not a significant issue in early prediction of response and is not hindering the response to treatment.

KEYWORDS: anti-interferon-α NAb, chronic hepatitis C, non-response, pegylated interferon-α2a, MxA, indirect ELISA, qRT-PCR and neutralizing antibody assay.

INTRODUCTION
Hepatitis C virus (HCV) is one of the main reasons of chronic liver disease around the world.[1] Egypt has a great incidence level of HCV infection; 10% - 20% of the overall inhabitants are infected, HCV is the primary cause of hepatocellular carcinoma (HCC) and chronic liver disease in the Egyptian population, nearly 90% is genotype 4.[2] The management of chronic HCV is centered on using PEG- IFN-α and ribavirin.[3] Forty-eight weeks is the typical period of treatment in patients with genotype 4. Response to IFN differs with viral genotype and grade of fibrosis suggesting that the virus encodes proteins that may directly or indirectly attenuate the antiviral actions of IFN-α. The response of HCV genotype 4 to interferon treatment is lesser than other subtypes.[4,5]

The response of hepatitis C patients taking IFN-α is classified into: (i) Responders (R) (more than 2 log10 reduction in HCV RNA load compared with the baseline or HCV RNA negative) and (ii) Non- responders (NR) display no difference of the HCV RNA before and after treatment. Early virological responders (EVR) are patients attaining an early virological response at week 12, rapid virological responders (RVR) (HCV RNA negative at treatment week 4), sustained virological responders (SVR) are defined as undetectable HCV RNA at 24 weeks after completion of therapy. RVRs have a better chance of maintaining a SVR. Even though a significant percentage of patients with chronic hepatitis

genotypes 4 require 48 weeks of therapy, those with RVR might be treated for 24 weeks. Relapsers are those which HCV RNA decreases and remains below the limit of detection (<50 IU/mL) during treatment but becomes detectable after cessation of treatment.[6][7]

Several elements are associated with non-response: (i) Viral factors: viral load and viral genotypes. (ii) Host factors: steatosis, obesity, insulin resistance, age, male sex and (iii) Molecular mechanisms induced by HCV proteins to inhibit the IFN signaling pathway.[8] Amongst the numerous factors which have been stated in the course of IFN treatment is possibly the development of antibodies to IFN by the patients after a time of IFN use and this may be one of the reasons for the low cure rates other than HCV genotype.[7][9] NAbs are associated with failure of IFN therapy they inhibit the biological activity of IFN by binding to epitope(s) within the active site(s) of the molecule terminating the antiviral and immunomodulating properties of the IFN.[10][11] It was first described in 1981 by Vallbracht *et al.,* who pronounced presence of NAbs in the course of IFN treatment in a patient having nasopharyngeal carcinoma.[12]

There are numerous IFN-stimulated genes (ISGs) that are specifically induced by type I IFNs (IFN- α/β) such as 2′-5′ oligoadenylate synthetase, dsRNA-activated protein kinase and MxA. Consequently, type I IFNs signaling could be demonstrated by the detection of MxA protein. MxA functions as a powerful antiviral protein both *in vitro* and *in vivo*.[13] It is a precise and reliable indicator for IFN-α bioactivity in the course of treatment.[14] The existence of anti-IFN NAbs may be associated with the lack of MxA induction after initiation of IFN-α treatment, i.e; binding of NAbs to active sites on IFN-α blocks the IFN-α signaling pathway and hence induction of MxA is stopped. HCV treatment in Egypt is expensive and genotype 4 is difficult to treat and treatment duration is long. This study is done after 12 weeks trying to find factors or markers for early detection of response to figure out the best treatment regimen for patients and reduce treatment costs. Only limited data are available on the development of NAbs and the expression of MxA gene in CHC genotype 4 patients treated with PEG-IFN-α2a/RBV during early treatment response after 12 weeks. Therefore, the aim of this study is to assess the presence of Anti-IFN-α2a IgG Abs and Anti-IFN-α2a NAbs in chronic hepatitis C (CHC) Egyptian patients treated with PEG-IFN-α2a/RBV for 12 weeks, to evaluate the role of these antibodies in early response to treatment using the obtained data. And to correlate the MxA gene expression with response status of these patients to ensure the value of it as a predictive factor for early response to PEG-IFNα2a in CHC patients. In addition, MxA gene expression could be an indirect way to assess if PEG-IFNα2a function is blocked at molecular level by NAbs.

MATERIALS AND METHODS

1- Patients and samples collection

This study was conducted on fifty-two Egyptian patients (28 males and 24 females) between ages 20 and 60 years; all patients treated for HCV chronic Hepatitis, genotype 4. They had been treated with combined therapy: PEG-IFN-α2a at a dose of 180μg/week, administered subcutaneously, once a week during three months and ribavirin at a dose of 800mg-1200mg/day according to body weight.[15]

Sixteen cases proved negative for HCV by PCR were used as controls. Patients have been identified as EVRs and non-EVRs, depending on the early responsiveness to the treatment. EVRs, the criterion of treatment response was undetectable HCV RNA at week 12 and non-EVRs those were tested detectable RNA at week 12. The inclusion criteria was: adult men or women (20–60 years old) with proven chronic hepatitis C genotype 4, elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, positive serum HCV-RNA by quantitative PCR. The exclusion criteria was: HCV infected patients under the age of 20 and over the age of 60 years, co-infected with human immunodeficiency virus (HIV), autoimmune hepatitis or hemochromatosis, 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 were excluded from the prospective study. Written informed consent obtained from all subjects in this study was approved by the Ethics Committee of Theodor Bilharz Research Institute (TBRI) according to the institutional committee for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland.

From each patient and healthy controls, 2x 5ml venous blood were drawn into two sterile tubes, one containing ethylenediamine-tetraacetic acid (EDTA) which was aliquoted and stored at -20°C and the other sample was drawn in non-additive tube and left to clot at room temperature for 30 min, centrifuged at 2000 rpm at 4°C for 10 min. Serum was removed, aliquoted and stored at -80°C.[16]

2- Measurement of viral load and clinical parameters

Characteristics including age, gender, HCV viral load (baseline and week 12) and liver associated enzymes ALT and AST (baseline and week 12) were done for HCV patients and controls.

HCV RNA extraction was done using Abbott *m*-sample preparation system kit (Abbott Molecular, Inc., Des Plaines, Illinois, USA) and the HCV viral loads in serum were measured by real-time reverse transcription polymerase chain reaction using the Abbott Real-time HCV Amplification Reagent Pack (Abbott Molecular, Inc., Des Plaines, Illinois, USA) according to the manufacturer's instructions and 7500 RT-PCR system

(AB Applied Biosystems, Foster City, California, USA).

3- Quantitation of MxA gene expression by real time PCR

MxA gene expression was quantified after 3 months of patients' treatment and control cases. RNA was extracted from 100µl blood from patients and controls according to manufacturer's instructions using Ambion MagMAX™-96, total RNA Isolation Kit catalog number: AM184 (Applied Biosystems). Quantification of MxA gene was performed using TaqMan, GeneExpression (Applied Biosystems Inc, Foster City, CA, USA). The following protocol has been applied. The used Primers and probes sequences according to Dirk *et al.,* 2007 are shown in Table 1.[17] The following cycling parameters: 55°C for 30 min, 95°C for 10 min and then 40 cycles of 95°C for 15 sec & 56°C for 1min have been used. In parallel glyceraldehydes 3-phosphate dehydrogenase (GAPDH) housekeeping gene quantification was performed for each sample as an internal reference control.[18] Fractional threshold cycles (CT) were expressing the initial concentration of target sequence. Relative mRNA quantification was calculated using the arithmetic formula $2^{-\Delta C}$,[19] where Δ C is the difference between the Ct of MxA mRNA and the endogenous reference (GAPDH). Positivity measured when the values surpassed the means plus three times the standard deviations of 16 negative controls (negative for HCV by PCR).

Table 1: Primers used for q-RT-PCR of MxA and GAPDH.

MxA probe	FAM 5' – CGG CTT GCT TTC ACA GAT GTT TCG ATA AA - 3' TAMRA
MxA forward	ACA CAC CGT GAC GGA TAT GGT
MxA reverse	AAT TTT GGA CTT GGC GGT TCT
GAPDH probe	FAM 5' – CCC ATC ACC ATC TTC CAG GAG CGA G - 3' TAMRA
GAPDH forward	ATT CCA CCC ATG GCA AAT TC
GAPDH reverse	AGC ATC GCC CCA CTT GAT T

4- Detection of anti-IFN-α2a IgG Abs in HCV infected patients using indirect Enzyme linked immunosorbent assay.

After 3 months during this study the indirect ELISA technique has been used to estimate the anti- IFN-α2a IgG Abs in sera of all patients and controls. Controls were used to estimate the cut off value required for determination of positivity and negativity of the tested cases. Samples considered positive for anti-IFN-α2a IgG Abs when their OD492 values exceeded the mean OD492 value of these negative controls plus three times the standard deviations for them. The titers of anti-IFN-α2a IgG Abs in treated patients have been estimated following these criteria**.**

Procedure of indirect ELISA as described by Antonio *et al.,*[20] Polystyrene 96-well ELISA plate (Microtitre®) was coated with 50µl/well of (2 µg/ml) PEG-IFN-α2a (Roche®) as the capture Ag and were incubated overnight at 4°C. Afterward, wells washed 5 times with PBS-containing 0.05% Tween-20 to remove non-binding protein, wells were blocked with 50µl blocking buffer containing 2% bovine serum albumin (BSA) to block the non specific binding sites and incubated at room temperature for 2 hours. After the disposal of the excess BSA, the serum (1ry antibody) samples, diluted 1/100 in blocking buffer, were added and kept overnight at 4°C. Non-binding antibodies were removed by washing 5 times with PBS-containing 0.05% Tween-20, and then 50µl of the 2ry antibody (Goat anti-Human IgG Horseradish peroxidase) diluted in blocking buffer (0.2µg/5ml), was added and kept at room temperature for 1 hour. After washing 5 times with PBS-containing 0.05% Tween-20, 50µl of the chromogenic substance o-phenylenediamine dihydrochloride (OPD) was added and the plate was kept at room temperature for 15 minutes in the dark. 50µl of 3N HCL acid was used to stop the reaction. Absorbance was measured at 492nm using Bio-Tek ELISA reader.

5- *In vitro* detection of anti-IFN-α2a NAbs in chronic Hepatitis C patients using neutralizing antibody assay

The neutralizing antibody assay which has been pronounced before Oberg *et al*., (1989) is a quantitative virus-induced cytopathic effect (CPE) assay, that is useful to detect and quantify antibodies with neutralizing effect.[21] The cell/virus system used in this study was the Vero cells (Adult African green Monkey Kidney epithelial Vero cells) ATCC No.CCL-81, and the Vesicular Stomatitis virus (VSV) ATCC®VR-185™ passage 4. (Both cell stock and virus were kindly obtained from VACSERA, Egypt). Vero cell line was grown in liquid growth medium of Dulbecco's modification of Eagle medium. VSV cultivated in Vero cells stored frozen in MEM Earle's media at -70ºC. Detecting the CPE of VSV on the Vero cell line was seen as morphological changes of cells such as rounding, fusion with adjacent cells and detachment from surfaces to which cells were attached and grown.

The assay principle is that when the serially diluted serum samples are preincubated with optimized concentration of PEG-IFN-α2a and if NAbs do exist, up to certain dilution, the IFN is not capable to bind to its target Vero cells. Thus, cells are not protected and this is seen as CPE to Vero cells by the VSV. The point at which 50% reduction of antiviral activity of the PEG-IFN-α2a by added NAb containing serum is the end point.[36] Rabbit polyclonal anti-human IFN-α2a antibody

(KOMABIOTECH), was used as the positive reference control antibody (figure 2). To choose the optimal PEG-IFN-α2a (Roche®) concentration to be used, three different concentrations of PEG-IFN- α2a 20IU/ml, 86IU/ml and 860IU/ml were preincubated with 2-fold serial dilutions of the rabbit polyclonal anti-human IFN-α antibody for 60 minutes in a polystyrene 96-well tissue culture plate. Then IFN/antibody mixtures were transferred using a multichannel pipette to Vero cells grown in another polystyrene 96-well tissue culture plate (30000cells/well). After incubation at 37°C for 24 hrs, the cells were infected with 10000 pfu of VSV per well. The plate was incubated for 24hrs. Neutralization of antiviral activity was assessed on the basis of the virus induced CPE. Cells were stained with 10µl of 0.5% (wt/vol) crystal violet (5 g/Liter in 70% methanol) for about 1 minute. Cells were washed 3 times with distilled water to remove excess dye. The dye taken up by the viable cells (protected by PEG-IFN-α2a stimulated anti-viral factors) were quantified by elution with 33% acetic acid and its absorbance was measured in an ELISA reader at 540nm. A virus control row was included; each well contained cells and virus only. A cell control row containing Vero cells only was also included. A standard curve was generated from OD540 readings of the 2-fold serial dilutions of the rabbit polyclonal anti-human IFNα2a aganist the neutralizing units/ml for each corresponding antibody dilution.

The neutralizing antibody assay was performed for all serum samples of patients. PEG-IFNα-2a concentration used was 860 IU/ml. Serum samples were serially diluted starting from 1:20 and then 2-fold serial dilution 1:40, 1:80, 1:160, 1:320 and 1:640. The titre of NAb in positive samples was calculated by substituting in the equation obtained from the standard curve with OD540 reading at which 50% reduction of antiviral activity of PEG-IFNα2a was detected.

6- Statistical methods

Results were expressed as mean ± SD or number (%). Comparison between the data [number (%)] in the two groups was done using Chi square test. In ELISA comparison between the incidence of anti-IFN-α2a IgG Abs in treated patients and early virological response in the EVR and non-EVR HCV patients was performed using unpaired t test. Comparison between mean values of MxA mRNA expression in blood samples of the three studied groups was performed using ANOVA test followed by LSD test if significant results were recorded. SPSS computer program (version 16 windows) was used for data analysis. A P value ≤ 0.05 was considered significant.

RESULTS

Clinical data and virological response

A total of 52 patients treated with PEG-IFN-α2a/RBV and 16 control individuals included in this study have physical and clinical characteristics given in Table 2. Determination for HCV-RNA in plasma has been carried out for all patients at the beginning of treatment and at week12. According to the clearance of HCV RNA in plasma, EVRs were characterized by negative HCV-RNA at week 12 after starting the treatment, while non-EVRs were characterized by persistence of positivity of HCV RNA in plasma. Results showed that, 26 (50%) out of 52 patients were EVRs for treatment, while 26 (50%) patients were non-EVRs (Table 2).

As shown in Table 3, no statistical significant difference between EVRs and non-EVRs as regard to age sub-categories ($p \leq 0.351$). As regard to the gender, there was no statistical significant difference in gender distribution among EVRs and non-EVRs groups (($p \leq 0.578$)). There is a significant increase ($p \leq 0.012$) in the abnormal level of AST (>38IU) in non-EVRs [19(73.1%)] when compared with the EVRs [10(38.5%)].

Table 2: Physical and clinical characteristics of HCV patients and healthy controls.

	Controls (n= 16)	EVRs (n= 26)	Non EVRs (n= 26)
Age (yrs.)	36.67 ± 9.16	39.81 ± 10.11	38.65 ± 9.16
Gender (F/M)	9/7	11/15	13/13
ALT(IU/L) week 12	23.0 ± 4.06	40.96 ± 10.28	50.42 ± 12.04
AST(IU/L) week 12	23.6 ± 7.34	39.58 ± 12.12	48.08 ± 16.35
Log HCV PCR week 0	Negative	2.364±1.20	2.38±1.60
Log HCV PCR week 12	Negative	negative	4.4 ± 2.31

Data are expressed as mean ± SD or number. EVRs= early virological responders, non-EVRs= non-early virological responders, n= number of patients, F:female, M:male, ALT: alanine aminotransferase and AST: aspartate aminotransferase.

Table 3: Relation between personal characteristics, laboratory data and the response to combined PEG-IFN-α2a/RBV among patients with chronic HCV

Patients characteristics	EVR (N=26)	Non-EVRs (n= 26)	P-value
Age (yrs.)			
Up to 30 yrs	1 (3.8%)	4 (15.4%) 0.351	0.351
31-50 yrs	22 (84.6%))	20 (76.9%)	
> 50 yrs	3 (11.5%)	2 (7.7%)	
Gender			
Female	11 (42.3%)	13 (50%)	0.578
Male	15 (57.7%)	13 (50%)	
ALT			
Normal level (17-49IU/L)	19 (73.1%)	14 (53.8%)	0.150
Abnormal level (> 49 IU/L	7 (26.9%)	12 (46.2%)	
AST			
Normal level (8-38 IU/L)	16 (61.5%)	7 (26.9%)	0.012*
Abnormal level (> 38 IU/L)	10 (38.5%)	19 (73.1%)	

*Data are expressed as number (percent). *p> 0.05= not significant. p ≤ 0.05= significant.*

MxA mRNA expression in EVR and non-EVR patients.

MxA mRNA expression was quantified using qRT-PCR in HCV patients' samples and controls. Comparison between mean values of MxA mRNA expression in blood samples of controls, EVRs and non-EVRs to PEG-IFN-α2a/RBV treatment showed a statistical significant difference between the three groups ($p \leq 0.001$).

There was a statistical significant elevation in the mean value of MxA mRNA in EVRs (4.10±1.54) when compared with both control (3.19±0.89, $P \leq 0.026$) and non-EVRs (2.56±1.09, ($p \leq 0.001$)). On the other hand, there was no statistical significant difference between control and non- EVRs ($p \leq 0.0115$). table 4. Data in table 5 show the different parameters analyzed for MxA mRNA between EVRs and non-EVRs.

Table 4: Comparison between mean values of MxA mRNA expression in patients samples of controls, EVRs and non- EVRs to PEG-IFN-α2a/RBV therapy.

	Controls (n= 16)	EVRs (n= 26)	Non-EVRs (n= 26)	*P*-value
Mean ± SD	3.19 ± 0.89	4.10 ± 1.54	2.56 ± 1.09	0.001*
P value vs. controls	---	0.026*	0.115	
P value vs. responders	---	---	0.001*	

p> 0.05= not significant.
**p ≤ 0.05= significant.*

Table 5: The different parameters for the validation of MxA mRNA diagnostic test

Parameters	MxA mRNA
Sensitivity	75%
Specificity	83.33%
Cut off value	≥ 5.86
PPV	76.51%
NPV	82.15%

Positive predictive value (PPV), Negative predictive value (NPV).

Anti-IFN-α2a IgG antibodies in EVR and non-EVR patients

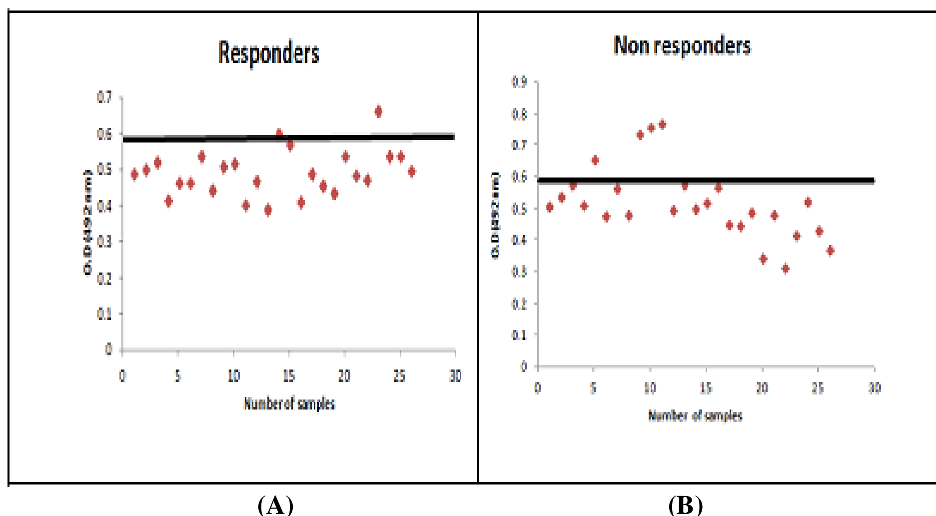
After 3 months of treatment with PEG-IFN-α2a/RBV results showed that, anti-IFN-α2a IgG production was seen in only a few number of patients during the course of treatment, the total positive rate of anti-IFN-α2a IgG was 11.5% (6/52).

According to the cut-off value = 0.59 calculated from mean of OD_{492} of controls ± 3×SD of them, the percentage of positivity of anti IFN-α2a IgG antibodies in EVRs was 2/26 (7.7%) and in non-EVRs was 4/26 (15.4%). They were statistically comparable ($p \leq 0.385$), table 6 and figure 1. There was no statistical significant difference between the mean values of anti-IFN-α2a IgG antibodies in EVRs (0.46±0.06) and non-EVRs (0.52±0.11), ($p \leq 0.321$) (table 7).

Table 6: Percentage of positivity of anti-IFN-α2a IgG antibodies in EVR and non-EVR HCV patients classified according the cutoff value deduced from the control group.

	EVRs (n= 26)	Non-EVRs (n= 26)	*P*-value
Negative (≤ 0.59)	24 (92.3%)	22 (84.6%)	0.385
Positive (> 0.59)	2 (7.7%)	4 (15.4%)	

p> 0.05= not significant.



(A) **(B)**

Fig. 1: Histogram of ELISA OD readings for anti-IFN-α2a Abs. (A) in EVR patients. (B) in non-EVR patients. The bold line at O.D 0.59 is the cut off value.

Table 7: Comparison between mean values of OD492 of anti-IFN-α2a IgG antibodies in EVR and non-EVR HCV patients.

	EVRs (n= 26)	Non-EVRs (n= 26)	*P*-value
Mean ± SD	0.49 ± 0.06	0.52 ± 0.11	0.321

p> 0.05= not significant.

***In vitro* detection of anti IFN-α NAbs using neutralizing antibody assay in EVR and non-EVR patients.**

Results in Figure 2: showed that the addition of PEG IFN-α2a at 20IU/ml and 86IU/ml with 2-fold serially diluted rabbit polyclonal anti-human IFN-α2a resulted in complete loss of antiviral activity in all dilutions, while the addition of PEG IFN-α2a at 860IU/ml with the antibody displayed antibody concentration-dependent loss of antiviral activity, i.e. quantity of PEG IFN-α2a neutralized corresponds directly to the quantity of antibody.

Using the standard curve (PEG IFN-α2a at 860IU/ml), for the total of 52 patients who had received PEG-IFN-α2a/RBV treatment, two out of 52 proved positive for anti-IFN-α NAbs using the neutralizing antibody assay. The percentage of positivity of anti-IFN-α2a NAbs in non-EVRs was [2/26 (7.7%)], while in EVRs it was [0/26 (0%)], ($p \leq 0.149$), table 8. The same was done for the 16 control cases, which showed no NAbs. Results were 138 NU/ml and 152 NU/ml for these two samples, while the rest of serum samples showed no neutralizing effect.

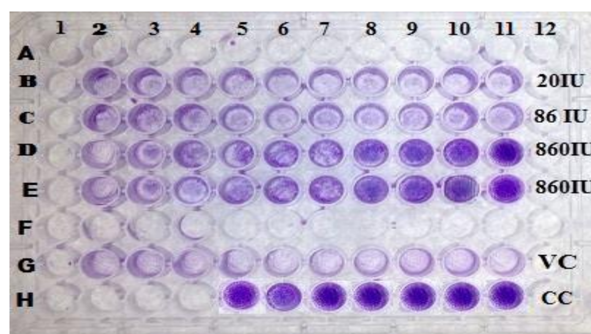


Fig. 2: Neutralizing antibody assay: Photo of determination of the optimum concentration of PEG IFN-α2a used in Neutralizing antibody assay in 96-well tissue culture plate. Two-fold dilutions of rabbit polyclonal anti-human IFN-α2a starting from 200 neutralizing units per ml (NU/ml) (column 2) titrated against three PEG IFN-α2a dilutions: 20IU/ml (row B), 86IU/ml (row C) and 860IU/ml (rows D & E), VC: virus control (row G) and CC: cell control (row H).

Table 8: Percentage of positivity of NAbs to PEG IFN-α2a in HCV patients (EVRs and non- EVRs).

NAbs	EVRs (n= 26)	Non-EVRs (n= 26)	*P*-value
Negative	26 (100.0%)	24 (92.3%)	0.149
Positive	0 (0.0%)	2 (7.7%)	

p> 0.05= not significant.

DISCUSSION

From 2004 until 2011, pegylated-IFN-α plus RBV combination therapy came to be a standard treatment, which provided a SVR in about 40%-50% of the patients with HCV infections.[8] Despite that the curative effect of IFN is not enough high, it remains an effective antiviral treatment for hepatitis C. It interferes with the biosynthesis of HCV. With this course of therapy, patients infected with HCV genotype 1 had SVR rates of about 40% and 50% to 80% SVR rates were accomplished in patients infected with genotype 2, 3, 5 and 6. Lower SVR rates of 40% to 60% were reached in patients with HCV genotype 4.[22]

Egypt represents the world's highest prevalence of HCV infection.[2] Genotype-4a of the virus is the common strain in Egypt. It responds poorly to the PEG-IFN-α2a/RBV combination therapy and had SVR rates of about 40%.[4][5] In late 2014, triple therapy with sofosbuvir (SOF), pegylated interferon (PEG) and ribavirin (RBV) (SOF-PEG-RBV) has presented higher SVR rates in clinical trials that involved HCV-genotype 4 Egyptian patients. But still the real-life results of this therapy for HCV genotype 4 is not known.[23] Moreover, SOF price is a clear obstacle to access to treatment.

As mentioned before, many factors affect non-response.[8] One of the issues concerning non- response to treatment is that, patients receiving IFN therapy may develop NAbs which were reported to be associated with a poor response to treatment.[11] Several studies suggested that anti-IFN-α NAb were more frequently detected in the sera of non-responders than in that of responders.[24] The association between anti-IFN antibodies and non-response to IFN therapy is still controversial. Positive correlations between antibody formation and non-response have been reported,[24][25][26] while negative correlation was reported by others.[27][28] This discrepancy may be explained by differences in IFN preparations used, dosage, route of administration and population's genetics.

Assessment of response after 12 weeks of treatment is used for many years for HCV treatment decision making and defined as early virological response, viral load decline >_2 logs or undetectable HCV-RNA at week 12. The HCV epidemic in Egypt is of a socioeconomic nature. It is mostly prevalent among lower social and economic segments of the population. A 48-week treatment full course with PEG- INFα/RBV from Roche and Schering-Plough cost a patient approximately EGP75,000.[29] Therefore, studying the cases after 12 weeks aimed to exclude factors for non-response and figure out the best treatment regimen for patients and reduce treatment costs. The aim of this study was to determine the incidence of anti-IFN-α2a Abs and the role of anti-IFN-α-2a NAbs in early response to PEG-IFN-α2a/RBV therapy in HCV patients treated for 12 weeks. Also, we aimed to correlate the gene expression of MxA with early response status of these patients, and to evaluate MxA gene expression as a predictive factor for early response in these patients.

In this study, regarding the viral load of CHC patients at week 12 (table 2), 26 patients were EVRs and 26 were non-EVRs. Data from table 3, showed a significant increase in AST level in non-EVRs when compared with EVRs ($p \leq 0.012$). The present MxA mRNA gene expression has been performed on whole blood not PBMCs for simplicity of extraction. Results of MxA gene expression after 12 weeks treatment showed that there was a statistical significant elevation in the mean value of MxA mRNA in EVRs (4.10±1.54) when compared with both control (3.19±0.89, $P \leq 0.026$) and non-EVRs (2.56±1.09, $P \leq 0.001$), (table 4). According to cut-off point for MxA (table 5), the patient will be responder to treatment when the patient has MxA ≥ 5.86. Hamdi and colleagues found that MxA mRNA levels in PBMCs after 12 weeks of therapy of genotype 4 patients is greater in early responders than non early responders.[30] Another study supported these findings it showed that global induction of ISGs in cultured PBMCs was significantly greater in responders than in nonresponders.[31] Other studies by Antonelli *et al.* and Francois *et al*,.[32][33] described a positive correlation between IFN-α induced expression of MxA mRNA in PBMC and the response of patients with chronic HCV infection to IFN-α therapy. Thus, MxA gene expression could be used as predictive factor for early response to IFN treatment as well as final response.

Indirect ELISA Assay with low background has been used for detection of anti-IFN-α2a binding IgG Abs. Results (Figure 1 & table 6) showed that six (11.5%) of the 52 patients developed Abs for PEG IFN- α2a. The incidence of development of these antibodies was 2/26 (7.7%) in EVRs and 4/26 (15.4%) in non- EVRs (p=0.385). Results in (Table 7) showed that, there was no statistical significant difference between the mean values of anti-IFN-α2a IgG antibodies in EVRs (0.46±0.06) and non-EVRs (0.52±0.11), ($p \leq 0.321$). These results are similar to study conducted by Loggie and others (2015),[7] who reported that, 21 patients out of 90 CHC patients (23.3%) tested anti-IFNα-Ab positive at week 12. The prevalence of anti-IFNα-Ab positivity in the present study is almost consistent with that assessed by Halfon and others (2010),[34] who reported that only few non-responders had antibodies to IFN-α-2a, 6/23(26%) at week 12. Also, a study by Wang *et al.,*[35] showed that

little anti-IFN α-2b IgG is produced in early treatment and positive rate was only 5% (5/94).

The data available in literatures by Matsuda *et al.,*[36] and Scagnolari *et al.,*[37] reported that, pegylated preparations of IFNα have lower immunogenicity compared to standard preparations. Although IFN-α 2a has certain antigenicity, anti-IFN-α 2a IgG production is seen in only a few patients during the course of treatment.

Antiviral assay as described before by Oberg *et al*., (1989) is used to distinguish antibodies with neutralizing effects.[21] Vero cells a virus-susceptible cell line was treated with patient serum and PEG- IFNα-2a prior to treatment with the VSV. Cells that were stimulated by the PEG IFNα2a to produce antiviral factors remain viable and were quantified. NAb titres were calculated.[38][39][40]

Results showed that none of the 26 EVRs had developed anti-IFN-α2a NAbs, while anti-IFN-α2a NAbs were detected in two of the 26 non-EVR patients (2/26: 7.7%) ($p \leq 0.149$) table 8. This was in agreement with Huma *et al.* (2007) who reported that of 134 sera checked for interferon antibodies only 17 were found to have titers of over 50 units and of these only five showed no response (6%) and that antibody production with alpha interferon in patients receiving treatment for chronic HCV infection after 3 months treatment is low and is not hindering the response to treatment.[41] Similar to our results a study conducted by Matsuda *et al.,* (2012) reported that in the PEG-IFN-α2b/RBV-treated patients after end of treatment 15% of NR were positive for anti-IFN-α NAbs.[36] Also, Carl *et al.,* (2006) detected anti-IFN-α NAbs in the sera of 3 of 38 (7.9%) chronically HCV infected patients who failed to respond to therapy but none in sera of patients who cleared HCV after IFN therapy.[42] Barone *et al*., (2004), detected that, no significance difference $p>0.05$ between anti-IFN Abs (which developed in only 6/72 (4.3%) non- responding HCV patients but none in sera of responding HCV patients) and response to treatment.[43]

Studies by Van der Eijk *et al.,*(2006) and Santantonio *et al.,*(2006) reported the actual detection of anti-IFN-α NAbs in PEG-IFN-a2a/RBV treated patients.[44][45] Basso *et al.,* (2007) and Poynard *et al.,* (2009) showed an association of NAbs with treatment failure.[46][3]

From our results, there is a positive correlation between Anti-IFNα Abs, NAbs and MxA expression. For the two samples positive using neutralizing antibody assay results were 138 NU/ml and 152 NU/ml, they were also positive using indirect ELISA and OD492nm were 0.768 and 0.736 respectively (According to cut-off point for indirect ELISA (table 6), positivity ≥ 0.59). MxA expression values for these two samples were 1.75 and 1.07 respectively (According to cut-off point for MxA (table 5), negative< 5.86).

Taking these reports together with our results, the NAb-emergence rate appears to be low in PEG-IFN-α2a treated patients. The antigenicity of PEG-IFN-α2a is low this may be attributed to polyethylene glycol, which may hamper the host immune system from recognizing the IFN antigen. We did not find NAb-positive EVRs in our PEG-IFN-α2a/RBV-treated patients. Also, there is a potential of interference by serum factors that could enhance or inhibit the drug's activity resulting in confounding results. IFN is still a safe and effective medicine for the treatment of the patients with CHC.

MxA protein expression is a sensitive biological marker for presence of exogenous type 1 IFN. These results highlight the importance of the detection of MxA expression during therapy as a predictable factor for assessing the likelihood of HCV genotype 4 to acheive a response for treatment with PEG-IFN- α2a/RBV.

It has been suggested that host genetics might be more useful for predicting drug response, as genome association studies have reported associations between IL28B single nucleotide polymorphisms (SNPs) and the response to antiviral treatment in individuals infected with HCV genotype 1,[47][48] and genotype.[49][50][51]

Early prediction of treatment outcome during therapy is of both clinical and economical interest. The present study, showed no association between the response to a 12-week PEG-IFNα-2a/RBV treatment and NAb to PEG IFNα-2a. Thus, the absence of NAbs to PEG-IFN-α2a might not explain the absence of response. It is probable that some cases might have been under reported as that might have produced low level antibodies at that time. For that reason, it would be advisable to collect consecutive samples in few cases and see if values go up each month or are unchanged irrespective of the duration of therapy.

CONCLUSION

The present study shows that antibody production with PEG-IFNα2a in patients receiving treatment for chronic HCV infection is low and is not hampering with the treatment response. In this study single blood sample was taken from each patient after 3 months of initiation of the therapy, therefore it is possible that some cases might have been under reported as that might have produced low level antibodies at that time. It would be worthwhile to collect serial samples in few cases and see if values go up each month or are stable irrespective of the duration of therapy. Also it shows that MxA protein expression is a sensitive biological marker for presence of exogenous type 1 IFN and the importance of the analysis of MxA expression during therapy which would be a practical approach to assess the individual molecular efficacy of the used combination PEG IFN-α2/RBV therapy, especially in genotype 4, which has not been previously investigated on the molecular level and the probability to acheive a response for treatment.

Furthermore, studies using a larger number of patients would be very helpful in confirming our data.

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