

Detection of bcl-2 Translocation in Patients with Chronic Hepatitis C and its Possible Relation to Antiviral Therapy: Preliminary Study

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ABSTRACT

It has been suggested that t(14;18) translocation of bcl-2 to the immunoglobulin heavy chain (IgH) locus may contribute to the pathogenesis of lymphoproliferative disorders (LPD) related to hepatitis C virus (HCV) infection. The present study aimed to assess the prevalence of bcl-2 translocation in Egyptian chronic HCV patients and to investigate the effect of combination antiviral therapy of interferon α and ribavirin on t(14;18). Fifty five chronic HCV patients were studied for the prevalence of t(14;18). These patients were classified into 2 groups, 33 non treated HCV patients and 22 treated HCV patients with antiviral therapy as well as control group of age and sex matched individuals. The bcl-2/IgH rearrangement was detected in peripheral blood mononuclear cells (PBMCs) by nested polymerase chain reaction. All patients have undergone HCV viral determination by real time PCR. Bcl-2/IgH translocation was detected in 21 (38.2%) of all 55 chronically infected HCV patients. Considering all patients with chronic HCV-infection, bcl-2 rearrangement was slightly more frequent in the non treated group than in those who underwent treatment with interferon plus ribavirin but the difference was not statistically significant, although treated patients showed biochemical and virologic response at the end of 6 months of antiviral therapy. In conclusion, t(14;18) in PBMCs is a frequent finding in chronic HCV infection.

Key Words: *Hepatitis C virus – Lymphoproliferative disorder – t(14;18) – Interferon.*

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem worldwide, with a global prevalence of 170 million people affected [1]. Egypt has one of the world's highest prevalences

of HCV infection [2]. Clinical studies in Egypt showed that 70% to 90% of patients with chronic hepatitis, cirrhosis, or hepatocellular carcinoma had HCV infections [3]. Extrahepatic disease manifestations may provide subtle complications of chronic infection, or in turn dominate its course. These extrahepatic manifestations of HCV infection include autoimmune phenomena as well as an association with B-cell lymphoproliferation and non-Hodgkin's lymphoma (NHL) [4].

Rearrangement of bcl-2 has been proposed to be involved in the multistep mechanism of lymphomagenesis [5]. In t(14;18), the bcl-2 protooncogene on chromosome 18q21 is inserted next to the immunoglobulin heavy chain gene (IgH) on chromosome 14q32 by a process frequently involving IgH joining segments. Rearrangement of bcl-2 results in disrupting the IgH locus while markedly increasing transcription of functional bcl-2. The normal role of the bcl-2 protein is to act at the mitochondrial membrane to impair apoptosis [6].

In humans, the B-lymphocyte specific chromosomal rearrangement, t(14;18) is not itself a transforming agent, but is considered an important step in the pathogenesis of certain NHL, being present in 80-90% of follicular lymphomas and some diffuse lymphomas [6]. The t(14;18) may be favored by sustained, strong antigenic stimulation [7], thus it has been suggested that t(14;18) may contribute to the pathogenesis of lymphoproliferative disorders related to hepatitis C virus [8].

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In addition to its being hepatotropic, HCV is also a lymphotropic virus that is able to infect and replicate within peripheral blood mononuclear cells (PBMCs) [7]. HCV contains an RNA genome, which replicates in the cytoplasm, does not contain an obvious oncogene and does not integrate into host genomes [9]. An interaction between genetic and environmental stimuli during lymphomagenesis has been suggested and HCV infection may serve as an environmental stimulus that supports development of lymphomas harboring the *bcl-2* rearrangement [10].

Also, the clonal proliferation of B cells in patients with chronic HCV infection appears to be responsive to antiviral treatment that may prevent or treat HCV-related LPDs. This phenomenon may be related to a direct effect of interferon on the proliferating clone or to an indirect effect by eradicating the antigenic stimulus [11].

Therefore, the present study was conducted to evaluate the prevalence of *bcl-2* rearrangement in peripheral blood mononuclear cells in a sample of Egyptian patients with chronic hepatitis C virus infection compared to healthy control subjects. We aimed to investigate whether an association exists between the presence of t(14;18) translocation and HCV viral detection, alanine aminotransferase (ALT) levels and liver histopathology. Also, the current study aimed to investigate the effect of combination antiviral treatment of interferon α and ribavirin on *bcl-2* translocation in patients chronically infected by HCV.

PATIENTS AND METHODS

Patients:

We studied 55 patients with chronic HCV infection referred to the Tropical Medicine Department, Cairo University. Patients were selected based on their diagnosis of chronic hepatitis C by biochemical liver profile and the presence of positive HCV antibody.

Chronic HCV patients were classified into 2 groups. Group I comprised 33 patients (27 men and 6 women; mean age \pm SD, 41.6 \pm 8.4 years) with chronic hepatitis C and no previous antiviral or immunosuppressive treatment. All patients had circulating anti-HCV antibodies (AxSYM, Abbott Labs), no evidence of other

causes of liver disease, nor evidence of HCV-associated lymphoproliferative disease. None of the patients tested positive for hepatitis B surface antigen (AxSYM, Abbott Labs). A "liver panel" (alanine and aspartate aminotransferases, serum albumin and globulin, bilirubin, alkaline phosphatase and prothrombin time) was performed. Liver biopsy and histopathological examination were done to detect the activity (grade) and fibrosis (stage) of chronic hepatitis according to the Ishak modification of the Knodell score [12,13] to all non treated HCV patients in group I.

Patients of group I were compared with 22 recruited HCV patients (group II) who had received antiviral treatment. Patients (16 men and 6 women; mean age \pm SD, 42.3 \pm 8.0 years) were treated with pegylated interferon α -2a (IFN α) at 180 μ g given subcutaneously once/week plus oral ribavirin (1-1.2g/d according to body weight). Group II were selected at the end of 6 months of antiviral treatment and showing virologic and biochemical response to therapy. Response to antiviral therapy was characterized by normalization of ALT and undetectable serum HCV RNA.

In addition, to evaluate the prevalence of t(14;18) translocation in a healthy, age- and sex-matched population, an additional group of 10 healthy subjects (control group) were analyzed. These subjects had no symptoms or signs suggestive of chronic liver disease, normal biochemical liver profile and negative hepatitis markers for HBV and HCV.

HCV RNA determination and load as well as t(14;18) detection were performed to all non treated chronic HCV patients (group I) and to patients who received antiviral treatment for 6 months (group II). Control subjects underwent t(14;18) detection.

Assessment of HCV RNA load by real time PCR:

Blood samples for HCV RNA load determination were extracted from sera using a Qiagen Viral RNA kit, according to the manufacturer's instructions (Hilden, Germany). Real-time one-step Reverse Transcriptase Polymerase Chain Reaction for detection (RT-PCR) of HCV RNA was performed using a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany). RNA Master Hybridization Probes were

used (LightCycler RNA Master Hybridization Probes Kit; Roche Diagnostics GmbH, Mannheim, Germany).

Amplification primers for HCV were 5' primer K78F (CAAGCACCC TATCAGGCAGT) and 3' primer K80R (AGCGTCTAGC-CATGGCGT). Hybridization probes FL 5'(GCAGCCTCCAGGACCCCC)3' and LC 5'(CCCGGGAGAGCCATAGTGGTCTG)3' were used to detect the product. Reaction mixtures included 7.5µl of Lightcycler RNA Master HybProbe, 3.25mM Mn(OAc)₂, 0.5µM concentration of each primer, 0.4µM of hybridization probe mix and 1µl of the RNA template in a total volume of 20µl. HCV RNA was first reverse-transcribed at 61°C for 20 minutes. Following denaturation for 30 seconds at 95°C, the LightCycler amplification was performed for 45 cycles, each cycle consisting of 5 seconds at 95°C, annealing at 62°C for 15 seconds and extension at 72°C for 10 seconds. Fluorescence was monitored at 530/640nm.

Detection of bcl-2/IgH translocation by nested PCR:

The mononuclear cell fraction was obtained from the peripheral blood by Ficoll-Hypaque centrifugation (Biochrom KG, Berlin). Genomic DNA was extracted from PBMCs according to a GFX Genomic Blood DNA purification Kit (Amersham Biosciences). Polymerase chain reaction was performed according to Gribben et al., [14] with slight modifications by Zuckerman et al., [11]. Each sample was amplified by PCR using nested oligonucleotide primers.

PCR was performed in a 25µl final volume using 0.5µg of DNA, 20nM of oligonucleotide primers and 12.5µl of 2x PCR master mix (Fermentas, Life Sciences) containing 0.6U Taq polymerase, 400nM of each of dNTPs; dATP, dCTP, dGTP and dTTP, in PCR buffer containing 4mM magnesium chloride.

The initial amplification was performed using 5' primer MBRout (CAGCCTTGAAA CATTGATGG) and 3' primer JHout (ACCTGAGGAGACGGTGACCAGGGT). The mixtures were heated at 95°C for 5 minutes followed by 30 cycles of 1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C, with a 5-minute final extension at 72°C. The second PCR amplification was performed under identical con-

ditions using 1µL of first PCR product and oligonucleotide primers internal to the original primers with 5' primer MBRin (TATGGTG-GTTTGACCTTTAG) and 3' primer JHin (GTGACCAGGGTCCCTTGGCCCCAG). All PCR reactions were performed in a thermal cycler. Amplification products were analyzed on 2% agarose gels stained by ethidium bromide.

Statistical analysis:

Data were statistically described in terms of range, mean, standard deviation (\pm SD), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables between the study groups was done using Mann Whitney U test for independent samples while comparison of age was done using Kruskal Wallis analysis of variance (ANOVA) test with posthoc multiple 2-group comparisons. For comparing categorical data, Chi square (χ^2) test was performed. A probability value (*p* value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) statistical program.

RESULTS

We studied 55 patients with chronic HCV infection, 33 of these patients did not receive treatment while 22 had received combination antiviral therapy with interferon α and ribavirin for 6 months. None of these patients had clinical symptoms or signs suggesting lymphoma. Table (1) summarizes the clinical, biochemical and virologic data of the chronic HCV patients in the non treated and treated groups. Both groups were matched with respect to age and gender. Risk factors for HCV infection (blood transfusion, parenteral antischistosomal therapy, injection drug use, surgical or dental procedures) were present in both groups.

The current study verifies bcl-2 rearrangement in patients with chronic HCV infection. The t(14;18) was significantly more prevalent in the 55 chronic HCV infected patients compared to the healthy control subjects (*p*=0.014) as shown in Table (2). The bcl-2 translocation was found in 21 (38.2%) of all chronically infected HCV patients (Fig. 1).

Table (1): Characteristics of non treated and treated hepatitis C virus-infected patients.

Parameter	Non treated HCV (n=33)	Treated HCV (n=22)	<i>p</i> value
<i>Age:</i>			
Mean \pm SD (years)	41.6 \pm 8.4	42.3 \pm 8.3	0.783
<i>Sex:</i>			
Male/female (n)	27/6	16/6	0.146
Abnormal ALT (n)	28	0.0	0.00
ALT mean \pm SD (IU/L)	68.9 \pm 33.6	34.4 \pm 5.9	0.00
HCV RNA detection (n)	28	0	0.00
Mean HCV viral load (copies/ml)	9.1x10 ⁵ \pm 1.8x10 ⁶	Undetectable	
<i>Liver biopsy (HAI):</i>			
Activity (grade); median	6.0	ND	
Range of grade	2-10	ND	
Fibrosis (stage); median	2.0	ND	
Range of stage	0-5	ND	

HAI: Histological activity index.
ND : Not done.

Table (2): Detection of t(14;18) in chronic HCV patients and normal controls.

Parameter	HCV patients (n=55)	Control (n=10)	<i>p</i> value
t(14;18) negative n (%)	34 (61.8)	10 (100.0)	0.014
t(14;18) positive n (%)	21 (38.2)	0 (0.0)	

p value is considered significant if ≤ 0.05 .

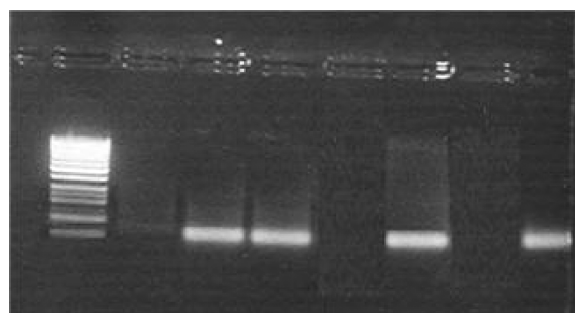


Fig. (1): Detection of t(14;18) translocation by *bcl-2*/IgH nested PCR in peripheral blood mononuclear cells from patients with chronic HCV infection and healthy control. Lane 1, 100bp molecular weight marker. Lanes 2,5,7 HCV patients negative for t(14;18) and lanes 3,4,6,8 HCV patients positive for t(14;18). PCR products were separated on 2% agarose gel.

Thirteen of 33 (39.4%) non treated HCV infected patients had evidence of *bcl-2*/IgH translocation in peripheral blood mononuclear cells (Table 3). This was significantly higher than the rate in the healthy control group ($p=0.047$). Eight of 22 (36.4%) treated patients carried the *bcl-2*/IgH translocation in their leukocytes. Compared to the healthy control group the rate of *bcl-2* translocation in treated HCV patients did not show statistically significant difference ($p=0.078$). No one of the control group had *bcl-2* translocation. Considering all patients with chronic HCV infection, *bcl-2* rearrangement was slightly more frequent in the non treated group than in those who underwent treatment with interferon plus ribavirin but the difference did not show statistical significance ($p=0.821$) (Fig. 2).

Table (3): Detection of t(14;18) in non treated and treated chronic HCV patients and normal controls.

t(14;18)	Non treated HCV (n=33)	Treated HCV (n=22)	Control (n=10)
Number	13	8	0
%	39.4	36.4	0
<i>p</i> value	0.047	0.078	

p value is compared to control group and considered significant if ≤ 0.05 .

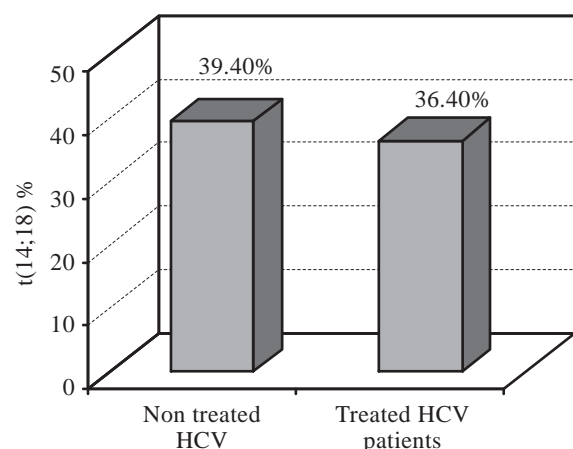


Fig. (2): Comparison between non treated and treated chronic HCV patients as regards t(14;18) percentage (p value=0.821).

In the non treated group of chronic HCV patients ($n=33$), there was no significant difference between the individuals with bcl-2 translocation positive and the bcl-2 negative patients with respect to age, gender, presence of hepatomegaly or splenomegaly, or history of bilharziasis. All liver panel parameters including ALT did not show significant differences between the t(14;18) positive and negative chronic HCV patients. Furthermore, no significant association was found between t(14;18) and the severity of liver disease determined by the histological activity index (HAI).

In the series of HCV infected patients who did not receive antiviral treatment, HCV RNA sequences were detected in the serum from 28 of 33 patients (85.0%) (Table 4). HCV viraemic patients showed mean viral loads of $9.1 \times 10^5 \pm 1.8 \times 10^6$ copies/ml. In the 28 viraemic patients, 11 were positive while 17 were negative for t(14;18). No significant association was found between the presence of bcl-2 translocation and the detection of HCV RNA in the serum ($p=0.976$). Moreover, 2 of the 5 nonviraemic patients had t(14;18).

The group of HCV infected patients who received antiviral treatment by combination therapy with interferon plus ribavirin ($n=22$) was strongly associated with biochemical and virologic response after 6 months of therapy. The treated patients showed complete clearance of serum HCV RNA and normalization of ALT (Table 1). Peripheral blood mononuclear cell samples were collected at the end of 6 months of treatment.

The duration of HCV infection to the detection of t(14;18) translocation could not be determined among patients of both non treated and treated groups as 76.0% of patients were discovered accidentally.

Table (4): Biochemical, virologic and molecular studies t(14;18) of non treated and treated chronic HCV patient

	Non treated HCV (n=33)	Treated HCV (n=22)
Abnormal ALT; n (%)	28 (85)	0 (0)
HCV viral detection; n (%)	28 (85)	0 (0)
t(14;18); n (%)	13 (39.4)	8 (36.3)

DISCUSSION

The mechanism of lymphomagenesis of hepatitis C virus related B cell lymphoma is unknown. Recently, it has been suggested that HCV may induce B cell clonal proliferation and bcl-2 (14;18) translocation in patients chronically infected with the virus. Thus, the present study aimed to assess the prevalence of bcl-2 translocation in Egyptian patients with chronic HCV infection and to investigate the effect of combination antiviral therapy of interferon α and ribavirin on t(14;18). Fifty five chronic HCV patients were studied for the prevalence of t(14;18). These patients were classified into 2 groups, 33 non treated HCV patients and 22 treated HCV patients with antiviral therapy. A control group of age and sex matched individuals was included in the study. The bcl-2/IgH rearrangement was detected in peripheral blood mononuclear cells by nested polymerase chain reaction.

The present study demonstrates a significant prevalence existing of bcl-2 rearrangement (14;18 translocation) in peripheral blood mononuclear cells of patients chronically infected with HCV (38.2%) compared to the healthy control subjects. The results of the current study support that HCV may play a role in predisposing to clonal proliferation and malignant transformation of B cells in patients with chronic HCV infection. However, a slight decrease in the frequency of bcl-2 translocation was observed in treated chronic HCV patients with combined interferon and ribavirin compared to non treated patients but the difference was not statistically significant.

Other studies also have found t(14;18) to be prevalent in HCV patients [15,16]. Zuckerman et al. [17] demonstrated that HCV-infected patients had a significantly higher rate of *bcl-2* rearrangement even in the absence of cryoglobulinaemia. Clonal B lymphocytes were frequently detected in the blood of patients with chronic HCV infection, in the absence of overt B cell malignancy [18]. The frequency of *bcl-2*/IgH translocation in PBMCs from NHL patients with chronic HCV infection was found to be higher than that of other NHL patients [8]. Sasso et al. [6] concluded that the increased prevalence of t(14;18) in HCV patients occurred with a high frequency of JH6 gene usage. In this regard, HCV-associated t(14;18) more closely resembled t(14;18) in lymphomas than t(14;18) from normal subjects.

However, in contrast to our findings, Sansonno et al. [19] failed to demonstrate specific *bcl-2*/IgH amplicons either in liver tissue or in PBMCs in HCV infected patients. Their findings supported the concept that production of IgH gene rearrangements was not associated with *bcl-2*/IgH chromosomal translocation. Also, low frequency of *bcl-2* rearrangement in HCV-associated NHL tissue was reported by Libra et al. [20].

Although t(14;18) presence does not necessarily correspond to malignancy, it represents an important substrate predisposing to lymphomagenesis [21]. *Bcl-2* recombination is interpreted as an error during the VDJ-gene rearrangement process in immunoglobulin genes during sustained B-cell proliferation. B-cell proliferation may take place as a consequence of strong viral antigenic stimulation as well as specific binding of HCV E2 protein to CD81, a B cell surface receptor [22]. The chronic stimulation of the B-cell compartment by HCV may contribute to the higher frequency of *bcl-2* recombination in patients with HCV infection observed in the present study.

No association was found between the presence of *bcl-2* translocation in chronic HCV patients in the present study and age, sex, clinical and biochemical parameters or liver histology. Our findings are in agreement with Franzin et al. [23] and Pozzato et al. [24] who found absence of a correlation between B-cell monoclonality and severity of liver disease, serum levels of

aminotransferases, age and gender as well as HCV genotype.

The findings of the present study may support the role of HCV in inducing clonal proliferation and malignant transformation of B cells in patients with chronic HCV infection. The demonstration by Fiorilli et al. [25] that splenic B cell lymphomas associated with HCV infection may regress after successful antiviral therapy confirms a role for this virus in B-cell lymphomagenesis. Emerging data from other studies suggested that interferon plus ribavirin is an attractive therapeutic option for some HCV-related low-grade lymphomas [21]. Thus, the second major objective of the present study was to investigate if the eradication of HCV by antiviral therapy may result in disappearance of the t(14;18).

The results of the current work showed a trend towards a slight decrease in the rate of *bcl-2* rearrangement in chronic HCV patients treated with combination therapy of interferon α and ribavirin for 6 months compared to the non treated patients but the difference was not statistically significant. However, treated patients demonstrated biochemical and virologic response characterized by normalization of ALT and disappearance of serum HCV RNA.

Our findings are in accord with Saadoun et al. [26] who observed that monoclonal immunoglobulin gene rearrangement persisted after antiviral treatment of splenic lymphoma with villous lymphocytes in patients with HCV regardless of the hematologic and virologic response although the majority of their patients achieved a sustained complete hematologic response after clearance of HCV RNA.

However, the effects of antiviral therapy on t(14;18) have been different in studies by Zuckerman et al. [11] and Giannelli et al. [27]. Zuckerman et al., [11] demonstrated the disappearance of t(14;18) in 6 of 7 treated patients that was strongly associated with virologic response to treatment. The close association between the virologic response and the loss of B-cell monoclonality and t(14;18) indicated that the more effective the antiviral therapy is, the more likely is the loss of B-cell clonality. In addition, translocated B lymphocyte clones have been shown to reappear after virologic relapse at the end of treatment [22]. Giannelli et al. [27] evaluated the

effects of interferon and ribavirin on t(14;18) in 30 HCV positive, t(14;18) positive patients. At the end of treatment, t(14;18) was no longer detected in 50% of patients with complete or partial virologic response, whereas it was persistently detected in non responders.

In this respect, data obtained by Giannini et al. [28] may explain the findings of the present study as regards the absence of a significant difference in the frequency of t(14;18) in treated HCV patients compared to non treated patients in spite of biochemical and virologic response at the end of 6 months of treatment. They analyzed HCV positive patients who showed sustained virologic response after treatment. An extensive follow-up showed persistent HCV RNA negativity both in serum and liver samples. In contrast, PBMCs were HCV RNA positive in 55% of the cases. Interestingly, isolated lymphatic infection was strictly associated with the persistence of t(14;18) bearing B cell clones [28]. Thus, these cells could represent a reservoir of virus and may play a major role in viral persistence. The persistence of HCV in PBMCs may result in chronic stimulation of B cells, favoring bcl-2 rearrangement in the present study in spite of HCV negativity in the serum after 6 months of treatment and suggesting the need for detection of HCV in PBMCs as a possible marker of virologic response.

Another explanation for the discrepancy between our findings and those of other studies [11,27] of t(14;18) in treated patients compared to non treated HCV patients may be due to the different HCV genotype prevalent in Egypt. Egypt has one of the world's highest prevalences of HCV infection, with a majority of genotype 4 infections [2]. Genomic variability of HCV may influence both the efficacy of the host immune response and the interferon treatment response [29]. Also, it has been observed that lymphotropism of HCV varies with different viral strains [30]. An additional elucidation of the discrepant results could be that the treated patients in the present study were recruited at the end of 6 months of therapy compared to 1 year treatment protocols of other studies [11].

In conclusion, t(14;18) in lymphoid cells is a frequent finding in chronic HCV infection. These data are in agreement with the hypothesis that t(14;18) may be involved in the complex multistep mechanisms leading from chronic

HCV infection to B-cell lymphoproliferative diseases. However, antiviral therapy for 6 months did not significantly decrease the frequency of t(14;18) compared to non treated HCV patients in spite of biochemical and virologic response to therapy. Further investigations in large scale studies are needed to establish whether t(14;18) in PBMCs could be used as a noninvasive marker of an increased risk to develop B-cell LPDs in patients with HCV infection. Also, additional studies are recommended to evaluate the effect of extended duration of therapy on t(14;18) in HCV infection and to assess HCV RNA sequence detection in PBMCs as a marker of response to antiviral therapy.

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