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Associations Between Human TRIM22 Gene Expression and the Response to Combination Therapy with Peg-IFNα-2a and Ribavirin in Iranian Patients with Chronic Hepatitis C

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Interferons are able to exert an antiviral effect against hepatitis C virus (HCV) infection via induction of interferon-stimulated genes (ISGs). This study tested whether differential expression of an important ISG with antiviral properties, tripartite motif 22 (TRIM22), correlates with a response to Peg-IFNα-2a/RBV combination therapy in treatment-naive patients with chronic hepatitis C. A total of 32 patients with chronic hepatitis C were enrolled in this study and received standard Peg-IFNα-2a/RBV combination therapy. HCV viral load was measured during treatment, at the end of treatment, and 6 months later to determine the treatment outcome. Quantitative real-time PCR was used to assess the expression levels of TRIM22 in peripheral blood mononuclear cells (PBMCs) of the patients before antiviral therapy. Of the 32 patients, 26 (81.3%) were males. In this study, there were 16 (50%) individuals with a sustained virologic response (SVR), and a virologic relapse was observed in the remaining half of the subjects. Testing for the presence of genomic HCV RNA in blood during therapy revealed a rapid virologic response (RVR) in 10 (31.2%) and a partial and complete early virologic response (EVR) in 8 (25%) and 24 (75%) of the cases, respectively. TRIM22 mRNA levels were significantly higher in patients with a sustained virologic response than in relapsers ($P = 0.002$) and in patients with a rapid virologic response than in the others ($P = 0.040$). No statistically significant difference was seen in the expression of TRIM22 between patients with a partial early virologic response and a complete early virologic response. This study showed that pretreatment upregulation of TRIM22 may be associated with responsiveness to Peg-IFNα-2a/RBV combination therapy. ***J. Med. Virol. 86:1499–1506, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: hepatitis C virus; pegylated IFNα-2a; ribavirin; tripartite motif 22

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of liver disease-related morbidity and mortality worldwide and represents a major public health problem [Alavian, 2010]. Hepatitis C virus infection leads to chronic liver disease in more than 70% of patients and results later in cirrhosis and hepatocellular carcinoma [Poynard et al., 1997]. To date, one of the approved treatments of chronic hepatitis C is a combination of pegylated interferon α-2a (IFNα-2a) and ribavirin (RBV) therapy. This expensive treatment strategy is successful in approximately 48–88% of cases with different HCV genotypes, and long-term treatment may lead to serious side effects [Fried et al., 2002; Poynard et al., 2003; Alavian et al.,

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2012]. Thus, there is an urgent need to find predictive biomarkers of the response to HCV treatment.

Several lines of evidence suggest that both host and viral factors affect the responsiveness or resistance to HCV antiviral therapy. Well-established viral factors responsible for a response to interferon-based antiviral therapy include viral genotype (response rates for individuals infected with HCV genotypes 2 and 3 are higher than for those with genotype 1), baseline viral load (lower starting HCV RNA levels in blood mean a better response), and HIV infection (individuals with HIV coinfection are less responsive) [Bell et al., 1997; Alberti, 2009]. Host factors influencing the response to interferon-based antiviral therapy include age (people under 40 years of age are more responsive), gender (females appear to respond better than males), ethnicity (Asians tend to be more responsive than Caucasians and African Americans), body weight (lower weight means a better response), liver damage severity (the greater the stage of liver disease and abnormal hepatic transaminases levels, the weaker is the response to antiviral therapy), and single nucleotide polymorphisms around the IL-28B gene. The patients with IL28B major genotype (rs8099917 TT and rs12979860 CC) response better than patients with IL28B minor genotype (rs8099917 TG/GG and rs12979860 CT/TT) [Backus et al., 2007; Asselah et al., 2010; Honda et al., 2010].

Although the exact mechanism of action of interferon (IFN) in HCV patients is still poorly understood, it is thought that IFNs are able to exert an antiviral effect by inducing expression of IFN-stimulated genes (ISGs) that have antiviral properties and boost the host's immune response against the virus [Manns et al., 2007]. Recently, it was shown that many members of the tripartite motif (TRIM) superfamily are upregulated in response to IFNs and exhibit antiviral effects [Ozato et al., 2008]. These proteins are characterized by the presence of a tripartite motif consisting of the ring ("really interesting new gene"), B-Box, and coiled-coil domains (RBCC motif) [Reymond et al., 2001; Nisole et al., 2005]. It has been hypothesized that the TRIM proteins may constitute a new and widespread class of antiviral molecules that restrict replication of a number of viruses [McNab et al., 2011]. There are presently approximately 100 known TRIM genes in the human genome and perhaps the most intensely studied TRIM protein to date is TRIM5α, which has been shown to restrict the replication of many different retroviruses in a species-specific manner [Stremlau et al., 2004; Kaiser et al., 2007]. A recent study showed that TRIM22 is a strongly induced TRIM family molecule in human hepatoma HepG2 cells after treatment with IFN, and this molecule can block the HBV gene expression and replication in cultured cells and in mice [Gao et al., 2009]. In addition, microarray analysis of HCV-infected chimpanzees showed TRIM22 upregulation during viral clearance [Wieland et al., 2004]. Furthermore, TRIM22 is significantly upregulated in patients with mild chronic HCV infection and no fibrosis [Folkers et al., 2011]. Also a recent study on pharmacodynamic effects of pegIFN-α in the liver, showed the superior therapeutic efficacy of pegIFN-α therapy is attributed to sustained overexpression of ISGs in liver-infiltrating immune cells [Dill et al., 2014].

These observations led us to analyze the differential expression of TRIM22 in the samples of peripheral blood mononuclear cells (PBMCs) from treatment-naive chronic hepatitis C patients with different responses to antiviral therapy with Peg-IFNα-2a plus ribavirin.

MATERIALS AND METHODS

Study Patients

The subjects were 32 treatment-naive chronic hepatitis C patients who were referred to the Clinical Department of Baqiyatallah Research Center for Gastroenterology and Liver Disease from March 2010 to June 2013. All the patients had a history of hemophilia or thalassemia. The exclusion criteria included coinfection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV) [Namazee et al., 2012].

The study was approved by the Ethical Committee of Iran University of Medical Sciences, and written informed consent was obtained from all the subjects. Baseline and clinical features of the patients are presented in Table I.

The Treatment Protocol

Subjects were treated with standard combination therapy using pegylated IFNα-2a (Peg-IFNα-2a; Pegasys, Roche, Basel, Switzerland), 180 μg/week, and ribavirin (RBV) (Copegus, Roche), 1,000–1,200 mg/day. The patients infected with HCV genotypes 1 and 4 were treated for 48 weeks, while genotype 3-infected patients were treated for 24 weeks based on standard protocols for HCV treatment [Alavian et al., 2010].

Description of the Response

To determine the end of treatment and post treatment responses, the presence of genomic HCV RNA in blood was evaluated at the end of treatment and 6 months later. According to the presence of viral RNA at both of the aforementioned time points, the cases were categorized into two groups, namely, individuals with a sustained virologic response (SVR) and those who relapsed (virologic relapse). Hepatitis C virus RNA clearance 24 weeks after treatment completion was defined as a sustained virologic response. A virologic relapse occurs when HCV RNA reemerges in a patient who has attained an end-of-treatment response. Also, the HCV RNA responses were evaluated during therapy. Based on these assays, the subjects were classified into three groups.

TABLE I. Demographic, Clinical, and Virological Features of Iranian Patients With Chronic Hepatitis C Before Anti-Viral Therapy With PEG-IFN2α plus Ribavirin

Variables	Patients				
HCV genotype and subtypes	1a	3a	4	Total	*P*. val
No. %	18 (56.2%)	12 (37.5%)	2 (6.2%)	32 (100%)	
Age[a]	31.7 ± 8.8	31.5 ± 6.9	35 ± 0.7	31.8 ± 7.7 (21–53)	0.846
Laboratory parameters					
ALT (IU/L)[a]	43.4 ± 19.3	70.0 ± 84.9	28.0 ± 1.3	52.4 ± 54.5 (9–250)	0.355
AST (IU/L)[a]	40.6 ± 15.5	46.3 ± 40.5	30.0 ± 0.6	42.1 ± 27.0 (15–130)	0.703
HCV Viral Load (IU/ml)[a]	$3.3 \times 10^6 \pm 6.9 \times 10^6$	$4.4 \times 10^5 \pm 3.8 \times 10^5$	$1.4 \times 10^4 \pm 0.8 \times 10^4$	$2 \times 10^6 \pm 5.3 \times 10^6$ ($14140–2 \times 10^7$)	0.314

ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a]Continuous parameters are presented as mean ± standard deviation.

Undetectable HCV RNA in blood at week 4 was defined as a rapid virologic response (RVR); early virologic response was defined as a ≥2 log10 decrease in the HCV RNA level compared with baseline (partial early virologic response); or HCV RNA undetectable during treatment week 12 (complete early virologic response).

Sample Collection and Processing

Approximately 6 ml of peripheral blood was collected from each patient into sterile EDTA-containing vacutainer tubes and then plasma was separated and frozen at −70˚C until use. Peripheral blood mononuclear cells (PBMCs) of the blood samples were isolated using Ficoll (Lympholyte H, Cedarlane, Hornby, Canada) density gradient centrifugation, and after washing with phosphate-buffered saline (PH = 7.3 ± 0.1), the cells were counted and resuspended in 200 μL of RNALater (Ambion, Inc., Austin, TX) and stored at −20˚C.

RNA Extraction and cDNA Synthesis

HCV RNA extraction from plasma samples was performed using the QIAamp viral RNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Total cellular RNA was extracted from approximately $3–5 \times 10^6$ PBMCs isolated from the pretreatment sample using easy-RED™ Total RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Korea) according to the manufacturer's instructions. Concentrations of RNA were determined on a nanodrop spectrophotometer (Thermo Scientific, Wilmington, MA), and samples with a ≥1.90 ratio of optical density A_{260}/A_{280} were used. Synthesis of cDNA was performed using 0.5 μg of total RNA with previously published protocols and reaction conditions [Bokharaei-Salim et al., 2011; Keyvani et al., 2013].

Virologic Testing

Plasma HCV RNA was detected using a qualitative PCR assay. Viral load was measured using the COBAS TaqMan HCV Test, v2.0 (Roche Molecular Diagnostics, Mannheim, Germany) real-time PCR. HCV genotyping was performed using a restriction fragment length polymorphism (RFLP) assay of the 5′ noncoding region (5′-NCR) of the HCV genome as described previously [Pohjanpelto et al., 1996].

Real-Time Polymerase Chain Reaction

This assay was performed on a CFX-96 real-time system (Bio-Rad, Hercules, CA) with the primer sets for TRIM22 as a target gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene [Singh et al., 2011]. A 25 μl PCR reaction mixture consisted of 1 μg cDNA, 12.5 μl Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD, USA), and 20 pmol/μl of each primer. For amplification of TRIM22, the PCR cycling conditions were as follows: 10 min at 95˚C, followed by 40 cycles of 6 sec at 95˚C, 6 sec at 60˚C, and 10 sec at 72˚C. For amplification of GAPDH, the PCR cycling conditions were as follows: 10 min at 95˚C, followed by 40 cycles of 6 sec at 95˚C, 6 sec at 65˚C, and 6 sec at 72˚C. To verify amplification specificity, a melting curve analysis was carried out. Nonspecific products were not observed. TRIM22 mRNA relative expression was normalized to the expression of GAPDH using Q-Gene software [Muller et al., 2002].

Statistical Analysis

All calculations were performed using the SPSS version 16 software (SPSS, Inc., Chicago, IL). Analysis of continuous variables was carried out using the independent-samples *T*-test and the Mann–Whitney *U* test. The χ^2-test was utilized to assess associations between categorical variables. Differences with a *P*-value of ≤0.05 were considered statistically significant.

RESULTS

Thirty-two patients with chronic HCV infection were followed up in this prospective study. Of the 32 subjects, 22 had a history of hemophilia, and the rest

of them were thalassemia patients. The mean age of the patients (male 26, female 6) was 31.8 ± 7.7 years (range 21–53 years). Pretreatment clinical and virological features including ALT, AST, HCV genotype, and HCV viral load are presented in Table I.

In this study, there were 16 (50%) individuals with a sustained virologic response who exhibited HCV RNA clearance 24 weeks after treatment cessation. In addition, the virologic relapse defined as reappearance of HCV RNA after the end of treatment was observed in the remaining half of the subjects. All of the virologic relapsers were male and they had a significantly higher baseline ALT level than did individuals with a sustained virologic response ($P = 0.025$). It was noteworthy that more patients with a relapse (57.1%) had high baseline viral loads (>600,000 IU/ml) compared with subjects with a sustained virologic response (42.9%), but this difference was not statistically significant ($P = 0.476$).

No significant differences were found between the HCV treatment response and age, HCV genotypes, risk groups (hemophilia–thalassemia), and the baseline AST level (Table II).

Testing for genomic HCV RNA during therapy revealed a rapid virologic response in 10 (31.2%) patients. In addition, all of the subjects showed an early virologic response (EVR). A partial early virologic response (pEVR) and a complete early virologic response (cEVR) were observed in 8 (25%) and 24 (75%) subjects respectively. In the sustained virologic response group, 14 (87.5%) patients achieved a complete early virologic response and 2 (12.5%) patients showed a partial early virologic response. Among the relapsers, 10 (62.5%) patients achieved a complete early virologic response and 6 (37.5%) patients showed a partial early virologic response. Notably, 90.9% (n = 20) of the 22 patients with a nonrapid virologic response were males ($P = 0.038$) and had a higher baseline AST level than did the patients in the rapid virologic response group (45.2 ± 32 vs. 35.2 ± 6.9; $P = 0.046$). Of the 22 subjects with a nonrapid virologic response 16 (72.7%) and 6 (27.3%) showed a relapse and a sustained virologic response respectively, and all of the patients with a rapid virologic response yielded a sustained virologic response ($P < 0.001$).

All of the patients with baseline viral loads ≤600,000 IU/ml (n = 18) showed a complete early virologic response. Of the 14 patients with a baseline viral load >600,000 IU/ml 6 (42.9%) subjects attained a complete early virologic response and 8 (57.1%) experienced a partial early virologic response ($P < 0.001$).

In the present study, a quantitative real-time PCR assay was used to assess expression levels of TRIM22 in PBMCs from patients with chronic HCV infection before antiviral therapy with Peg-IFNα-2a/RBV. The Q-Gene software [Muller et al., 2002] was utilized for normalization of the TRIM22 expression to the housekeeping gene GAPDH. Median normalized expression levels of TRIM22 with respect to treatment outcomes were compared using the Mann–Whitney *U* test. TRIM22 mRNA levels were significantly higher in patients with a sustained virologic response than in relapsers (Fig. 1). Similarly, expression of TRIM22 was significantly higher in patients with a rapid virologic response than in those without a rapid virologic response (Fig. 1). No statistically significant difference was seen in the expression of TRIM22 between patients with a partial early virologic response and complete early virologic response.

Although TRIM22 expression was notably higher in patients with HCV genotype 3a in comparison with individuals infected with HCV genotypes 1a and 4, this difference was not statistically significant (Fig. 2). Patients with viral load ≤600,000 IU/ml

TABLE II. Comparison of Baseline Demographic, Clinical, and Virological Characteristics Between Sustained Virological Responders and Relapsers

Patients features	SVR	Relapsers	Total	*P*-value
Number of patients (%)	16 (50%)	16 (50%)	32	—
Age (years)	34.2 ± 8.7	29.5 ± 6.0	31.8 ± 7.7	0.459
Gender (male/female)	10/6	16/0	26/6	0.007[a]
Risk groups				
Hemophilia	12 (54.5%)	10 (45.5%)	22 (68.8%)	0.446
Thalassemia	4 (40%)	6 (60%)	10 (31.2%)	
HCV genotype and subtypes				
1a	8 (44.4%)	10 (55.6%)	18 (56.3%)	0.329
3a	6 (50%)	6 (50%)	12 (37.5%)	
4	2 (100%)	0 (0%)	2 (6.3%)	
RVR/NoRVR	10/6	0/16	10/22	<0.001[a]
EVR (cEVR/pEVR)	14/2	10/6	24/8	0.102
Baseline HCV viral load >600 000 IU/ml No (%)	6 (42.9%)	8 (57.1%)	14 (43.7%)	0.476
Baseline ALT level (IU/L)	44.1 ± 17.6 (25–82)	60.7 ± 75.3 (9–250)	52.4 ± 54.5 (9–250)	0.025[a]
Baseline AST level (IU/L)	38.2 ± 17.2 (15–76)	46 ± 34.4 (18–130)	42.1 ± 27 (15–130)	0.221

SVR, sustained virologic response; RVR, rapid virologic response; EVR, early virologic response; cEVR, complete early virologic response; pEVR, partial early virologic response; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a]Statistically Significant.

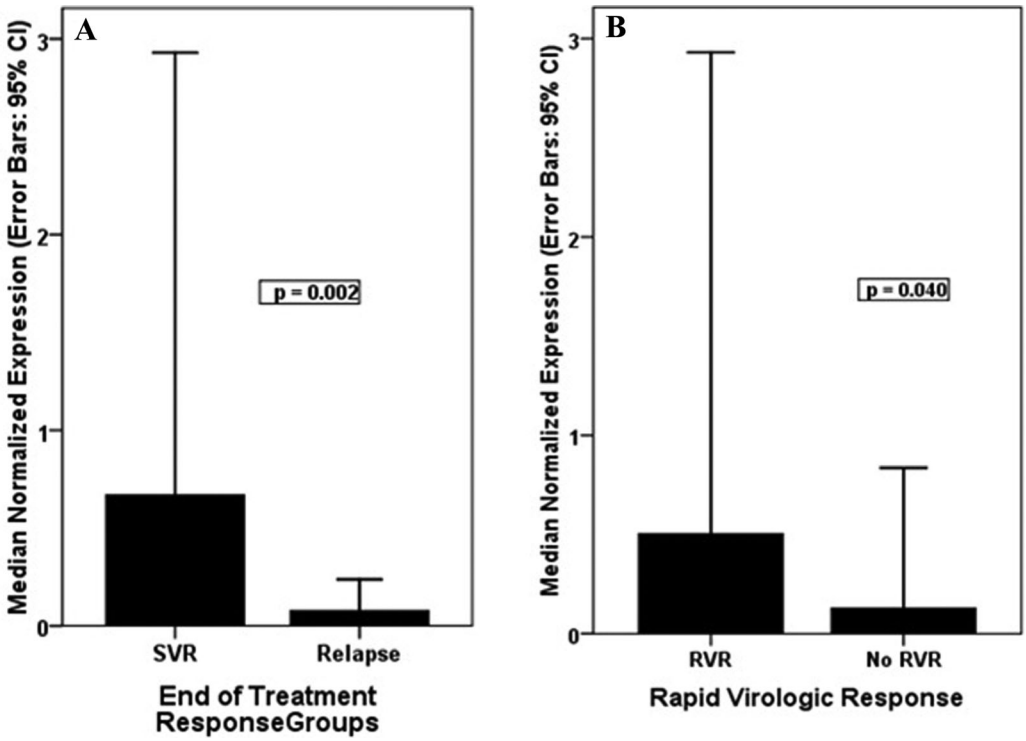

Fig. 1. Human TRIM22 gene expression levels in PBMC samples of patients: (**A**) End of treatment response goups [Sustained virologic response (SVR) vs. Relapse] and (**B**) Rapid virologic response (RVR) groups (RVR vs. no RVR). Error bars indicate standard error. The *P*-values were determined by the Mann–Whitney *U* test.

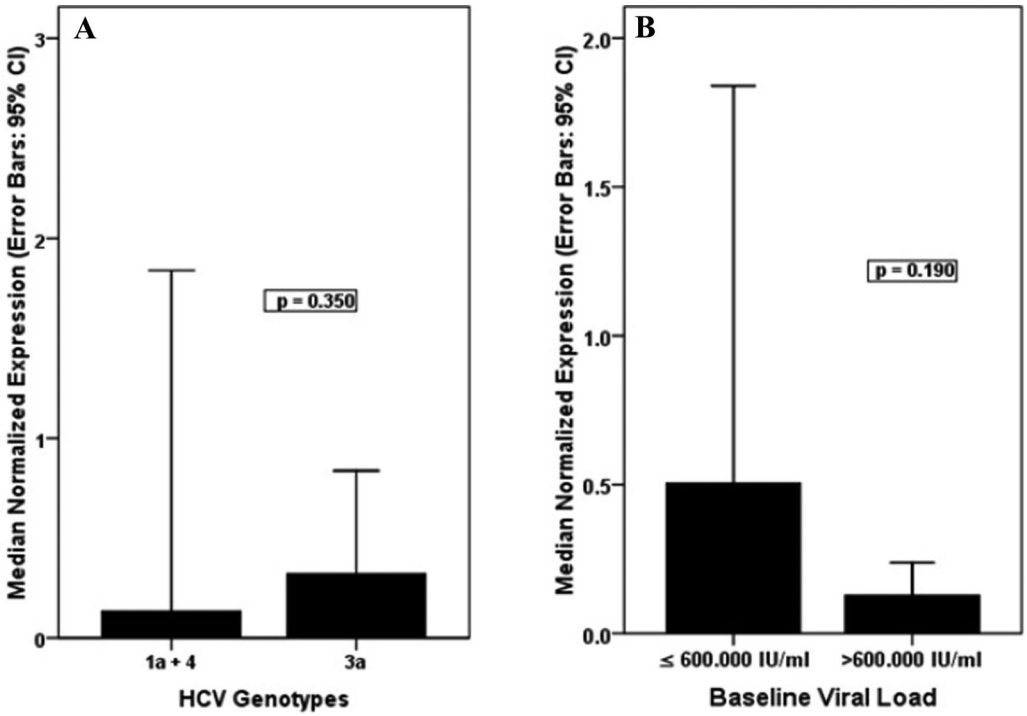

Fig. 2. Association between human TRIM22 gene expression levels in PBMC samples and (**A**) Hepatitis C virus (HCV) genotypes, and baseline HCV viral load. Error bars indicate standard error. The *P*-values were determined by the Mann–Whitney *U* test.

exhibited elevated expression of TRIM22 compared with subjects with >600,000 IU/ml (Fig. 2).

DISCUSSION

Patients with HCV infection who showed sustained response to interferon and ribavirin based antiviral therapy likely reflects differences in the viral and host factors in comparison with relapsers and non responders. On the Host side, genetic variation in IL28B and ISGs expression before and during IFN treatment have been shown to be associated with rates of response to treatment. Members of the tripartite motif (TRIM) genes are one of the ISGs with broad antiviral features. Approximately 100 TRIM genes have been recognized in the human genome and most of them are induced by interferon [Hattlmann et al., 2012]. Several of TRIM proteins including TRIM5α, TRIM19, and TRIM22, have antiviral properties [Kawai and Akira, 2011]. There are a number of studies that reported significant upregulation of TRIM22 in response to interferon in various human tissues [Hattlmann et al., 2012]. To date, antiviral activity of TRIM22 has been demonstrated against several viruses including human immunodeficiency virus type 1 (HIV-1), encephalomyocarditis virus (EMCV), influenza A virus, and hepatitis B virus (HBV) [Di Pietro et al., 2013]. The first evidence of antiviral activity of TRIM22 against HIV-1 was reported in 1995. It was shown that overexpression of TRIM22 restricts HIV-1 transcription from LTR [Tissot and Mechti, 1995].

A recent work demonstrated that expression of TRIM22 and type 1 interferon was concordant in peripheral blood mononuclear cells (PBMCs) of HIV-1–infected patients. Expression of TRIM22 was also associated with remarkably lower viral loads and higher CD4+ T-cell counts [Singh et al., 2011].

It is known that TRIM22 may act as a protective factor for liver against hepatotropic viruses. A recent study indicated that TRIM22 is upregulated by interferon in HepG2 cells and TRIM22 overexpression leads to suppression of HBV replication via core promoter inhibition [Gao et al., 2009]. Furthermore, TRIM22 is strongly upregulated during clearance of HBV and HCV in chimpanzees [Su et al., 2002; Wieland et al., 2004]. Little is known, however, about TRIM22's anti-HCV activity in a clinical context and its possible role as an interferon-stimulated gene (ISG) in response to HCV antiviral therapy. Because of undesirable treatment outcomes in some cases, expensiveness, and side effects of Peg-IFNα-2a/RBV antiviral therapy, identification of predictive markers of a response to antiviral therapy is desirable.

The present study demonstrated that pretreatment of PBMCs from patients who achieved a sustained virologic response resulted in significantly higher TRIM22 mRNA levels compared with patients with a relapse. In addition, a similar significant difference was observed in patients with a rapid virologic response compared with the nonrapid virologic response. Pretreatment upregulation of TRIM22 in both the rapid virologic response group and the sustained virologic response group is consistent with the literature, namely, that a rapid virologic response predicts a high probability of achieving a sustained virologic response [Ghany et al., 2009].

The results of the present study are inconsistent with a number of reports, which indicate that patients with no response to treatment tend to have high levels of pretreatment ISG expression [Feld et al., 2007; Sarasin-Filipowicz et al., 2008; Asselah et al., 2009; Chen et al., 2010]. It should be noted that these studies examined liver biopsies, and TRIM22 was not evaluated.

The direct effects of Peg-IFNα-2a/RBV therapy on TRIM22 expression in hepatocytes are more difficult to study than PBMCs because of the requirement of liver biopsies from patients undergoing standard therapy. Fundamentally, IFN-α exerts its antiviral effect both through the induction of antiviral state in infected hepatocytes and through continuous stimulation of circulating immune cells. Interestingly a study by Dill et al. [Dill et al., 2014] revealed that PegIFN-α induces sustained overexpression of a set of genes involved in cellular immune responses. They proposed a model in which the superior therapeutic efficacy of pegIFN-α is not the result of continuous upregulation of the Jak/STAT pathway in hepatocytes, but rather caused by an indirect mechanism involving the prolonged induction of ISGs in liver nonparenchymal cells including liver-infiltrating immune cells. Also a recent study demonstrated that differential cell-type specific ISG gene expression prior to treatment initiation is associated with treatment outcome. It was reported that ISG15 and MxA expression was more pronounced in macrophages of treatment responders than non-responders [Chen et al., 2010]. These findings might be relevant with TRIM22 expression in PBMCs.

Upregulation of other ISGs in patients with chronic HCV infection compared with a control group has been reported by different investigators [Smith et al., 2003; Honda et al., 2006; Ura et al., 2009]. It has been reported that some ISGs including ISG-16 and ISG-56 can block HCV replication; ISG-16 intensifies the anti-HCV activity of IFNα and ISG-56 inhibits HCV IRES–mediated translation [Wang et al., 2003; Zhu et al., 2003]. Remarkable upregulation of TRIM22 was reported during clearance of HCV and HBV in chimpanzees [Su et al., 2002; Wieland et al., 2004].

The ENCODE tiling array analysis was used to examine differential gene expression in control, mild chronic hepatitis C (no fibrosis), and chronically infected hepatitis C (cirrhotic) human liver biopsies [Folkers et al., 2011]. Their results revealed overexpression of TRIM22 in liver biopsies of both HCV cirrhotic and mild hepatitis C patients compared with control group. Regardless of difference in sample type

(PBMCs versus liver biopsy) these data are in line with the findings of the present study. However to argue TRIM22 role in the kinetic of HCV infection, further experimental validation would be necessary.

Considering the potent inhibitory effects of TRIM22 on transcription of HIV and HBV [Tissot and Mechti, 1995; Gao et al., 2009], examination of the impact of TRIM22 on HCV seems to be worthwhile.

In conclusion, the present study revealed a potential association between pretreatment upregulation of TRIM22 and responsiveness to Peg-IFNα-2a/RBV combination therapy. This study is a preliminary work on the predictive value of baseline TRIM22 expression for response to HCV antiviral therapy. The exact role of TRIM22 in HCV infection is a subject of an upcoming project in our laboratory.

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