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Original article

Interferon lambda-3 polymorphism and response to pegylated interferon in patients with hepatitis D

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Abstract

Background: Specific single nucleotide polymorphisms (SNPs) near the interferon lambda 3 (IFNλ3) gene (formal interleukin 28B), influence the response to treatment with interferon in hepatitis C patients. We aimed to investigate such an influence in hepatitis D patients.

Methods: Study population consisted of hepatitis D patients who were previously treated with pegylated interferon for one year and spontaneous clearers of the virus post recent superinfection. SNP of IFNλ3, rs12979860 was determined by PCR-RFLP protocol.

Results: The total number of patients were 64; median age 30.5 years, male 53. Patients with sustained virologic response one year post-treatment were 17, non-responders 29, relapsers 11, and spontaneous clearers post superinfection 07. Cirrhosis was present in 28 (44%). IFNλ3, rs12979860 genotype CC was present in 41 (64.1%), CT 21 (32.8%), and TT 02 (3.1%). There was no difference in the body mass index, baseline ALT, HBeAg and HBV DNA status among patients with sustained response and response failure (no response or relapse). The median age of response failures was 33.5 years compared to 26 in responders (p=0.024). They had higher GGT levels (p=0.030) and cirrhosis (0.003). Genotype CC was present in 29/40 of response failures compared to 9/17 of the responders (p=0.152). Logistic regression analysis showed that cirrhosis was the independent risk factor for failure to have a response (p=0.001). 4/7 patients with spontaneous clearance had genotype CC.

Conclusions: IFNλ3 rs12979860 SNP does not have any significant influence on the long term hepatitis D clearance. Presence of cirrhosis may influence the response.

Introduction

Genome-wide association studies (GWAS) studies have shown that a single nucleotide polymorphism (SNP) rs12979860 near the interferon lambda-3 (IFNλ3) gene, (formally known as interleukin 28B), on chromosome 19, is associated with variable responses to the interferon therapy in hepatitis C. The CC genotype of IFNλ3 rs12979860 is associated with two to three fold increases in sustained virologic response as compared with either CT or TT genotype [1,2]. In case of hepatitis B, the association of IFN-λ3 polymorphism with response to interferon has not been confirmed [3]. A recent study has shown that IL28-B polymorphisms are related to differing response to pegylated interferon (Peg-IFN) in the treatment of HBeAg-negative chronic hepatitis B, which may also be hepatitis B virus genotype dependent [4].

Although the prevalence of hepatitis D has decreased in Europe, it is still prevalent in many countries of Asia Pacific region including Pakistan [5–7]. Hepatitis D is a difficult-to-treat infection. Interferon, standard or pegylated, is the only treatment option available to hepatitis D patients. Peg-IFN for hepatitis D is of limited efficacy [8,9] and the addition of a nucleoside analog does not improve the response [10]. The agent seems effective in suppressing viral and liver disease activity in some patients, but this improvement is not sustained in the majority of patients.

Not enough data is available on the association between IFNλ3 polymorphisms and hepatitis D virus (HDV) response to Peg-IFN based therapy and spontaneous clearance rate. This study aimed to find out IFNλ3 rs12979860 genotypes in responders, non-responders and relapsers to the Peg-IFN therapy and in spontaneous clearers of hepatitis D infection.

Methods

Patients

Study population consisted of HBsAg and anti-HDV antibody positive patients with compensated liver disease who visited our clinic in a period of two years for whom longitudinal observations were available from the medical records. Patients were considered to have chronic hepatitis D when HDV-RNA was detectable in patients reactive for anti-HDV antibodies for more than six months. Subjects were considered to have achieved spontaneous viral clearance post superinfection if they had two consecutive negative HDV-RNA results six months apart without any antiviral treatment. Patients with detectable HDV RNA who had been previously treated with Peg-IFN for one year, were categorized as responders, non-responders and relapsers to the therapy. Responders were defined as patients who had negative HDV RNA at the end of treatment and one year post treatment.

Informed consent was taken from each patient. Baseline characteristics at the time of commencement of therapy were noted from the records, including age, sex, body mass index (BMI),

HBeAg status, HBV DNA and HDV RNA, qualitative or quantitative, and type and duration of therapy received.

Exclusion criteria were treatment with standard interferon, duration of treatment less than or more than one year, concomitant or previous nucleos (t) ide consumption, co-infection with hepatitis C or HIV, decompensated cirrhosis, alcohol abuse, autoimmune disorders or hepatocellular carcinoma. We also excluded patients where end of treatment and follow up HDV RNA status was not documented. The study was approved by the Ethics Committee of the hospital.

Blood samples were taken for HDV RNA and SNP. SNP of IFNλ3 rs12979860 was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocol as mentioned below. HDV RNA clearance was compared in lambda-3 rs12979860 "CC" with "non CC".

IL28B genotyping

7 mL of blood will be drawn into EDTA (lavender top) tube. Genotyping for the IL-28B rs12979860 C/T polymorphism was performed by polymerase chain reaction based restriction fragment length polymorphism assay. Genomic DNA was extracted from whole blood samples by means of the QIAamp DNA blood mini kit (Qiagen, Milan, Italy) according to manufacturer's instruction. A 242 base pair (bp) product was obtained with the forward primer 5′-GCTTATCGCATACGGCTAGG-3′ and the reverse primer 5′-AGGCTCAGGGTCAATCACAG-3′, previously described [11]. PCR amplification was carried out in a total volume of 10 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, Tween-20 0.01%, 0.2 mM deoxyribonucleotides, 2–4 pmol of each primer, 2.0 mM $MgCl_2$, 0.5 units hot-start Taq DNA polymerase (Promega). Samples containing 10 ng of genomic DNA were subjected to 40 cycles of denaturation (at 95 °C for 30 s), annealing (at 62 °C for 30 s), and elongation (at 72 °C for 30 s) using a Perkin Elmer 9700 thermal cycler. In a total volume of 20 μl, 10 μl of the amplified products were digested with 1 unit of the BstU-I restriction endonuclease (New England Bioloabs, UK) at 60 °C overnight. The fragments digested were, respectively, 135 + 82 + 25 bp for the C allele and 160 + 82 bp for the T allele variant. The fragments were resolved by electrophoresis in 3.5% agarose gel after staining with ethidium bromide. In 5 patients the genomic region encompassing the IL-28B rs12979860 C/T polymorphism was sequenced with results confirming those obtained by the RFLP assay.

HBV DNA

Serum HBV-DNA levels were quantified with the Real Time PCR COBAS AmpliPrep/COBAS TaqMan HBV Test 2.0 (Roche Molecular Systems, NJ, USA). HBsAg, HBeAg, anti-HBs, anti-HBe were detected by the Elecsys instrumental platform (Roche Diagnostics, Italy).

HDV RNA extraction

Viral RNA was extracted from 600μl of the sample (serum) using an automated procedure (Qiasymphony, QIAGEN, Hilden, Germany). The manufacturer's protocol was followed, and the purified RNA was eluted in 60μl of RNase-free water.

Reverse transcription

The reverse transcription reaction (RT) was performed using first strand M-MLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 5μl out of the extracted RNA was added to 15 μl of RT mixture containing RT buffer, dNTP mix (10 mM each of four deoxynucleoside triphosphate stocks), 50 μM random hexamer primer and 75 U of M-MLV reverse transcriptase. The RT reaction mixture was incubated at 37°C for 120 min, and then at 85 °C for 5 min to inactivate the enzyme. The resulting 20 μl of cDNA was kept at −20°C until use.

Statistical analyses

Statistical analyses were performed using SPSS, release 20 (IBM Corporation, Chicago, IL, USA). Categorical variables were expressed as frequencies (%) while continuous variables were presented as mean ± standard deviation or median with 25th-75th percentiles inter-quartile range (IQR). Differences between categorical variables were assessed by means of chi-square or Fisher's exact test, while those between continuous variables were evaluated using the Mann-Whitney U test. Multivariate logistic regression analysis with the stepwise forward selection was performed with p-values of less than 0.05 as the criteria for model inclusion. For this model, continuous variables were expressed as dichotomous using median values.

Results

The total number of patients were 64; mean age 31.8±10.1 years, median 30.5 (range 13-55 and interquartile range 23.2-40.0), male 53, median body mass index (BMI) 22.6 (IQR: 20.1-27.7), alanine aminotransferase (ALT) 80 (IQR: 51-118), gamma glutamyl transferase (GGT) 55 (IQR: 30-100). Patients with sustained virologic response were 17, non-responders, relapsers 11, and spontaneous clearers post recent superinfection 07. Cirrhosis of the liver as evident from histology, ultrasound or clinical examination was present in 28 (44%) and two patient was diabetic. IFNλ3, rs12979860 genotype CC was present in 41 (64.1%), CT 21 (32.8%), and TT 02 (3.1%).

There was no difference in the age, BMI, baseline ALT, HBeAg and HBV DNA status among patients with sustained response one year post-treatment and response failures (no response and relapse) to the treatment (Table 1). The median age of response failures was 33.5 (IQR: 25.7-42.7) years compared to 26 (IQR: 21.0-33.5) in responders (p=0.024). They had higher GGT levels (p=0.030) and cirrhosis (0.003). Genotype CC was present in 16/28 patients who showed end of treatment virologic

response (ETR) compared to 22/29 who could not achieve ETR (p=0.134). These figures were 9/17 for sustained responders versus 29/40 for response failures (0.152) (Figure 1). IFNλ3, rs12979860 SNP was not associated with any clinical parameter (cirrhosis, ALT and GTT levels). Regression analysis showed that cirrhosis was the independent risk factor for failure to have a sustained response at one year of post treatment follow up (p=0.001). 4/7 patients with spontaneous clearance had genotype CC.

Discussion

We could not find any beneficial effects of favorable IFNλ3 genotype CC on treatment response in hepatitis D in spite of substantial evidence reported with hepatitis C. Two recently published papers have discussed the role of IFNλ3 polymorphism in hepatitis D [12,13]. However, any association of this polymorphism with treatment response still remains in debate. The mechanism by which interferon lambda3 genotypes regulates the antiviral response to the interferon alpha remains unclear. Unlike type I interferons, the target cell populations of interferon-lambda is restricted and mainly include epithelial cells and hepatocytes [14]. Like interferon alpha, interferon lambda upregulates interferon stimulated genes (ISGs) by inducing JAK-STAT Pathway. However, interferon lambda binds to a different receptors than interferon alpha [15,16]. Guedj J, et al showed that after initiation of Peg-IFN therapy, a median delay of 9 days was observed with no significant changes in HDV level. Thereafter, HDV declined in a biphasic manner, where a rapid first phase lasting for 25 days was followed by a slower or a plateau second phase. None of the patients with flat second phase in HDV achieved complete viral clearance. The interferon lambda-3 polymorphism (rs12979860) was not associated with kinetic parameters [17].

Genetic analysis of the host may predict which patients are more likely to respond to treatment. IFNλ3 genotypes are only one of many factors that might influence the response to Peg-IFN and should be interpreted in the context of other clinical factors. Other factors that play a role in modulating response to the Peg-IFN therapy, may include HDV and HBV genotypes, viral loads, and baseline liver fibrosis. In our country hepatitis D genotype 1 is prevalent [18] and hepatitis B genotype D is present in more than 90% cases [19]. These genotypes are less responsive to treatment to treatment with interferon. There might be a role of IFNλ3 CC genotype in influencing response to Peg-IFN therapy in hepatitis B genotype D patients [4]. However, it is apparent from our study that even having a favorable IFNλ3 CC polymorphism was not helpful in our hepatitis D patients (figure 1).

Our patients who cleared the virus in response to treatment were younger, had lower GGT levels and were not having clinical cirrhosis. It has been shown previously that age at the time of diagnosis and the presence of cirrhosis are associated with disease progression in hepatitis D [20]. One way of interpreting our data may be that as the degree of fibrosis in liver progresses with time, the chances of clearing virus become less.

This study has some limitations of our study. The study included patients who were treated retrospectively. There may be a selection bias as polymorphism was checked in patients who visited the clinic during the study period, not all treated patients as we did not have preserved samples and took samples as they visited us. The number of patients were small. However, sample size is bigger than other two recent studies [12,13], and we included patients treated with Peg-IFN only, not the standard interferon. Though the study relied on the baseline laboratory results done in the past, the tests were done in a single center. Baseline quantitative PCR and HBsAg levels, and early virological response at week 12 were not available in all patients so these variables could not be analyzed.

Spontaneous clearance of both viruses is seen in patients with HBV and HDV confection, but seven patients with spontaneous clearance in our study were HBsAg positive even before HDV exposure suggesting that these patients most likely had superinfection and then cleared the virus as documented from their records. The number is small to draw any conclusion and the natural comparator group for spontaneous clearers in a larger study would be all those patients who did not clear the virus spontaneously. The long-term follow-up studies indicate that several patients defined as responders after a follow-up of six months relapse in the long run [21]. We defined responders who had undetectable HDV RNA one year after stopping the treatment. There is a possibility that some of the responders would have become relapsers if had followed up for a longer duration. The study cohort does not include any patient who had cleared HBsAg as well. The lack of effect of rs12979860 SNP would not rule out other the role of other SNPs which need to be addressed in future studies.

In conclusion, IFNλ3 rs12979860 SNPs in patients infected with HDV does not have any significant influence on the long term virologic clearance. The degree of fibrosis may influence the response to the Peg-IFN therapy.

Disclosure statement

There are no conflicts of interest to declare relevant to this article.

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Figure 1. IFNλ3 rs12979860, CC or non-CC genotypes, and response to pegylated interferon in patients with hepatitis D.

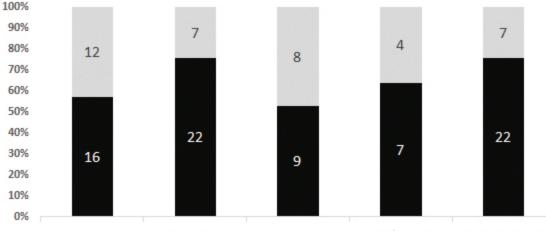ETR: end of treatment virological response; VR-1: virological response persisting one year post-treatment.

Table 1: Characteristics of patients who received pegylated interferon therapy

	Responders N=17	Non-responders and relapsers n=40	P value

Gender
  male
  female
	
	14 (82%)
	03
		
		34 (85%)
		06
			
			1.00

Age
  mean±S.D.
  Median (IQR)
	
	27.2±7.6
	26 (21.0-33.5)
		
		34.0±10.6
		33.5 (25.2-42.7)
			
			0.024*

Body mass index (kg/m^2)
  mean±S.D,
  median (IQR)
	
	24.0±4.7
	23.2 (20.1-28.3)
		
		23.7±5.6
		22.5 (20.0-27.0)
			
			0.711

Cirrhosis	2	22	**0.003***

Alanine aminotransferase (IU/L)
  mean±S.D.
  median (IQR)
	
	147±180
	87 (56.5-137.5)
		
		103±78
		94 (49-118)
			
			0.545

Gamma glutamyl transferase (IU/L)
  mean±S.D.
  median (IQR)
	
	51±39
	36 (30.0-64.5)
		
		103±97
		80.5 (31.7-132.2)
			
			0.030*

HBeAg reactive	4 (24%)	16 (40%)	0.364
HBV DNA detected	3 (18%)	14 (35%)	0.224

Interferon λ3 type
  CC
  non-CC
	
	9 (53%)
	8
		
		29 (73%)
		11
			
			0.152

P values by Fisher exact test or Man-Whitney U test. *statistically significant p values S.D.: standard deviation; IQR: interquartile range

rs12979860 polymorphism

■ CC ■ non-CC

Category	CC	non-CC
ETR	16	12
No ETR	22	7
VR-1	9	8
Relapse	7	4
No response	22	7