T29C genotype polymorphism of estrogen receptor alpha is associated with initial response to interferon-alpha therapy in chronic hepatitis B patients

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BACKGROUND: **Virological clearance, delayed progression to cirrhosis or liver cancer, and increased survival are the long-term goals of antiviral therapy in chronic hepatitis B patients. Identification of host factors correlated with therapeutic response may contribute greatly to individual treatment. This study aimed at investigating whether T29C genotype polymorphism of estrogen receptor alpha (ESR1) is associated with the initial response to interferon-alpha (IFN-α) therapy in chronic hepatitis B patients.**

METHODS: **The initial responses of 100 patients to IFN-α therapy were evaluated and compared by classifying them into three groups according to T29C genotype polymorphism of ESR1: T/T, T/C, and C/C genotype groups. Polymerase chain reaction-restriction fragment length polymorphism was used to analyze the genotype polymorphism in T29C.**

RESULTS: **The frequency of initially combined response was markedly higher in both the T/T and T/C groups than in the C/C group (Z=10.326, P=0.006 and Z=26.247, P=0.000, respectively). In addition, the initial virological response was higher in the T/T and T/C groups than the C/C group (χ^2=5.674, P=0.017 and χ^2=4.980, P=0.026, respectively). In 78 initially HBeAg-positive patients, however, the frequency of initial e-antigen disappearance or seroconversion among the T/T, T/C, and C/C genotype groups was 34.15%, 27.78% and 15.79%, respectively, which were not significantly different.**

CONCLUSION: **The T29C genotype polymorphism of ESR1 is associated with the initial response to IFN-α in patients with chronic hepatitis B, and might be a significant marker for predicting the initial response to IFN-α, at least in this study population.**

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KEY WORDS: **estrogen receptor; polymorphism; chronic hepatitis B; initial response; interferon-alpha**

Introduction

Hepatitis B virus (HBV) infection constitutes a public health menace, and the number of HBV carriers worldwide is estimated to be 350 million. HBV-associated hepatitis, liver cirrhosis, and hepatocellular carcinoma lead to more than one million deaths annually and antiviral therapy plays an important role in prevention through persistent suppression of HBV replication.[1] Interferon-alpha (IFN-α) is one of the currently conventional drugs for anti-HBV treatment. However, some patients become non-responsive to antiviral treatment due to the influence of several factors such as age, gender, alanine aminotransferase (ALT), hepatitis B virus surface antigen (HBsAg) serum level, post-treatment response, HBV DNA level,[2-5] and host genetic factors including HLA-Ⅰ, Ⅱ, and Ⅲ, as well as polymorphisms of A (MxA)-88 and eIF-2a of regulatory region 2.[2, 6-8] Therefore, there is an urgent need to find more factors related to the response to interferon therapy in order to select better individual treatment for chronic hepatitis B (CHB) patients. T29C genotype

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polymorphism of ESR1 is associated with chronic HBV infection[9] and HBV-related hepatocellular carcinoma.[10] Our study aimed to evaluate whether the T29C gene polymorphism of ESR1 is associated with the response to IFN-α therapy in CHB patients.

Methods

Study population

Between June 2008 and February 2009, 100 patients with CHB from clinics and wards in our hospital were prospectively included in this study. Patients were not enrolled if they had ever received nucleoside or nucleotide analogue therapy. All selected patients volunteered to participate in the study and their initial response was evaluated after follow-up for 12 weeks from the beginning of the therapy. Informed consent was given by all patients who took part in the study.

Criteria for diagnosis and antivirotic response

All patients were confirmed according to the criteria in the *2005 Guide on the Prevention and Treatment of Chronic Hepatitis B in China*.[11]

There are several definitions of IFN-α therapy in patients with CHB: (1) initial response: response after 12 weeks of therapy; (2) virological response: serology HBV DNA level undetectable or ⩾lg2 lower than baseline; (3) HBeAg seroconversion: disappearance of HBeAg and seroconversion to anti-HBe; and (4) combined response, which is divided into complete response (meeting the standard of serological response, virological response, and normalization of serum ALT levels at the same time in patients with HBeAg-positive CHB, or normalization of serum ALT levels and virological response at the same time in patients with HBeAg-negative CHB), partial response (between complete response and no response), and no response (patients who show none of the above responses).[12]

DNA extraction

From each patient before a meal, venous blood was drawn into vacuum tubes containing EDTA and stored at -20 ℃. Genomic DNA was extracted within one week using the DNA Extract kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. HBV serologic markers were detected by ELISA (Kehua Biotech Co., Shanghai, China), HBV-DNA was detected by the quantitative polymerase chain reaction (PCR-fluorescence probing) (Da'an Biotechnology, Guangzhou, China), and liver function was assessed by a Modular-DPP automatic biochemical analyzer (Roche, Switzerland).

Genotyping

T29C genotype polymorphisms of ESR1 were characterized using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).[13] PCR was performed in a volume of 25 μl containing 0.5 μl Tap, 0.5 μl sense primer, 0.5 μl anti-sense primer, 0.5 μl dNTP, 2.5 μl 10×PCR buffer, 2.5 μl DNA template, and 18 μl dH_2O (Promega, USA). The sense primer (5'-GAC CAT GAC CCT CCA CAC CAA AGG ATC-3') and antisense primer (5'-ACC GTA GAC CTG CGC GTT G-3') were synthesized by Invitrogen Co., Shanghai, China. For PCR amplification, an initial denaturation at 94 ℃ for 5 minutes was followed by 35 cycles at 94 ℃ for 30 seconds, 61 ℃ for 40 seconds, and 72 ℃ for 30 seconds, and a final extension at 72 ℃ for 5 minutes. The PCR product of 5 μl was digested for 1-2 hours with 1 μl of BamHⅠ (TaKaRa Biotechnology) at 30 ℃ according to the manufacturer's instructions and then separated on 3% agarose gels. RFLP bands were visualized under UV light with ethidium bromide staining. Digested amplicons from the C/C genotype appeared as 197bp bands on agarose gel electrophoresis, while the T/T allele appeared as a 220bp band. The T/C genotype had both of these bands[13] (Fig.). To confirm the genotyping results, three genotypes of T29C were selected and PCR-amplified DNA samples were examined by DNA sequencing. DNA sequences of the PCR products were determined using the PCR sense primer with a GeneAmp PCR System 9700 (ABI, USA). The results of genotyping were 100% concordant.

Statistical analysis

Quantitative data were expressed as mean±SD. Statistical analysis was conducted using SPSS 10.0 (Chicago, IL). Genotypes were compared by using the Chi-square test or Fisher's exact test for categorical data and one-way analysis of variance (ANOVA) or

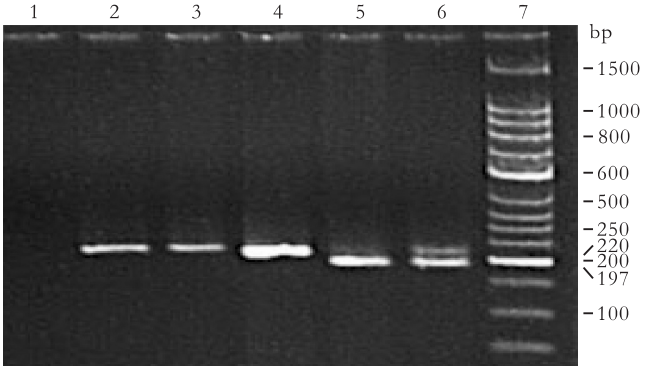

Fig. PCR-based restriction fragment length polymorphism genotyping of ESR1 T29C. Lane 1: negative control; lanes 2-4: T/T homozygotes; lane 5: T/C heterozygote; lane 6: C/C homozygote.

nonparametric test for continuous data. A *P* value less than 0.05 was considered significantly different, and a Bonfferoni correction was used to adjust for multiple amprisons, requiring a *P* value of 0.05/3=0.017 for statistical significance.

Results

Clinical and demographic characteristics of patients

One hundred and six patients with CHB were studied. Six patients were lost to follow-up because two of them were out of contact, two withdrew from the study, and another two discontinued therapy due to drug side-effects.

The T29C genotype polymorphism of ESR1 was genotyped in 100 patients with CHB, of 55 had the T/T genotype, 22 had T/C, and 23 had C/C. No significant difference among the three genotypes was found in terms of the distribution of gender (χ^2=1.148, *P*=0.563), age (*F*=1.153, *P*=0.320), initial ALT level (*F*=0.862, *P*=0.441), pretreatment HBV DNA (*Z*=1.961, *P*=0.161), pretreatment e-antigen status (χ^2=0.055, *P*=0.973), and type of IFN-α (χ^2=0.841, *P*=0.657). Overall, clinical and demographic characteristics showed no significant differences among the three genotypes in patients with CHB (Table 1).

Initial combined response among T/T, T/C, and C/C genotypes

The frequencies of the initial combined response in the T/T, T/C, and C/C groups are shown in Table 2. The respective frequency in no response, partial response, and complete response in the T/T group was 14.55%, 56.36% and 29.09%. This was 27.27%, 54.54% and 18.19% in the T/C group, and 73.91%, 17.40% and 8.69% in the C/C group. The initial combined response between the T/T and T/C groups was not significantly different (*Z*=2.078, *P*=0.354). However, the initial combined response among the T/T, T/C, and C/C groups was significantly different (*Z*=35.558, *P*=0.000; not shown in Table 2). Furthermore, the frequency of the initial combined response was markedly higher in the T/T and T/C groups than in the C/C group (*P*=0.000 and *P*=0.006, respectively) (Table 2). The results showed that patients with CHB who carried the T/T or T/C genotypes were more likely to achieve an initial combined response.

Initial virological response among T/T, T/C, and C/C genotypes

The respective frequencies of the initial virological response among the T/T, T/C, and C/C groups were 60.00%, 63.64% and 30.43%, respectively. No significant difference was found between the T/T and T/C groups (χ^2=0.087, *P*=0.768). However, the initial virological response among the T/T, T/C, and C/C genotype groups was significantly different (χ^2=6.751, *P*=0.034; not shown in Table 3). In addition, the frequencies of initial virological response in the T/T and T/C groups were higher than in the C/C group (χ^2=5.674, *P*=0.017 and χ^2=4.980, *P*=0.026, respectively) (Table 3). The data

Table 2. Initial combined response to IFN-α therapy among the three genotype groups of patients with CHB (*n*, %)

Genotype	*n*	Initial combined response (*n*, %)			*Z*	*P*
		NR	PR	CR		
T/T	55	8 (14.55)	31 (56.36)	16 (29.09)	2.078*	0.354[a]
T/C	22	6 (27.27)	12 (54.54)	4 (18.19)	10.326*	0.006[b]
C/C	23	17 (73.91)	4 (17.40)	2 (8.69)	26.247*	0.000[c]

NR: no response; PR: partial response; CR: complete response. *: Mann-Whitney test. a: vs. T/C genotype group; b: vs. C/C genotype group; c: vs. T/T genotype group.

Table 3. Initial virological response to IFN-α therapy among the three genotype groups of patients with CHB (*n*, %)

Genotype	*n*	Initial virological response (*n*, %)		χ^2	*P*
		(−)	(+)		
T/T	55	22 (40.00)	33 (60.00)	0.087*	0.768[a]
T/C	22	8 (36.36)	14 (63.64)	4.980*	0.026[b]
C/C	23	16 (69.57)	7 (30.43)	5.674*	0.017[c]

*: Chi-square test; a: vs. T/C genotype group; b: vs. C/C genotype group; c: vs. T/T genotype group.

Table 1. Clinical and demographic characteristics of the study population

Genotype	*n*	Gender (M/F)	Age (mean±SD, yr)	Initial ALT (mean±SD, IU/L)	Initial HBV DNA (mean±SD, lg)	HBeAg status (*n*) (Positive/Negative)	Peg a-2a IFN (*n*) (+/-)
T/T	55	42/13	25.20±9.54	283.26±135.98	6.49±1.130	39/16	27/28
T/C	22	19/3	29.00±11.34	301.41±203.19	7.00±1.00	16/6	9/13
C/C	23	19/4	26.48±8.96	274.61±235.63	6.74±1.15	16/7	9/14
χ^2/*F*		1.148*	1.153#	0.862#	1.961#	0.055△	0.841△
P		0.563	0.320	0.441	0.161	0.973	0.657

*: Chi-square test; #: ANOVA test, △: Kruskal-Wallis test.

Table 4. Initial HBeAg disappearance or seroconversion with IFN-α therapy among the three genotype groups of patients with HBeAg-positive CHB (*n*, %)

Genotype	*n*	Initial HBeAg disappearance or seroconversion (*n*, %)		χ^2	*P*
		(−)	(+)		
T/T	41	27 (65.85)	14 (34.15)	0.232*	0.630[a]
T/C	18	13 (72.22)	5 (27.78)	0.236*	0.627[b]
C/C	19	16 (84.43)	3 (15.79)	2.155*	0.142[c]

*: Chi-square test. a: vs. T/C genotype group; b: vs. C/C genotype group; c: vs. T/T genotype group.

indicated that patients with CHB who carried the T/T or T/C genotype were more likely to achieve an initial virological response.

Initial HBeAg disappearance or seroconversion

Of the 78 initially HBeAg-positive patients, only 22 achieved initial HBeAg disappearance or seroconversion, and the remaining 56 achieved neither (Table 4). The respective frequencies of initial HBeAg disappearance or seroconversion among the T/T, T/C, and C/C groups were 34.15%, 27.78%, and 15.79% (χ^2=2.163, *P*=0.339; not displayed in Table 4). However, the initial HBeAg disappearance or seroconversion between the T/T and T/C groups was not significantly different (χ^2=0.232, *P*=0.630). In addition, no significant difference was found between the T/C and C/C groups (χ^2=0.236, *P*=0.627), and the difference between the T/T and C/C groups was not significant (χ^2=2.155, *P*=0.142). The data indicated that the T29C genotype polymorphism of ESR1 was not related to initial HBeAg disappearance or seroconversion.

Discussion

As one of the important antiviral treatments, IFN-α therapy excerts its anti-viral effect indirectly through the immune system. However, patients react differently to IFN-α therapy. With regard to individualized treatment, it is desirable to predict the treatment response. In this study, we evaluated the initial response to IFN-α therapy in 100 patients and analyzed the genotype polymorphism in T29C by PCR-RFLP. All patients were classified into three groups according to the T29C genotype polymorphism of ESR1: T/T, T/C, and C/C genotype groups. Studies have shown that some characteristics of patients, i.e., age, gender, ALT, HBV DNA level, and pretreatment e-antigen status[2-5] may affect the response to IFN-α therapy. In our study, these factors were distributed uniformly among the T/T, T/C, and C/C groups, which may have removed them as confounding factors. Furthermore, the distribution of initial combined response and initial virological response were evidently different among the three genotypes. In conclusion, our results suggested that patients with CHB who possessed the T/T genotype were more likely to achieve an initial combined response, and patients with the T/C genotype were more likely to achieve an initial virological response, whereas those with the C/C genotype were less likely to develop these responses.

The association between T29C genotype polymorphism of ESR 1 and initial response to IFN-α therapy in CHB patients has never been reported. In addition, the exact mechanism of this association is not clear. The IFNs are a family of natural glycoproteins and regulatory cytokines with pleiotropic cellular functions, such as anti-viral, anti-proliferative, and immunomodulatory activities. It is known that IFN-α enhances anti-viral activity through immune regulation, which contributes to the result and clinical prognosis of HBV infection.[14] In addition, HBV dissemination is mainly limited by cellular immunity. Cytotoxic T lymphocytes play an important role in the host defense against HBV. T-cell receptors may also be activated by IFN-α. Higher absolute numbers of $CD4^+$ T-cells and higher levels of Th1 cytokines occur in females[15] and they show higher levels of antibodies and stronger T-cell activation than males after vaccination.[16] The estrogen/estrogen receptor axis can be simultaneously involved not only in the reproductive system, bone, and cardiovascular function but also in immune regulation.[17-21] The protective role of estrogen in the progress of chronic liver disease by inhibiting the replication of HBV has also been reported.[21]

Our study indicates that patients who have different T29C genotypes which react differently to IFN-α therapy, so we suggest that these genotypes may enhance the effect of IFN-α therapy by enhancing cellular immunity differently. This indicates a direct or indirect relationship and fine balance between the levels of ESR and IFN-α.

Estrogen carries out its function mainly through ESR1. The polymorphism of ESR1 affects the expression and function of estrogen.[22] The T29C genotype polymorphism of ESR1 which is caused by a C→T transition at codon 10 in exon 1[4] is a synonymous polymorphism and may not have functional consequences.[23] As there is no nonsynonymous SNP which results in amino acid change in the ESR1 gene, it is very likely that there exist(s) as-yet-unidentified causative regulatory polymorphism(s) associated with T29C.[9, 24] The yet-unidentified causative regulatory polymorphism(s) may be in linkage disequilibrium with the observed associated T29C in the promoter introns. For example, in PvuⅡ and XbaⅠ RFLP,[22] a

(TA)n variable number of tandem repeats[18] can affect gene transcription.[23] Therefore, these polymorphisms cannot be excluded from linkage disequilibrium with the observed associated T29C. In that case, the association between ESR T29C genotype polymorphism and the initial response to IFN-α in patients with CHB may be a superficial phenomenon. More studies are required to fully understand the mechanism of the T29C genotype polymorphism of ESR1 in the initial response to IFN-α in patients with CHB.

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