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Role of RANTES Polymorphism in Response to Treatment of Chronic Hepatitis C Genotype 3 - A Pilot Study

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ABSTRACT

Host genetic factor has been shown to be influential at least partly in determinating treatment responsiveness. For HCV infection, RANTES polymorphisms has been theoretically speculated and subsequently proven by few association studies to play a role in antiviral treatment response. We conducted a study to determine whether in Thai population, RANTES polymorphisms may also exert such effect. Using 40 HCV genotype 3 infected patients, we gathered demographic data and genotyped each individual at three separate RANTES SNPs. Results showed that one of the SNPs, IVS1 +306 T/C, was associated with treatment response variation. IVS1 +306 C allele was found to be statistically higher frequency in non-responder group. This result, if replicable and confirmable, may lead to an integrated molecular testing prior to HCV antiviral treatment similar to those beginning to be used in HIV infection.

Key words : Hepatitis C, Rantes polymorphisms, Treatment response

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BACKGROUND

Hepatitis C virus (HCV) is a major global health problem with 3 % of the world's population or approximately 170 million persons chronically infected.(1) The prevalence of infection in Thailand is 1-2% of which hepatitis C virus genotype 3a is the most common genotype (40-50%). Persistent hepatitis C virus infection has quite a variable outcome, with 5-20% of those infected developing cirrhosis within 20-25 years. Approximately 1-2% of cirrhotic patients per year will proceed to hepatocellular carcinoma. The major goal in the treatment of hepatitis C virus infection is to prevent the development of decompensated liver disease and death. This strategy includes eradication or prolonged suppression of viral replication, reduction of hepatic inflammation, and slowing of the rate of progressive liver injury. At present, the standard treatment of chronic hepatitis C should be a combination of interferon or pegylated interferon and ribavirin.(2) Recent study has shown that sustained virological response was seen in 84% of patients with genotype 2 or 3 virus who were treated with 24 weeks of combination of pegylated interferon α-2a and ribavirin, and in

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52% of patients with genotype 1 virus who were treated with 48 weeks of combination therapy.[3] While viral factors probably play a role in the outcome of infection, host factors including genetic susceptibility may contribute to the variable manifestations of the disease and treatment response.

Chemokine and chemokine receptor genes are strong candidate genes for outcome of hepatitis C virus. They play important roles in leukocyte trafficking to site of infection, in regulating T helper cell polarization, and also in linking innate and adaptive immunity.[4,5] The most widely known, a 32-base-pair deletion in the open reading frame of CC chemokine receptor 5 (CCR5Δ32) is associated with protection against human immunodeficiency virus 1 infection and delayed progression to AIDS in white population.[6] The frequency of CCR5Δ32 homozygosity was recently reported to be dramaticly increased in patients with hepatitis C. It was suggested that individuals with CCR5Δ32/Δ32 genotype had increased suseptibility to HCV infection.[7] Interestingly, CCR5Δ32 has recently been linked to a negative response to interferon monotherapy in hepatitis C.[8]

The CC chemokine RANTES (regulated on activation normally T cell expressed and secreted; systematic name CCL5) is produced by T lymphocytes, monocytes, fibroblasts, and endothelial cells, and attracts T lymphocytes to inflamed tissue.[9] In hepatitis C virus infected liver, RANTES messenger RNA has been shown to be up-regulated, and its intrahepatic expression levels correlated with serum alanine aminotransferase activities.[10,11] The human RANTES gene spans 8.88 kb on chromosome 17q11.2-q12 and has the characteristic three exon/two introns organization of the CC chemokine family. The polymorphism at position -403 of the RANTES gene promotor appears to have functional difference with the -403 A allele leading to increased transcription of RANTES[12] and was associated with reduced portal inflammation.[13] Another study has shown that the haplotype (a distinct set of gene polymorphisms on a single chromosome) constructs harbouring the -403 A but not the Int 1.1 C allele was more prevalent in responders compared with nonresponders. In contrast, the haplotype harbouring the int 1.1 C polymorphism lead to a strongly significant down regulation of transcription in the RANTES gene and was associated with negative outcome of antiviral therapy.[14]

Using data from ThaiSNP project regarding RANTES gene, three single nucleotide polymorphisms (SNPs) were identified as shown in Figure 1. The first SNP was located in the 5' untranslated region (-96 C/G), the second SNP was located in the first intron (IVS1 +306 T/C), and the last novel SNP was located in the third exon and was found only in Thai population (31,223,286 T/C).[15] To our knowledge, no data regarding the correlation between these RANTES polymorphisms and responses to antiviral therapy in HCV infected patients has ever been reported. Our research goal is to assess the relationship between these RANTES polymorphisms and outcomes of treatment in patients with chronic hepatitis C (genotype 3).

PATIENTS AND METHOD

Forty patients with chronic hepatitis C (HCV genotype 3) were included in the study. The diagnosis of chronic hepatitis C was based on elevated serum

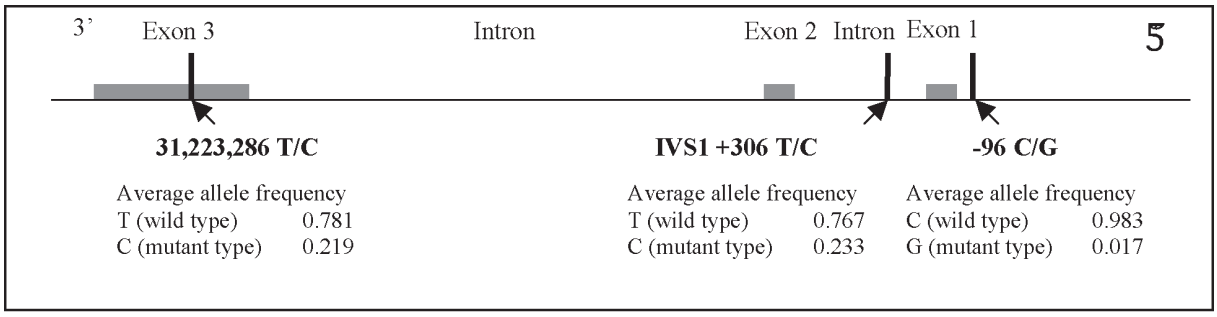

Figure 1 The genomic structure of the RANTES gene on chromosome 17 displays the characteristic three exons/ two introns organization of the CC chemokine family. 3 SNPs were identified in Thai population, as indicated by the small vertical bars. One SNP is located in the 5' untranslated region (-96 C/G), the other one is located in the first intron (IVS1 +306 T/C), and the last one is located in the third exon (31,223,286 T/C).

aminotransferase levels at least 2 times the upper limit of normal (40 U/L) for at least 6 months, histological examination, anti-HCV positive antibody status assessed by third-generation enzyme-linked immunosorbent assay version 3.0 (Abbott Axym), and consistently detectable serum HCV RNA using quantitative reverse-transcriptase polymerase chain reaction with the lowest detection sensitivity of 600 IU/ml (Cobas Amplicor Monitor HCV version 2.0, Roche diagnostics). HCV genotypes were determined using reverse hybridization assay (INNO LiPA HCVC-II, Innogenetics). Patients were excluded for any of the following reasons: other liver diseases, such as chronic hepatitis B, autoimmune hepatitis, alcohol-induced liver disease, drug-induced hepatitis, decompensated liver disease, coinfection with human immunodeficiency virus, current pregnancy or breast feeding, or previous antiviral therapy. Written informed consent was obtained from each patient, and the study was approved by the Ethics Committees of Siriraj hospital, Mahidol University, Thailand.

Antiviral Therapy

All patients received their first course with standard antiviral therapy in the hepatology outpatient clinic of Siriraj hospital. One of the three different recommended regimens were used as the standard treatment of hepatitis C virus infection in this study. These include 1) A combination of interferon α-2b 3 mU by subcutaneous injection thrice a week plus daily oral ribavirin (in divided doses administered twice daily; 1000-1200 mg, depending on weight for 48 weeks of therapy. 2) A combination of pegylated interferon α-2a 180 μg by subcutaneous injection once a week plus 800 mg daily of oral ribavirin (in divided doses administered twice daily) for 24 weeks of therapy. 3) A combination of pegylated interferon α-2b 1.5 μg/kg by subcutaneous injection once a week plus at least 10.6 mg/kg daily of oral ribavirin (in divided doses administered twice daily) for 24 weeks of therapy. Routine laboratory tests such as complete blood count and liver function tests were performed at baseline, 24 or 48 weeks (the end of treatment), and 48 or 72 weeks (24 weeks after the end of treatment) depending on the treatment regimens. The virological response was assessed by a qualitative HCV RNA and the patients were defined as (1) sustained virological responders (no detectable HCV RNA at the end of treatment and 24 weeks afterwards), (2) virological non-responders (detectable HCV RNA at the end of treatment or detectable HCV RNA within 24 weeks after the end of treatment response).

Genotyping of RANTES Single Nucleotide Polymorphisms

RANTES polymorphisms -96 C/G, IVS1 +306 T/C, and 31,223,286 T/C were determined using PCR-restriction fragment length polymorphism analysis. For SNP -96 C/G, polymerase chain reaction primers were 5'-CCTTTATAGGGCCAGTC-3' and 5'-GGTGCTTGGTCAAAGAGGAA-3'. For SNP IVS1 +306 T/C, polymerase chain reaction primers were 5'-GCCTTGAGGGTGTAGACCTC-3' and 5'-ATGCCTACCCCATCCCAATG-3'. For SNP 31,223,286 T/C, polymerase chain reaction primers were 5'-GTCTCGAACTCCTGACCTCG-3' and 5'-TGCCTGTTTCTGCTTGCTCT-3'. Each of the sense primers was modified to include restriction site for restriction enzyme Taq1. After PCR amplification using sample DNA, Taq1 (New England Biolabs) was used for restriction fragment length polymorphism analysis of all SNPs. The presence of specific SNPs in the sequence will enable cutting or abolish cutting by Taq1 depending upon each SNPs. Gel electrophoresis was performed to detect the sizes of restriction products and positive and negative control for each SNPs were run in parallel. Individuals were categorized as homozygote wild type, heterozygote and homozygote mutant.

Statistical Analysis

Patient characteristics were analyzed by descriptive statistics and reported as mean, range, and percent. For the results, we compared sustained virological responders and non-responders with Chi-Square test, Fisher's exact test, student t test, Mann-Whitney tests as appropriate. To determine cutoff levels of the quantitative factors with significant differences between the response groups, we also analyzed odds ratios with 95%-confidence intervals. Two sided P-values less than 0.05 were regarded as significant. All calculations were performed with SPSS software version 13.0 (SPSS Inc.)

RESULTS

Patient characteristics overall, out of 40 patients with chronic hepatitis C (genotype 3), 21 patients

(52.5%) achieved a sustained virological response, while 19 were non-sustained responders. Of these non-sustained responders, 11 patients (27.5%) relapsed after end of treatment response, and 8 patients (20.0%) never achieved end of treatment response. Baseline characteristics of all patients were summarized in Table 1. The major risk factor of HCV infection in this study was blood transfusion (60%). Thirty-five percent of patients had no risk factors.

RANTES SNPs

From restriction fragment length polymorphism analysis, 44 alleles of SNP IVS1 +306 T, 36 alleles of SNP IVS1 +306 C, 50 alleles of SNP 31,223,286 T, and 30 alleles of SNP 31,223,286 C were identified. (Table 2) Unfortunately, due to technical difficulty, we could not amplify polymerase chain reaction products from primers of SNP -96. The frequency of IVS1 +306 C allele was higher in non-responders compared with sustained responders and this had statistical significance (61.1% vs. 38.9%; P = 0.048; odds ratio of 2.75, 95% confidence interval 1.11-6.83). The statistical significance was not found in the 31,223,286 C allele frequencies (40.0% vs. 60.0%, respectively; P = 0.133).

A haplotype is defined as a distinct set of polymorphism on a single chromosome.[14] For the tested two SNPs, 4 possible haplotypes can be expected. Out of the four, 3 haplotypes were found in our patients at frequencies of 55.0%, 37.5% and 7.5%. However, we

Table 1 Patients Characteristics at Baseline (n = 40)

Age (years)	50.0 (21-68)
Sex: Male/Female	21/19 (52.5% vs. 47.5%)
Body weight (kg)	64.6 (41.8-91.0)
Risk factors*	
Blood transfusion	24 (60%)
Intravenous drug used	3 (7.5%)
Tattoo	2 (5%)
Unknown	14 (35%)
Aspartate aminotransferase (U/l)**	100.0 (21-267)
Alanine aminotransferase (U/l)**	119.9 (32-390)
Liver histology	
Inflammation	6.56 (1-12)
Fibrosis	1.79 (0-4)
HCV RNA viral load (× 103 IU/ml)	2,151 (0.61-16,400)
Virological response to therapy	
Sustained virological response	21 (52.5%)
Virological non-response (including relapse)	19 (47.5%)

Note: Continuous data are given as mean (range), * Some patients had more than 1 risk factors, ** Normal range: < 40 IU/ml

Table 2 RANTES Single Nucleotide Polymorphisms Frequencies in HCV Genoype 3 Infected Patients (n = 80)

RANTES gene	Responders (alleles)	Non-responders (alleles)	P value	Odds ratio
IVS1 +306			0.048	2.7 (1.1-6.8)
T	28	16		
C	14	22		
31,223,286			0.133	-
T	30	20		
C	12	18		

Note: The result of all alleles in this study showed 100% homozygosity

Table 3 RANTES Haplotype Distribution in HCV Genoype 3 Infected Patients

IVS1 +306	31,223,286	HCV patients (alleles)	Responders (alleles)	Non-responders (alleles)	P value
T	T	44 (55.0%)	28	16	0.084
C	C	30 (37.5%)	12	18	
C	T	6 (7.5%)	2	4	
T	C	0	-	-	

Table 4 Univariate Analysis of Variable Factors between the Response Groups

Univariate analysis	P value
Age (<40 years vs. >40 years)	NS
Body weight (<75 kg vs. >75 kg)	0.05
Sex	NS
Alanine aminotransferase	NS
HCV RNA viral load	NS
Liver histology	
Inflammation	NS
Fibrosis	NS

did not find an association between any of these haplotypes and antiviral treatment response (P = 0.084). (Table 3)

Age, sex, alanine aminotransferase, HCV RNA viral load, and liver histology were also not associated with response to antiviral therapy whereas body weight was borderline significant (P = 0.050). (Table 4)

DISCUSSION

Approximately one fifth of all cases of HCV infection (genotype 2 or 3) who were treated with pegylated interferon and ribavirin still exhibited detectable HCV RNA.[3] Patients who either spontaneously clear HCV RNA or respond to antiviral therapy display a predominantly proinflammatory (T helper 1 lymphocyte associated) cytokine profile. RANTES specifically binds to the chemokine receptor (CCR5) and attracts proinflammatory CD4 T helper 1 lymphocytes.[5] RANTES might be associated with treatment response to antiviral therapy, which is thought to depend on sufficient proinflammatory cytokine response. Promrat, *et al*. and Hellier, *et al*. both investigated two RANTES promotor polymorphisms (-28 C/G, and -403 G/A) in patients with chronic HCV infection and found no association of these polymorphisms with treatment response.[12,13] However, Wasmuth, *et al*. showed that RANTES haplotypes carrying the functional variants Int 1.1 C are significanly associated with a negative outcome of antiviral therapy in patients with chronic HCV genotype 1, and 4 infection and the haplotypes carrying the -403 A allele are more prevalent in responders compared to non-responders.[14] Although, seven SNPs of RANTES gene, -403 G/A, -109 C/T, -105 C/T, -28 C/G, Int 1.1 T/C, Int 1.2 G/A, and 3' 222 T/C, were identified on Caucasian and African individuals, only 3 SNPs, -96 C/G, IVS1 +306 T/C, and 31,223,286 T/C were demonstrated in Thai population.[15] In this study, the results provided evidence that RANTES polymorphisms IVS1 +306 C allele are associated with negative outcome of standard treatment. Our detection of a significance difference in RANTES gene variants between responders and non-responders should be confirmed in the future study employing a different method such as Denaturing High Performance Liquid Chromatography (DHPLC), in a separate and bigger population sample with normal population as a control group. Should this association hold true?, we can speculate that RANTES polymorphism can be used for predicting response to antiviral therapy in Thai population with HCV genotype 3 infection. This may then lead to treatment decision based upon host genetic factor that can be both timesaving and economical for this expensive and sometime cumbersome treatment regimen. That is those patients with RANTES SNP IVS1 +306 T allele would be more likely to be a virological responder and therefore should receive priority for this treatment, whereas those with C allele esp. IVS1 +306 C homozygote who may be less responsive and if become available, should be considered first for future available treatment.

CONCLUSION

In conclusion, this study identified RANTES gene polymorphisms in Thai population with genotype 3 HCV infection. We have shown for the first time the association between IVS1 +306 C and the decreased level of responsiveness to standard antiviral treatment. We have also demonstrated a novel SNP found in Thais and confirmed that the heterozygosity ratio is close to that reported in ThaiSNPs project.

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