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Mutation Analysis of Protein Kinase Binding Domain of HCV NS5A Gene Isolated from Patients with Chronic Hepatitis C

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Abstract: The majority of hepatitis C virus (HCV) isolates are resistant to antiviral therapy. Although, the molecular mechanisms of resistance against to antiviral therapy were investigated in such studies *in vivo* and/or *in vitro* and the significant results were obtained, the resistance problem has not been solved yet. The aim of this study was to investigate the relationship between therapy and response by detecting the mutations in $ISDR_{2209-2248}$ and $PKR\text{-}BD_{2209-2274}$ site among HCV positive patients in Gaziantep. Fifty nine patients (25 men and 34 women) with chronic hepatitis C diagnosed at Gastroenterology Department of Gaziantep University Sahinbey Research Hospital between 2009-2010 years were included. The 59 HCV RNA positive patients: 34 women and 25 men were included. In $ISDR_{2209-2248}$ site in $PKR\text{-}BD_{2209-2274}$ site, 25 of 89 amino-acid substitutions (28.09%) of the sustained virologic responder patients; 12 of 78 amino-acid substitutions (15.38%) in the non-responder patients were determined. The significant relation between the number of mutation in interferon sensitivity determining region- $ISDR_{2209-2248}$ and protein kinase binding domain- $PKR\text{-}BD_{2209-2274}$ of NS5A gene and the response to interferon-alpha+ribavirin combination therapy was not determined ($p>0.05$). The number of mutation in this region was not significant in predicting the response of chronic hepatitis C patients to the treatment. The evaluation of age, individual immunization, nutritional situations, psychological situation of people infected with HCV and other gene regions would be useful.

Key words: Hepatitis C virus, non-structural 5A gene, protein kinase binding domain, interferon sensitivity determining region, mutation

INTRODUCTION

Hepatitis C virus infection is a major public health problem in Turkey as well as in Australia, Italy, Romania, Mongolia (Dore *et al*., 2003; Raffaele *et al*., 2001; Kmieciak *et al*., 2006; Gheorghe *et al*., 2008). Approximately 80% of the hepatitis C infections become chronic and result in important complications such as cirrhosis and hepatocellular carcinoma (Aman *et al*., 2012). According to the estimations of World Health Organization (WHO); HCV infection prevalence is around 3% although it varies among country (Giannini and Brechot, 2003). Today, combination of Pegylated Interferon Alpha (PEG-IFN) and a nucleoside analogue Ribavirin is used for the treatment of chronic HCV infection. After treatment during 24-48 weeks Sustained Virologic Response (SVR) was determined in 54-56% cases (Manns *et al*., 2001; Fried *et al*., 2002). The majority of HCV isolates are resistant to antiviral therapy. The molecular mechanism of resistance to antiviral therapy have been investigated in many studies *in vivo* and *in vitro* and significant results were obtained but the molecular basis of the resistance has not been clarified yet (Pawlotsky, 2000). HCV is a unique known member of the Hepacivirus genus of Flaviviridae. It is single stranded and 50 nm positive-sense RNA virus and has six major genotypes (Brass *et al*., 2006).

HCV genotype 1b is the most resistant and the worst responder group to treatment (Hermida *et al*., 1997). There are various studies with different results about mutations of Interferon Sensitivity Determining Region (ISDR) of NS5A gene of HCV. For instance, it was reported that although mutations in this region were associated with response to treatment in Japanese studies, no relationship between mutations and treatment was determined in some studies in Europe (Enomoto *et al*., 1995; Zeuzem *et al*., 1997). In our country, studies at Ankara University and Dokuz Eylul University have shown that $ISDR_{2209-2248}$ mutations of NS5A gene were not associated with response to interferon treatment (Aygun, 2003; Aslan *et al*., 2004). These contradictory results related to $ISDR_{2209-2248}$ of HCV NS5A gene mutations show us that

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this issue is still controversial. However, some studies about PKR-$BD_{2209-2274}$ (Protein Kinase Binding Domain) region located in the same gene region as well as $ISDR_{2209-2248}$ indicated to relate mutations of this region and IFN-therapy (Macquillan *et al*., 2004).

The aim of this study was to investigate the relationship between therapy and response to HCV infection by detecting the mutations in $ISDR_{2209-2248}$ and PKR-$BD_{2209-2274}$ region among HCV positive patients in Gaziantep.

MATERIALS AND METHODS

Patients: Fifty nine patients (25 men and 34 women) with chronic hepatitis C whose positive anti-HCV anticore and HCV RNA diagnosed by ELISA and also detected quantitative HCV RNA levels at Gastroenterology Department of Gaziantep University Sahinbey Research Hospital between January 2009 and January 2010 years were included.

Before treatment, quantitative HCV RNA values and biochemical values of patients were determined. These patients were treated with combination of peg-interferon alpha-2a or peg-interferon alpha 2b once for every day and ribavirin for a week at Gastroenterology Department. Patients were classified according to number of mutations in the ISDR and PKRBD regions of NS5A of HCV gene; Wild type (no mutation), Intermediate type (1-3 mutations) and Mutant Type (4 and more mutations) (Enomoto *et al*., 1995).

RNA extraction: Ten microliter of peripheral venous blood samples were taken from each patient. HCV RNA was obtained with HCV RNA extraction kit (RocheHigh Pure Viral RNA Kit, Germany) as directed by the manufacturer. It was added 400 μL binding buffer supplemented with Poly (A) to 200 μL serum or plasma and mixed well and then transferred to high pure filter tube assembly. After then, it was centrifuged for 15 sec at 8,000 xg and discarded the flow through and collected into the collection tube. Five hundred microliter inhibitor removal buffer was added to the upper reservoir and centrifuged for 1 min at 8,000 xg and discarded the flow through and into the collection tube 450 μL Washing Buffer was added to the upper reservoir twice. After centrifugation for 1 min at 8,000 xg and then centrifuged repeatedly at max speed for 10 sec and discarded the flow through. And 50 μL Elution Buffer added to the upper reservoir into the collection tube and centrifuged for 1 min at 8,000 xg.

RT-PCR: It was performed RT-PCR procedure according to Saiz *et al.* (1998) with slight modification. Twenty microliter reaction mixture containing 4 μL Buffer, 5 μL dNTP (Fermantas), 0.05 μL AMV RT, 4 μL water with DEPC, 0.05 μL RNase inhibitor, 2 μL R_3 primer (Metabion, Germany) (5'-GCAATGGGCACCCGTGTACC-3') and pure RNA was prepared. This mixture was incubated for 1 h at 42°C in Minicycler MJ Research (United States of America) device.

Amplification of PKRBD (2209-2274) of HCV NS5A gene: Nested PCR was performed for amplification and it was studied in a 50 μL reaction mixture containing 5 μL Buffer, 5 μL dNTP, 3 μL $MgCl_2$, 2 μL Primer F_3 (5'-GGGCATGACCACTGACAACGT-3'), 2 μL Primer R_3 (5'-CAATGGGCACCCGTGTACC-3'), 0.25 μL *Taq* polymerase, 5 μL cDNA and 27.5 μL distilled water. Samples were subjected to denaturation by 1 cycle at 95°C for 5 min; followed by 30 cycles at 95°C for 1 min, at 55°C for 1 min and at 72°C for 2 min (annealing) and a final step by 1 cycle at 72°C for 7 min (elongation) in Minicycler MJ Research (United States of America) device.

In the second round of nested PCR was performed in a 50 μL mixture containing 5 μL Buffer, 5 μL dNTP, 3 μL $MgCl_2$, 2 μL Primer F_4 (5'-GCAGTGCTCACTTCCATGC TCAC-3'), 2 μL Primer R_4 (5'-GGACTCTAGCAGTGGA GGGTTGTA-3'), 0.25 μL taq polymerase, 1 μL DNA and 31.75 μL distilled water. The second PCR was conducted in the same way. The size, purity and approximate obtained yield of the DNA were verified by direct observation of 1% agarose gel.

Sequence analysis: The amplified PCR products were sequenced by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) as directed by the manufacturer within ABI 3130XL Genetic Analyzer. It was performed in a 20 μL reaction followed mixture: 8 μL big dye, 1-100 ng PCR template, 3.2 pmol primers and deionized water. HCV-genotyping was performed by the blast search program of the sequences with the HCV genotype gene bank. The phylogenetic analysis was used to differentiate the separate clusters of the identified HCV genotypes. Following sequences were used: AB056525 for 1b, AB056524 for 1b, HM042084 for 3b, HM042083 for 3b, FJ896370 for 1a, FJ896369 for 1a, AB056550 for 1c, AB056546 for 1c, AB056569 for 2a, AB056564 for 2a and AF339212 for 3a (GENBANK). PKR-$BD_{2209-2274}$ and $ISDR_{2209-2248}$ of HCV NS5A gene were aligned to the following reference sequence: HCV-CR (GenBank) for genotype 1b. For mutation and phylogenetic analysis MEGA 4.1 program was used.

Statistical analysis: For statistical analysis Chi-square (χ^2) test at SPSS 17.0 program was used (Yuan *et al*., 2010). The p-value of 0.05 or less was considered to represent statistical significance.

RESULTS

Twenty nine of 59 patients with chronic hepatitis C had sustained virologic responder against to interferon-ribavirin combination therapy while 30 patients had non-responder. Amino acid changes of PKR-$BD_{2209-2274}$ and $ISDR_{2209-2248}$ of HCV NS5A gene were determined by comparison with sequences of HCV genotype 1b of fifty-nine patients and the NS5A-CR (GeneBank) Reference Sequence (Fig. 1). The response of patients to IFN therapy by calculated mutation frequencies in $ISDR_{2209-2248}$ and PKR-$BD_{2209-2274}$ site of HCV isolates was shown at Table 1. In addition, changes in amio-acids caused by mutations were determined by based on universal genetic code. Some findings of the mutation and changes in the amino-acids were presented all together in Fig. 2-5. The most common amino-acid changes was 2270 codon in PKR-$BD_{2209-2274}$ region (from CTT (Leu) to GTT (Val)) (Fig. 2). Some amino-acid changes of PKR-$BD_{2209-2274}$ of HCV NS5A gene isolated from patients with chronic hepatitis C was 2217 codon (from ACC (Thr) to GCC (Ala)) (Fig. 3). Most amino acid mutations was detected on patients No. 29 (9 amino acid changes). Three of nine amino acid changes was 2223, 2224 and 2225 codon (Fig. 4) Two of amino acid changes was 2251 and 2253 codon (respectively; from GTA (Val) to ATA (Ile), from CTG (Leu) toATG (Met)). In our study, 24 amino-acids changes in PKR-$BD_{2209-2274}$ and 39 amino-acids changes in $ISDR_{2209-2248}$ were detected. Taking into consideration of mutation frequencies in $ISDR_{2209-2248}$ site of HCV isolates from patients with chronic HCV infection, 17 of 29 sustained virologic responder patients (58.6%) were wild type, 11 (37.6%) were intermediate type and only 1 (3.4%) was mutant type. And also 21 of non-responder patients to IFN combination therapy (70%) and 17 of responder patients (58.6%) were wild type. In addition, mutation frequencies in $ISDR_{2209-2248}$ site of HCV isolates from non-responder patients was lower than from responder patients.

To PKR-$BD_{2209-2274}$ site of HCV isolates, 21 of 29 sustained virologic responder patients to IFN combination therapy (72.4%) were intermediate type and the rest of them were mutant type. Twenty two of 30 non-responder patients were intermediate type and the rest were mutant type. Wild type PKR-$BD_{2209-2274}$ site was not determined among responder and also non-responder patients. The differences between two groups was not significant ($p>0.05$).

DISCUSSION

Hepatitis C virus has been a major public health problem in our country like as in all over the world. Current therapy for HCV infection has been a combination of pegylated interferon alpha- ribavirin and response of patients to therapy have been affected by several host and viral factors (Davis and Lau, 1997; Ferenci, 2004; Gonzalez and Keeffe, 2011). Major effective factor for therapy has appeared HCV genotype (Shiratori *et al*., 1997; Martinot-Peignoux *et al*., 1998). We investigated whether PKR-$BD_{2209-2274}$ and $ISDR_{2209-2248}$ of NS5A gene of HCV has been effective viral factors for response to therapy or not. In our study, the most common change was 2218. amino-acid in $ISDR_{2209-2248}$ of NS5A gene of HCV (from CAT (His) to CGT (Arg/R)). The finding was correlated with the result of Enomoto *et al*. (1995). The most common change was 2270. Amino-acid in PKR-$BD_{2209-2274}$ region (from CTT (Leu) to GTT (Val)). The frequency of non-responder patients with wild type $ISDR_{2209-2248}$ sequence was higher than responder patients. However, the frequency of non-responder patients with intermediate type $ISDR_{2209-2248}$ sequence was higher than responder patients. None of those patients were detected with wild type PKR-$BD_{2209-2274}$ sequence. There was no significance in the number of amino-acid changes in PKR-$BD_{2209-2274}$ sequence between non-responder patients and responder patients ($p>0.05$). Our result was consistent with the finding of Kmieciak *et al*. (2006). Initially Enomoto *et al*. (1995) suggested that there was a strong relationship between the frequency of mutations in $ISDR_{2209-2248}$ and response to treatment. Seventeen (44.7%) of thirty-eight patients with wild type $ISDR_{2209-2248}$ sequence were responder and 21 (55.3%) of the patients

Table 1: Comparison of the number of mutations in ISDR and PKR-BD with treatment response of patients

		Interferon sensitivity determining region (ISDR)			Protein kinase binding domain (PKR-BD)		
Site	n	Wild type (no mutation)	Intermediate type (1-3 mutations)	Mutant type (4 and more mutations)	Wild type (no mutation)	Intermediate type (1-3 mutations)	Mutant type (4 and more mutations)
SVR	29	17 (58,6%)	11 (37,9%)	1 (3,4%)	-	21 (72,4%)	8 (27,6%)
NR	30	21 (70%)	9 (30%)	-	-	22 (73.3%)	8 (26.7%)

SVR: Sustained virological responder, NR: Non-responder

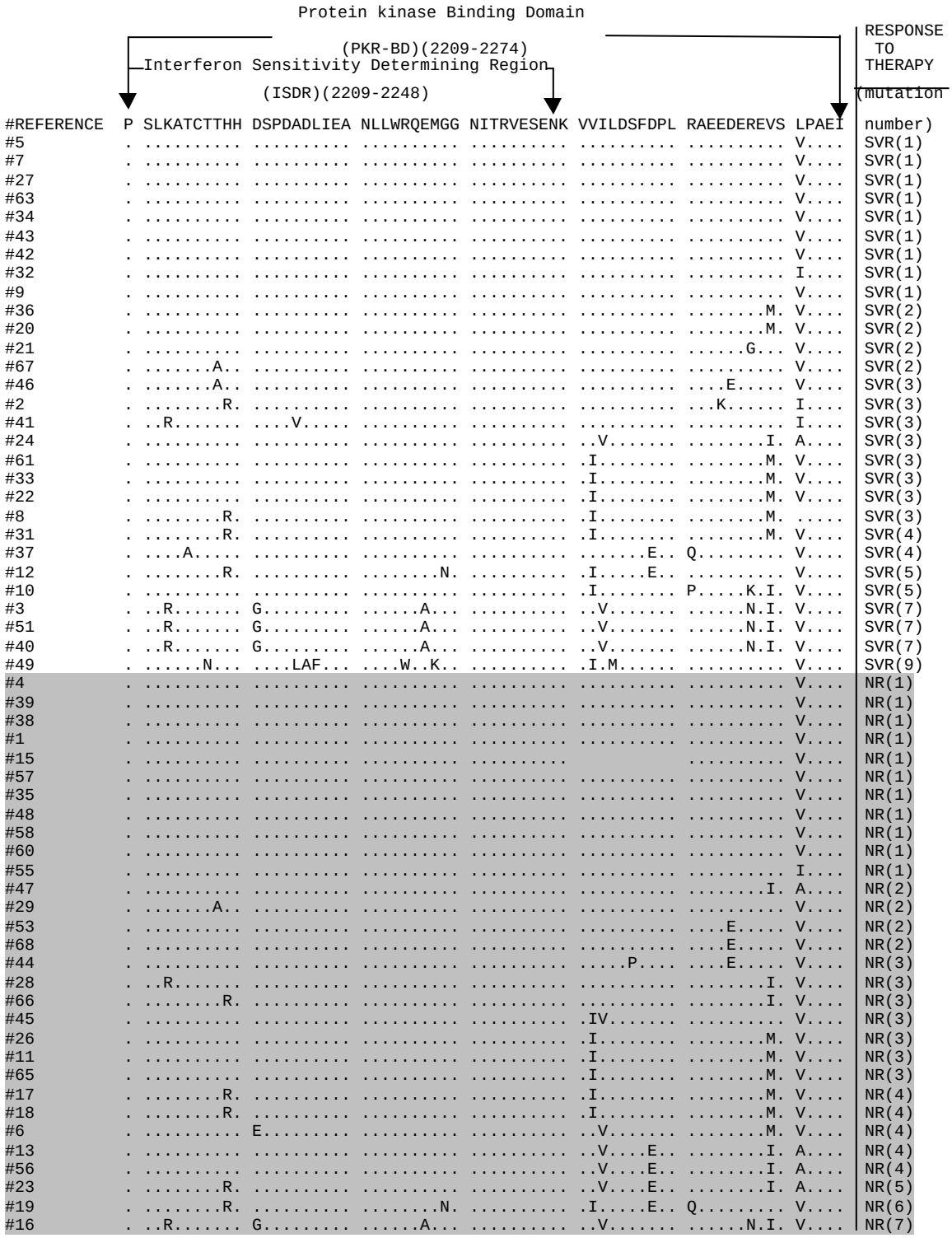

Fig. 1: Amino acid changes of PKR-BD and ISDR of HCV NS5A gene and reference sequence (SVR: Sustained virologic response, NR: Non responders)

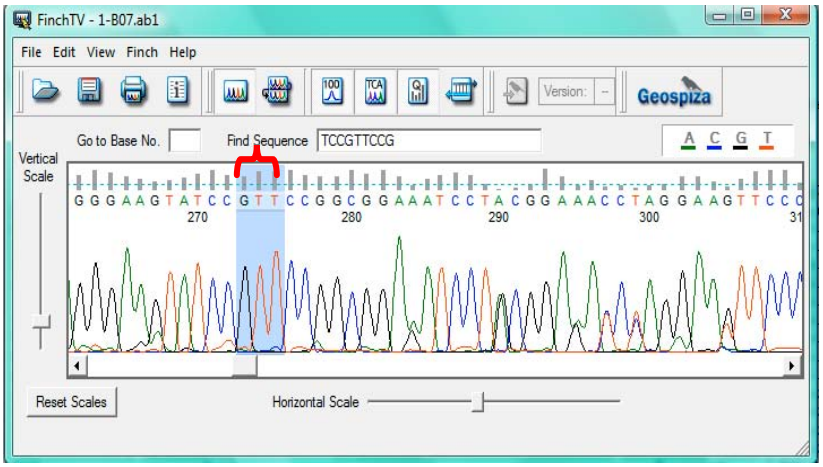

Fig. 2: L2270V mutation (from CTT (Leu) to GTT (Val))

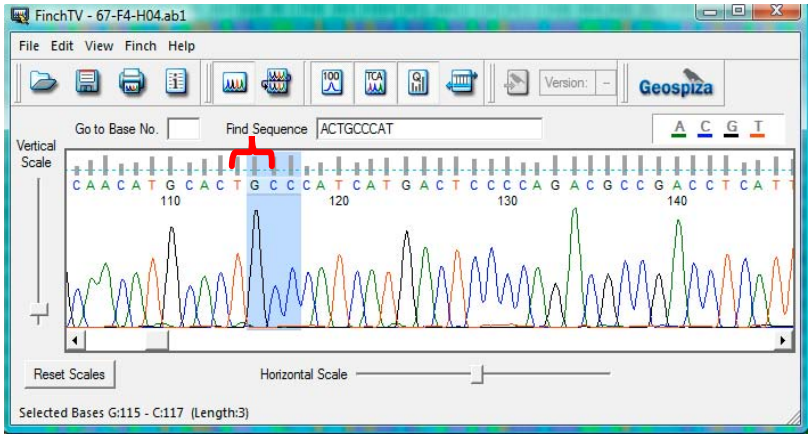

Fig. 3: T2217A mutation (from ACC (Thr) to GCC (Ala))

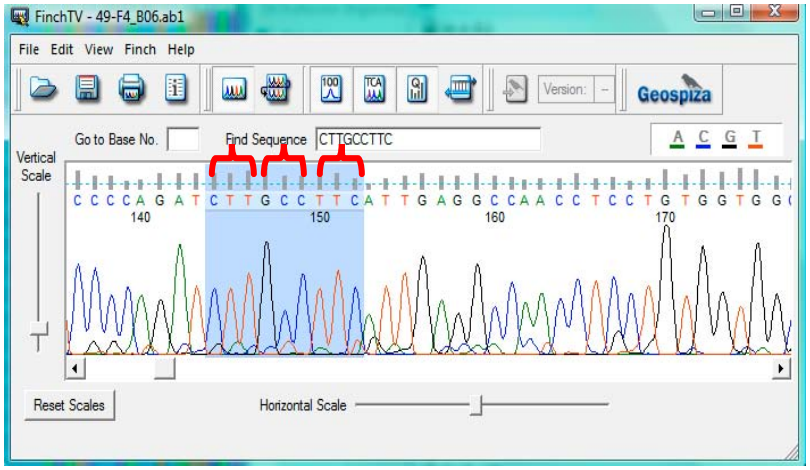

Fig. 4: A2223L, A2224A and L2225P mutations, respectively; from GCC (Ala) to CTT (Leu), from GAC (Asp) to GCC (Ala), from CTC (Leu) to TTC (Phe)

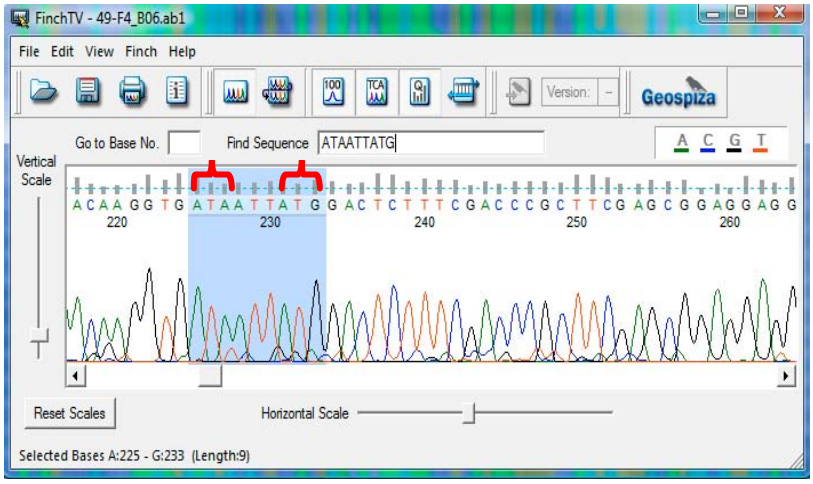

Fig. 5: V2251I and L2253M mutations, respectively; from GTA (Val) to ATA (Ile), from CTG (Leu) to ATG (Met)

were non-responder. As evaluation $ISDR_{2209-2248}$ site of responder patients, the frequency of intermediate type was higher than rate of wild type which seems to support that the positive relationship between sustained virologic response and the frequency of mutation. Japanese studies confirmed generally that there was a strong relationship between response to treatment and $ISDR_{2209-2248}$ mutations (Enomoto *et al*., 1995; Kurosaki *et al*., 2011). However, European and Asian studies were differed with them (Zeuzem *et al*., 1997; Hu *et al*., 2002). Several studies were performed the relationship between response to treatment and mutations in $ISDR_{2209-2248}$ by different groups of Japan, Europe and America (Hofgartner *et al*., 1997; Torres-Puente *et al*., 2008; El-Shamy *et al*., 2011).

Zeuzem *et al*. (1997) showed that no association between European $NS5A_{2209-2248}$ of HCV-1b isolates and initial-sustain response to IFN-alpha therapy. They also found no correlation between the number of amino acid changes in the NS5A2209-2248 region and the initial decline of serum HCV-RNA (Zeuzem *et al*., 1997).

We studied the same issue in 59 Turkish patients chronically infected with subtype HCV-1b and we obtained similar result with Zeuzem and colleagues (Zeuzem *et al*., 1997). We found no significant relationship between mutation number of $ISDR_{2209-2248}$ of NS5A gene of HCV-1b isolates and response to IFN-alpha therapy ($p>0.05$).

In accordance with the meta-analysis study based on geographical varieties; an obvious positive relationship between $ISDR_{2209-2248}$ sequence types and sustained virologic response was shown in European patients. However, the rate of sustained virologic response in Japanese patients infected with mutant $ISDR_{2209-2248}$ type HCV was more than in European patients. The rate of sustained virologic response increased as the number of mutation in $ISDR_{2209-2248}$ increased. However, it was suggested that this relationship was more apparent in Japanese patients (Pascu *et al*., 2004).

In a study at our country, Aslan and colleagues reported that the mutation in $ISDR_{2209-2248}$ of NS5A gene was not associated with response to interferon treatment in Turkish patients with chronic hepatitis C virus genotype lb infection (Aslan *et al*., 2004). In Spain, De Rueda *et al*. (2008) investigated that relationship between mutation of E2-PePHD, NS5A-PKRBD, NS5A-ISDR and NS5A-V3 of HCV Genotype 1 and PEGinterferon+ribavirin therapy. They reported that the presence of >4 mutations in the $PKR\text{-}BD_{2209-2274}$ region was associated with SVR and early virologic responses. In terms of this region, when intermediate type and mutant type were evaluated according to sustained virologic response, no statistically significant relationship had been detected (De Rueda *et al*., 2008). In our study, wild type $PKR\text{-}BD_{2209-2274}$ sequence in responder and also non-responder patients was not determined. In the same region, patients with intermediate type $PKR\text{-}BD_{2209-2274}$ sequence (72.9%) was more than patients with mutant type $PKR\text{-}BD_{2209-2274}$ sequence (27.1%).

Murphy *et al*. (2002) reported that no relationship between the frequencies of mutations in $PKR\text{-}BD_{2209-2274}$ and in intermediate type as correlation with us (Murphy *et al*., 2002). In contrary to our result, it was reported that the difference between the frequency of mutation in $PKR\text{-}BD_{2209-2274}$ and in intermediate type was not significant as compared with sustained virologic responder and non-responder patients (Gale *et al*., 1998;

Christoph *et al*., 2000). While our findings related to this region do not indicate any parallelism to the studies of Gale *et al*. (1998) and Christoph *et al*. (2000), they indicate conformity with the study of Murphy *et al*. (2002).

CONCLUSION

In conclusion, any relationship was not found between PKR-$BD_{2209-2274}$ of NS5A gene of HCV and response to interferon therapy. Thus, it was detected that the number of mutation in this region was not significant in predicting the response of chronic hepatitis C patients to the treatment. In addition to these regions, evaluation of age, individual immunization, nutritional situations, psychologic situation of people infected with HCV and other gene regions would be useful.

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