

# Association of SNPs in Interferon Receptor Genes in Chronic Hepatitis C with Response to Combined Therapy of Interferon and Ribavirin

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**Abstract-** Hepatitis C Virus is one of the main reasons for chronic liver disease and hepatocellular carcinoma. Combination therapy with Interferon (peg-IFN- $\alpha$ ) and Ribavirin (RBV) clear the virus more likely than the others. Different factors like virus and host characteristics influence on response to treatment. The most important viral factors include virus genotype and viral load; host factors like genetic, gender, race, age, weight and liver enzymes are also important. Previous studies have shown that single nucleotide polymorphisms (SNPs) in IFNR genes can regulate and influence on treatment with IFN. The purpose of this study is to investigate the association between SNPs in IFN- $\alpha$  receptor (IFNAR1 & IFNAR2) genes among subjects affected with chronic hepatitis C, who have treated with IFN and RBV, and also relationship between HCV genotypes and response to combination antiviral therapy. Peripheral blood mononuclear cells (PBMCs) were taken from whole blood of 61 patients affected with chronic hepatitis C who were treated with IFN and Ribavirin. Then, DNA was extracted from PBMCs and quality of DNA was assessed with Nanodrop finally two SNPs [Ex4-30G>C] and [Ivs1-4640 G>A] of IFN receptor genes (IFNAR1 and IFNAR2) were measured by TaqMan Real-Time PCR in ABI Prism 7900 system. Also to confirm the response rate to therapy, RNA was extracted then RT PCR was performed and final product was studied with gel electrophoresis and UV spectroscopy. Statistical analysis was performed using SPSS version 18.0 for Windows. The analysis of results from TaqMan SNP Genotyping has been shown that two SNPs (Ex4-30G>C and Ivs1-4640 G>A) of IFNAR1 and IFNAR2 did not show any relationship with response to combined therapy in subjects affected with chronic hepatitis C who have treated with peg-IFN- $\alpha$  and Ribavirin. 61 patients complete the treatment period. 54 patients (%88/5) of them responded to treatment and 7 patients (%11/5) did not. Research and data analysis have shown that there is no significant relationship between sex ( $P=0/7$ ) and age ( $P=0/2$ ). But there is a relationship between genotype-3a and response to combined therapy of IFN- $\alpha$  and RBV (0/02). Studies have shown that gene polymorphisms in IVSS1-22G location of IFNAR1 gene had a relationship with IFN treatment response. But current study has shown that there is no significant relationship between two SNPs Ex4-30G>C and Ivs1-4640 G>A and response to IFN therapy. In continue we suggest that it would be better to use this technique to evaluate other SNPs in IFN genes.

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**Keywords:** Hepatitis C Virus (HCV); Single nucleotide polymorphism (SNP);  $\alpha$ -Interferon receptor (IFNAR); Interferon; Ribavirin

## Introduction

Hepatitis C virus (HCV) infection is one of the most serious problems in many parts of the world and a global public health problem with an estimated 170 million individuals chronically infected worldwide (1,2) . The

center for disease control and prevention (CDC) reported that almost 1.8% of our population is anti-HCV-Ab positive (3) the evidence show that HCV infection occurs in %1 of Iranian people (4). Most patient (75%) with HCV infection have chronic liver disease and show a wide range of signs and symptoms

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(5,6). The chronic HCV is one of the major factors as the leading cause of increase in hepatic enzymes until the morbidity and mortality among liver cirrhosis, hepatic failure and hepatocellular carcinoma (HCC) in 20-30% of infected persons (7,8). Hepatitis C is a highly contagious virus transmitted by intravenous drug abuse (9,10), sexual intercourse (11) and direct per- mucosal contact, vertical transmission (12), needle-stick injury, Health care exposure (blood transfusion, blood products and organ transplantation without HCV screening) and body piercings (13) but today, the most common and major cause for Acute HCV in more than %75 of new cases in Iranian people is drug injection (14).

Interferon alpha is a major cytokine with characteristic of immune system stimulating and anti-viral effects. In the past, INF- $\alpha$  was prescribed alone to prevention of liver disease progress (15) and its efficacy was confirmed by several clinical trial studies (16,17). Current studies show that combination therapy (anti-viral drug ribavirin and peg-interferon alpha) causes an increase (40%) in stable response rate (18,19). Also the Pegylated INF- $\alpha$  has more molecular half life and less kidney clearance than INF- $\alpha$  and in combination with RBV cause a significant increase in sustained virologic response (SVR) (20).

Ribavirin has some major properties in treatment such as direct inhibition of HCV proliferation, make a mutation in virus proliferation process and induce of increase in Th1 cells in immune system, but monotherapy by RBV cannot decrease the viral load in treatment duration (21-23).

The genetic susceptibility, ethnic differences, environmental factors and virus factors such as virus load and virus genotype have influence on response or resistance to therapy with Interferon (24). In recent studies showed the kind of virus genotype has an important role in the response rate to the therapy; as a result, specifying that affects choosing the drug and usage duration (25). HCV has seven genotypes and major genotype is genotype 1 (40-80%) (26). The performed studies showed that sever liver disease and also HCC may be associated with genotype 1. According to the obtained documents, HCV genotype 1 dose not respond to therapy as well as other genotypes (27-29).

Host factors are other reasons of Hepatitis C virus resisting the therapy. There are several host factors that affect the response to interferon therapy. These factors include age, sex, race, obesity, and degree of hepatic fibrosis and duration of illness (30-32). On the other hand, everybody's genetic factors have major roles in

HCV infection risk and next immune responses. Several studies showed the probability of a positive relation between incidence of HCV disease and the host genetic polymorphism (**Error! Bookmark not defined.**).

Some of these researches claim that some genetic polymorphism may affect on the process of HCV infection (33) and the response rate to current treatment (combination anti-viral drug ribavirin and peg-interferon alpha) is dependent on genotype differences (34-35). Many of biological and genetic factors can set the interferon alpha therapy. These factors are human leukocyte antigens (HLAs), cytokine polymorphism, and ethnicity (36-37). Some of the host MHC genes especially inflammatory cytokine genes have basic role for response to therapy (38). The type 1-alpha interferon receptors exist in most human cells and have at least two subgroups which are coded with IFNAR1 and IFNAR2 genes that directly affect the signal transduction (39-41). Many of genes were identified by SNP analysis methods in disease, used for diagnostic methods as genetic markers (42-44). Investigations showed that single nucleotide polymorphisms (SNPs) of interferon receptor genes can set the alpha interferon therapy (45-46). Genetic tests have done on sequence of genes to predict the relation between polymorphisms and response to the therapy.

## Materials and Methods

In this research, using molecular methods, SNPs in interferon receptor genes (IFNAR1, IFNAR2) in patients suffering from chronic Hepatitis C and also the relationship between them to response to combined therapy of interferon and ribavirin were investigated.

### Study population and Clinical characteristics

The studied population in this research included patients suffering from chronic Hepatitis C who were recruited according to the documents of signs, symptoms, lab data (LFT) and imaging from Digestive Disease Research Institute, Tehran University of Medical Science. 72 patients who were infected by Hepatitis C virus were selected in zero time (before treatment). After we started the treatment, 11 patients refused to participate in the course of subsequent treatment and omitted from the list. So the genome of 61 patients was studied. The study was approved by the Ethics Committee of Tehran University of Medical Science. Written informed consent was obtained from every selected subject.

### Combination therapy

The patients were treated by combination therapy with Pegylated-alpha interferon (Peg-IFN $\alpha$ ) and Ribavirin (RBV: anti-viral drug). Peg-IFN $\alpha$  was injected subcutaneously once a week and the injected dose was 180mg, and RBV capsules were prescribed daily according to the patient's weight. For patients less than 75kg, 1000mg capsules and for those more than 75kg, 1200mg capsules were prescribed for a period of 24 weeks.

### DNA extraction and TaqMan real time genotyping

Separation of Peripheral blood mononuclear cells (PBMCs) from whole blood of 61 patients who received Peg-IFN $\alpha$  and RBV was done. Then Genomic DNA was extracted and was purified from peripheral blood leukocytes using the QIAamp DNA Mini Kit (Qiagen). The kit was designed for rapid sedimentation of patients' DNAs from biological liquids such as blood with high purity and exactly according to the kit leaflet. The procedures were performed with high speed and accuracy in a short time. All of the processes of nucleic acid extraction were done in RNAase and DNAase free condition. Nanodrop machine (N 1000, Thermo Company) was used to assess the quality of DNA. Then Taq Man SNP genotyping test was performed with Real-Time PCR [ABi Real Time Prism 7900 (47,48)] for two SNPs: Ex 4-30GC and IVS1-4640GA related to interferon receptor genes (IFNAR1 and IFNAR2). Topologic and thermodynamic properties of primers were obtained and were confirmed with Gene Runner software. Final volume for one reaction in real-time PCR was 25 $\mu$ l, including TaqMan universal PCR Master Mix (12/5  $\mu$ l), TaqMan Genotyping Assay Mix (0/75  $\mu$ l), DNA Template (8  $\mu$ l) and DEPC (3/75  $\mu$ l). The PCR protocol was as follows: 10 minutes at 95°C for initial step followed by 40 cycles of 15 seconds at 92°C (Denaturation) and 1 minute at 60°C (Annealing/Extension).

### RNA extraction and RT-PCR for confirmation of response to treatment

The RNA extraction was performed by QIAamp viral RNA Mini Kit (Qiagen). Then with the use of one step RT-PCR Kit (Qiagen), reverse transcriptase reaction and PCR were done at the same time. Final products were loaded on 2% Agarose gel electrophoresis and were studied with UV spectroscopy.

### Statistical analysis

Results were analyzed using SPSS software (version 18, SPSS, Chicago, IL, USA). P value of <0.05 was considered as significant. Mean  $\pm$  SD and relative frequency were used for quantitative and qualitative traits, respectively. SHAPIRO-WILK test was used to compare quantitative traits in subjects, before and after the treatment. Mean of quantitative traits in both groups was compared by t-test. The association of SNPs with the respond to therapy was tested using  $\chi^2$  analysis.

### Results

We conducted a case-control association study comprising 61 HCV-infected patients in two groups, before and after combination therapy to determine the relation between response to treatment and the kind of viral genotypes and IFNAR Gene SNP polymorphisms.

The first results of PCR experiment were consisted of 20 patients, which are displayed in diagrams in figure 2 which is included SNP proliferation diagrams and their proliferation to allele analysis. Every SNP has two alleles, IFNAR1 contains C and G alleles and the frequency of G allele is more than C and IFNAR2 has G and alleles that the frequency of G is more than A. To be more accurate, in this study, a negative control sample was evaluated for each SNP too.

To determine the response to therapy, 61 plasma samples of patients were selected for this study and RNA was extracted exactly according to RNA kit with QIAam Viral RNA Mini Kit (Qiagen). Then with using One Step RT PCR kit both RT and PCR were performed with materials and schedule at the same time. After that, the final product of PCR was studied on 2% agarose gel with ultraviolet light. As it is obvious in figure 1, for example, it is specified that only three of 35 patients that PCR was done for their plasma samples (no. 6, 8, 26) were positive in the case of having HCV viral genome which shows that they are resistant to combined therapy of Interferon and Ribavirin, and there is no response to therapy. Samples of other patients (32 patients) were negative in the case of having HCV viral genome, which indicates that these patients have responded to the combination therapy of Interferon and Ribavirin (Table 1).

Overall, in this research 72 patients with chronic Hepatitis C who received combination therapy of Interferon and Ribavirin were investigated and 11 of them did not take part in next treatments and were omitted from the research. Statistical analysis was performed on 61 patients. As mentioned in table1, 49 of them were men (%80.32) and 12 of them were women

(%19.67). The patients had a range of 22 to 65 years old, and average was 41.45 years. Their weight average was 77.40kg. Among these 61 patients, 29 of them had genotype-1a (%47.5), 27 of them had genotype-3a

(%44.3) and 5 of them had genotype-1b (%8.2) of Hepatitis C Virus, those 22 patients who had genotype-1a and all individuals who had genotype-1b and genotype-3a responded to the treatment.

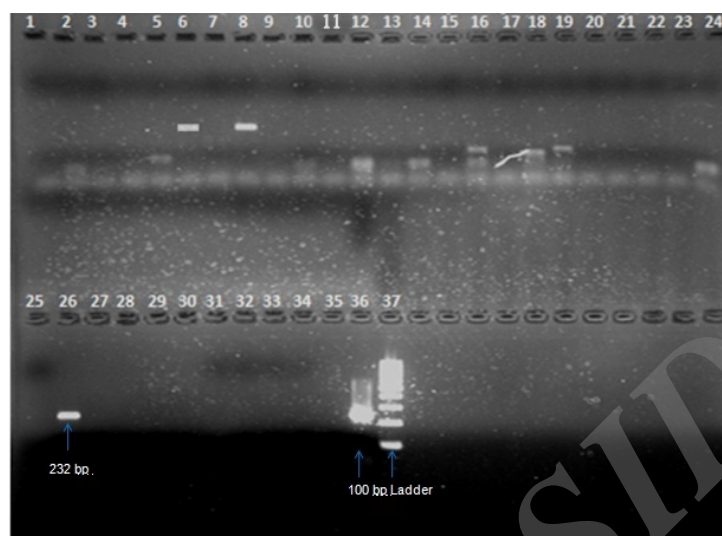


Figure 1. Patients' samples no.1 to 35- positive control no.36 –ladder no.37

Table 1. Data of 61 patients suffering from chronic Hepatitis C

SNP2	SNP1	Response to therapy	HCV Genotype	AGE	SEX	NO	SNP2	SNP1	Response to therapy	HCV Genotype	AGE	SEX	NO
NS	NS	Neg	3a	49	M	32	Hetero	Hetero	Pos	1a	44	M	1
Homo	Hetero	Pos	1a	51	M	33	Homo	Hetero	Neg	1b	52	M	2
Hetero	Hetero	Neg	3a	25	M	34	Homo	Hetero	Neg	3a	36	M	3
Homo	Homo	Neg	1a	58	M	35	Homo	Homo	Neg	1a	57	M	4
NS	NS	Neg	1b	56	F	36	Hetero	Hetero	Neg	3a	57	F	5
Hetero	Hetero	Neg	1a	57	F	37	Homo	Homo	Neg	3a	40	M	6
Hetero	Hetero	Neg	3a	43	M	38	NS	NS	Neg	1a	50	M	7
NS	NS	Neg	3a	65	F	39	Homo	Hetero	Neg	3a	30	M	8
Homo	Hetero	Neg	1a	37	M	40	Homo	Hetero	Neg	3a	26	M	9
Hetero	Hetero	Neg	3a	33	F	41	Hetero	NS	Neg	1a	40	F	10
Hetero	Hetero	Neg	1a	52	M	42	Homo	Hetero	Neg	1a	31	M	11
Hetero	Hetero	Neg	1a	47	F	43	Hetero	Hetero	Pos	1a	42	F	12
Hetero	Hetero	Neg	3a	44	F	44	Homo	Hetero	Neg	1a	32	M	13
Homo	Hetero	Neg	1b	24	M	45	Hetero	Hetero	Neg	3a	61	M	14
Homo	Hetero	Neg	1a	34	M	46	Homo	Homo	Neg	1a	32	M	15
Homo	Homo	Neg	1a	48	M	47	NS	NS	Neg	1a	34	M	16
Homo	Hetero	Neg	3a	39	M	48	Homo	Homo	Neg	1a	46	M	17
Hetero	Hetero	Neg	3a	22	M	49	Homo	Hetero	Neg	1a	29	M	18
Hetero	Hetero	Neg	3a	40	M	50	Homo	Hetero	Neg	3a	35	M	19
Homo	Homo	Neg	3a	30	M	51	Hetero	Hetero	Neg	3a	34	M	20
Homo	Hetero	Neg	1a	40	M	52	Homo	Homo	Pos	1a	56	M	21
NS	NS	Neg	3a	32	M	53	Hetero	Hetero	Neg	3a	35	M	22
Homo	Hetero	Pos	1a	33	M	54	Hetero	Hetero	Neg	1a	27	F	23
Hetero	Hetero	Neg	1a	31	M	55	NS	NS	Neg	3a	38	F	24
Hetero	Hetero	Pos	1a	42	M	56	Homo	Homo	Neg	3a	43	M	25
Homo	Homo	Neg	3a	35	M	57	Homo	Hetero	Neg	1a	36	M	26
Homo	Hetero	Pos	1a	60	M	58	Homo	Hetero	Neg	3a	31	M	27
Homo	Hetero	Neg	1b	65	F	59	Homo	Hetero	Neg	3a	31	M	28
NS	NS	Neg	1a	50	M	60	Homo	Homo	Neg	3a	39	M	29
Homo	Hetero	Neg	3a	57	M	61	Hetero	Hetero	Neg	1a	32	M	30
							Homo	Hetero	Neg	1b	52	M	31

NS: No SNP

Association of SNPs in interferon receptor gene

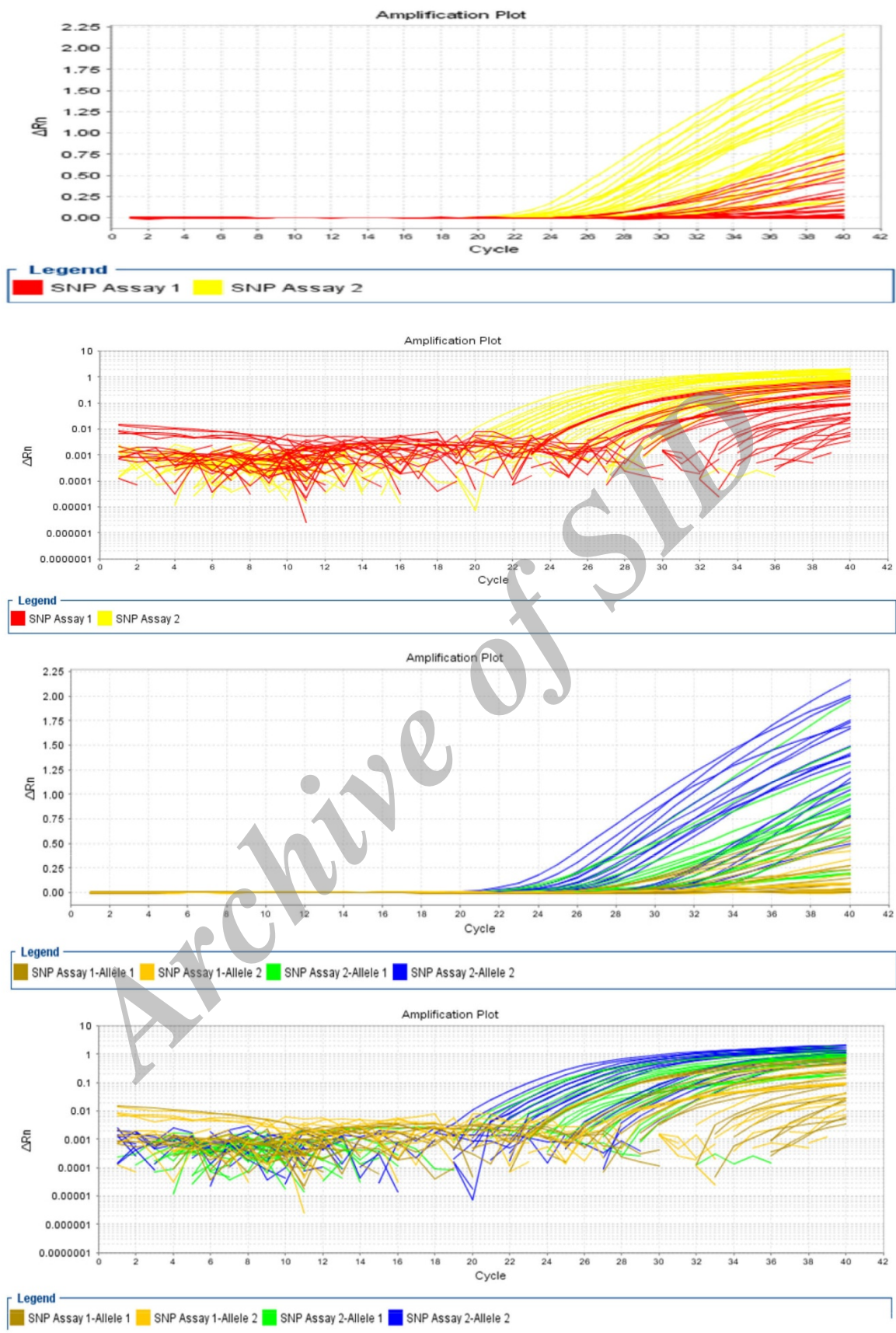


Figure2. Two first diagrams are the results of SNPs proliferations and two others are the results of their proliferation with allele's analysis

54 of the patients (%88.5) responded to the therapy and 7(%11.5) of them did not. Then using SPSS software, single nucleotide polymorphism Ex4-30GC in IFNAR1 gene and IVS1-4640GA in IFNAR2 gene and the relation between response to Interferon combination therapy and Ribavirin in chronic Hepatitis C patients was investigated. According to the results, there was no significant relation between investigated polymorphisms and response to the combined therapy ( $p=0.7$ ). The number and percentage of homozygote and heterozygote SNPs among patients were shown in table 2.

**Table 2. Number of homozygote and heterozygote percentage of patients in SNPs**

<b>Homozygous</b>	11(%18)	33(%54/1)
<b>Heterozygous</b>	41(%67/2)	20(%32/8)
<b>No SNP</b>	9(%14/8)	8(%13/1)

In this study the relation between HCV genotypes, sex, and age of patients with their response to the Interferon combined therapy and Ribavirin was evaluated with using SPSS software. Investigation and analysis of the results have shown that there is no significant relation between sex ( $p=0.7$ ), age ( $p=0.2$ ) and the response to the therapy, but there is a significant relation between genotype-3a of virus and response to Interferon combined therapy and Ribavirin ( $p=0.02$ ). That means patients who were infected by HCV genotype-3a respond better to the therapy than those that were infected by genotype-1a and genotype-1b.

## Discussion

In this study, we aimed to explore the association of the polymorphisms of the Interferon receptor gene and HCV genotypes with the response rate to combination therapy in Chronic HCV infected Iranian population.

Morgan *et al.*, in 2008 studied the effect of 8 polymorphisms among 638 patients with interferon therapy and showed that the gene polymorphisms were related to interferon treatment in IVSS1-22G of IFNAR1 gene (**Error! Bookmark not defined.**).

In another study they worked on 56 polymorphisms in 13 genes which were involved in interferon pathway and showed that the polymorphisms (IVS1-22G and Ex2-33 C) in IFNAR1 and IFNAR2 genes are associated with response to anti-viral therapy (49).

Matsuyama *et al.* in 2003 showed a relation between a microsatellite in IFNR gene receptor and response to therapy (50).

In this research we assumed that SNPs can cause

changes in cellular immune response affecting the tending of receptors to alpha-interferon molecule, but the results showed that there is no significant relation between single nucleotide polymorphisms (Ex4-30 GC) and (IVS1-4640 GC) in IFNAR1 and response to combined therapy of Interferon and Ribavirin .

We found a significant association between viral genotype-3a and response to combined therapy of Interferon and Ribavirin. This means that patients infected with HCV genotype-3a have better responded to therapy than those infected with genotype-1a and genotype-1b. We hope we could find patterns of resistant gene, as a result of our study, for better practical treatment strategy to cure the Iranian patients. In this study Genotyping has done by TaqMan PCR to find SNPs and Real Time PCR has done by ABi Prism 7900 HT, which can analyze both color types, specify the color quantity and determine the genotypes for large number of samples in just few minutes (51). So we have suggested that studying of other SNPs in alpha interferon receptor (one SNP for IFNAR1 and four SNPs for IFNAR2) and their relation with response to Interferon and Ribavirin therapy is needed.

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