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Interferon Lambda 4 Gene (IFNL4) Linked to Hepatitis C virus clearance, treatment

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ABSTRACT

Background: A designated IFNL4 gene, encoding the interferon-λ4 protein (IFNL4), which is moderately similar to IFNL3, is more strongly associated with HCV clearance in individuals of African ancestry, whereas it provides comparable information in Europeans and Asians.

Aim of the work: The study was attempted for the identification of interferon Lambda 4 (IFNL4) gene expression in the liver biopsy and the recombinant IFNL4 protein in the serum of CHCV patients.

Patients and methods: Eighty five patients with chronic hepatitis C virus infection (CHCV), whose age ranged between 19 and 57 years, were selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, before chronic HCV therapy, during the preparation of patients, and ten healthy individuals were included to serve as controls. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigations, CBCs. Liver biopsy was done to all patients and controls. Patients revealed mild fibrosis (Metavir fibrosis from F1 to F3). Using freshly frozen liver biopsies to identify gene (IFNL4) by real time-PCR and the detection of its serum protein levels by ELISA.

Results: Patients with CHCV have higher hepatic expression of IFNL4 before treatment and also recombinant IFNL4 protein expression was detectable in serum with high levels.

Conclusion: An inducible human protein-coding gene IFNL4, which is related to, known IFNs have been identified in genotype 4 CHCV patients.

Recommendations: The therapeutic inhibition of IFNL4 might represent a novel biological target for the treatment of HCV and HBV infection and possibly other diseases.

Keywords: HCV, IFNL4, clearance of HCV.

INTRODUCTION

Approximately 130-150 million people are estimated to be infected with HCV. Each year, an estimated 700 000 people die from HCV-related complications including fatty liver (cirrhosis), cancer (hepatocellular carcinoma) and liver failure. Unfortunately, many people with HCV only learn about their infection when they develop symptoms from cirrhosis or liver cancer [(1)]. Results from genome-wide association studies (GWAS) identified common inherited genetic markers that were associated with response to hepatitis C virus treatment and spontaneous clearance of the infection. Those markers are located on chromosome 19 near a known interferon gene, IFNL3. The National Institutes of Health (NIH), a division of the U.S. Department of Health and Human Services (HHS), announced in a press release dated 1/6/2013 that scientists from the National Cancer Institute (NCI) have discovered a gene mutation that affects the treatment of the hepatitis C virus [(2)]. The variant in this gene is directly responsible for the creation of the identified protein [(3)]. The gene belongs to what is a family of four interferon-lambda protein-encoding genes, three of which were discovered more than ten years ago (IFNL1, IFNL2 and IFNL3). The mechanism by which the IFNL4 protein impairs hepatitis C virus clearance remains unknown [(2)].

Single nucleotide polymorphisms (SNPs) in the interferon lambda 4 (IFNL4) - gene are predictors for treatment success in patients with hepatitis C virus (HCV) infection. For direct acting antiviral (DAA) combinations only weak

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association with IFNL4 SNPs was observed. Little is known about potential selections of resistance-associated variants (RAVs) by the IFNL4 genotype. The NS5A RAV Y93H is significantly associated with the presence of beneficial IFNL4 SNPs and a high baseline viral load in HCV Genotype 1-infected patients, which may explain a lack or even inverse correlation of treatment response with IFNL4 genotype in some NS5A inhibitor containing IFN-free regimens [(4)]. HCV infection is proposed to induce a more efficient antiviral response in individuals with the IFNL4 TT/TT genotype that result either in viral clearance or selection for viral adaptions. The association between IFNL4TT/TT genotype and Y93 substitutions may impact the risk of antiviral resistance in NS5A inhibitors in DAA therapy. Interaction between host genotype and amino acid substitutions might also influence the risk of antiviral resistance in interferon-free direct acting antiviral (DAA) therapies [(5)]. The dinucleotide variant ss469415590 (TT/ΔG) in a gene IFNL4 was identified as a stronger predictor of hepatitis C virus clearance in individuals of African ancestry compared with rs 12979860. The IFNL4 ss154949590 TT/TT genotype favors spontaneous clearance of HCV. IFNL4 is associated with treatment-induced clearance in patients with genotype 1b, but not 2a. ss469415590 (or rs 12979860) genotyping should be considered for patients with HCV genotype 1b and high viral load when making a choice between standard dual therapy and an IFN-free direct-acting antiviral regimen [(6)].

Prokunina-Olsson *et al.* [(7)] postulated that they performed RNA sequencing in primary human hepatocytes activated with synthetic double-stranded RNA to mimic HCV infection. Upstream of IFNL3 (IL28B) on chromosome 19q13. And that they discovered a new transiently induced region that harbors a dinucleotide variant ss46915590 (TT or ΔG), which is in high linkage disequilibrium with rs12979860, a genetic marker strongly associated with HCV clearance. Ss469415590 (ΔG) is a frameshift variant that creates a gene, designated IFNL4, encoding the interferon -λ4 protein (IFNL4), which is moderately similar to IFNL3. Compared to rs 12979860, ss469415590 is more strongly associated with HCV clearance in individuals of African ancestry, although it provides comparable information in Europeans and Asian [(7)]. It is thought that IFNL4 could lead to the development of a new universal genetic test to guide treatment in all ethnicities [(8)]. The study was attempted for the identification of interferon Lambda 4 (IFNL4) gene expression in the liver biopsy and the recombinant IFNL4 protein in the serum of CHCV patients.

Ethical consideration

Informed consent was obtained from each patient and control. The Research Ethical Committee of the General Organization for Teaching Hospitals and Institutes approved the study protocol. Liver biopsy was taken from healthy subjects during liver donation.

PATIENTS AND METHODS

Eighty five patients with chronic hepatitis C virus infection (CHCV), whose age ranged between 19 and 57 years, were selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, before hepatitis C therapy during the preparation of patients, and ten healthy individuals were included to serve as controls. All the patients and controls were subjected to the following: history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigations, CBCs. Liver biopsy was done to all patients and controls. Patients revealed mild fibrosis (Metavir fibrosis scores from F1 to F3).

Using freshly frozen liver biopsies to identify (IFNL4) gene by real time-PCR and the detection of its serum protein levels by ELISA.

Detection of Interferon Lambda-4(IFNL4) in serum by ELISA:

Reagent preparation:

Reagent A and B-Dilute to the working concentration using Assay diluent A and B (1:100), respectively.

Wash Buffer-if crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30mL of wash buffer concentrate into deionized or distilled water to prepare 750 mL of wash buffer.

Standard Preparation: Catalog No: E2028H. Reconstitute the standard with 1.0 mL of sample diluent. This reconstitution produces a stock solution of 1000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle prior to making serial dilutions (making serial dilution in the wells directly is not permitted). The undiluted standard serves as the high standard (1000 pg/mL). The sample diluent serves as the zero standard (0 pg/mL).

Assay procedure

Allow all reagents to reach room temperature (do not dissolve the reagents at 37 °C directly). All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at-20 °C until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments. 1-Add 100 uL of Standard, Blank, or Sample per well. Cover with plate sealer. Incubate for 2 hours at 37°C. 2-Remove the liquid of each well, don't wash. Add 100 uL of detection reagent A working solution to each well. Cover with the plate sealer. Incubate for 1 hour at 37°C. Detection reagent A working solution may appear cloudy. Warm to room temperature and mix gently until solution appears cloudy. Warm to room temperature and mix gently until solution appears uniform. 3-Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with wash buffer (approximately 400 uL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1-2 minutes. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels. 4-Add 100 uL of detection reagent B working solution to well. Cover with a new plate sealer. Incubate for 1 hour at 37°C. 5-Repeate the aspiration/wash process for 5 times as conducted in step 3. 6-Add 90 uL of substrate solution to each well. Cover with a new plate sealer. Incubate within 15-30 minutes at 37°C. Protect from light. 7-Add 50 uL of stop solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. 8-Determine the optical density of each well at once, using a microplate reader set to 450 nm [(9)].

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) in the liver biopsy

Total RNA was extracted from each sample using Qiagene kit (USA) according to a standard protocol) with catalogue number 04053228005940. The isolated total RNA was converted into complementary DNA (cDNA) using Moloney murine leukemia virus (M-MLV) reverse transcriptase kit (Qiagene , USA) with catalogue number 04053228014195 .Real-time PCR was performed using Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and an SYBR® Green PCR Master Mix (Qiagene ,USA) with catalogue number 204141 in a final volume of 10 µl with the following thermal cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The sequences of PCR primer pairs used for each genera shown in Table 4. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the beta actin genes as an invariant endogenous control (reference gene). The relative quantification was then calculated by the expression $2^{-\Delta\Delta Ct}$ [(10)].

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data was summarized using mean, standard deviation, median, minimum and maximum in quantitative data. Comparisons between quantitative variables were done using the non-parametric Mann-Whitney test [(11)]. Correlations between quantitative variables were done using Spearman

correlation coefficient [12]. ROC curve was constructed with area under curve analysis performed to detect best cutoff value of Interferon Lambda 4 for detection of cases. P-values less than 0.05 were considered as statistically significant.

RESULTS

The study included 85 patients with CHCV infection, before receiving treatment and 10 healthy controls.

We found that the mean level of serum IFNL4 in CHCV patients =39.43 which show statistically significant difference between the controls ($p< 0.05$) (table 1 and figure 1).

The mean level of IFNL4 in the liver biopsy = 21.27 which was significantly higher in CHCV patients than the healthy controls ($p=0.001$) (table 1 and figure 2).

Table (2) shows that there's no correlation between serum and biopsy levels of IFNL4.

Figure (3) illustrates the ROC curve for detection of IFNL4 gene levels in CHCV patients liver biopsy.

Table (3) illustrates the best cut off value = 1.14, sensitivity = 82.4%, specificity = 90%, with significant value (p value = 0.001) of IFNL4 gene in CHCV patients liver biopsy.

Table (4) shows the sequence of the primers used for measuring the real – time PCR.

Figure (4) illustrates the real-time amplification ploto quantitative PCR curve of lambada 4 gene in the liver biopsy of CHCV patients before treatment.

Table (1): The Comparison between CHCV patients and healthy controls as regarding IFNL4 gene levels in serum and liver biopsy.

Variables	Patients		Control		P value
	Mean	SD	Mean	SD	
serum Interferon Lambda 4	*39.43*	*8.27*	*49.85*	*4.12*	*0.033* *S
biopsy Interferon Lambda 4	*21.27*	*4.21*	*1.04*	*.10*	*0.001* *S

Statistically significant difference between both groups as regard (IFNL4 serum and liver biopsy).

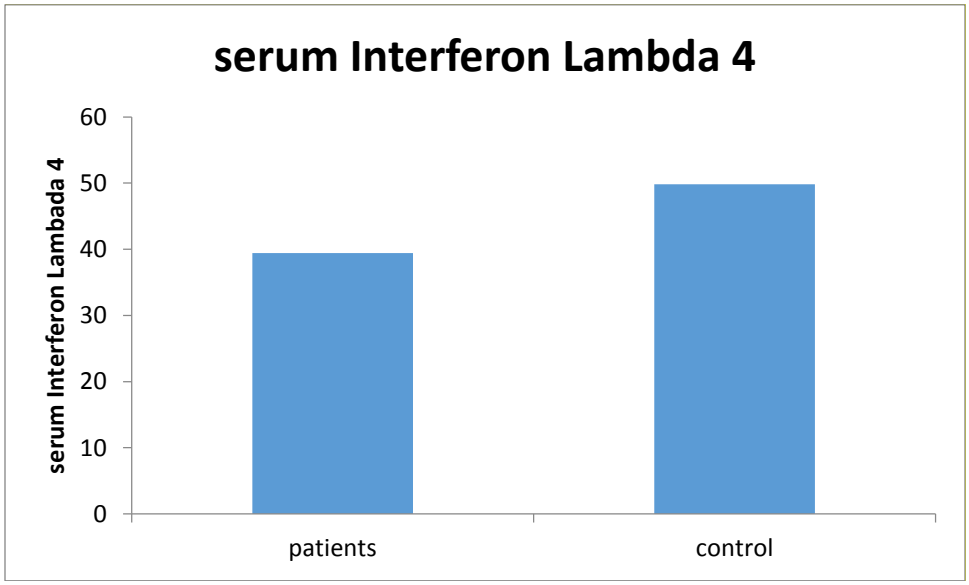

Figure (1): The comparison between CHCV patients and controls as regarding IFNL4 levels in serum.

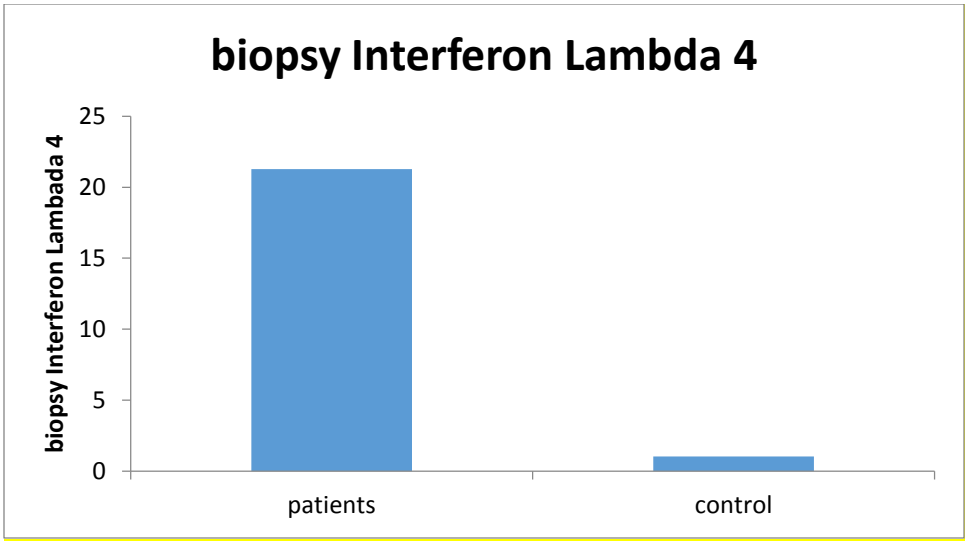

Figure (2): The comparison between CHCV patients and controls as regarding IFNL4 gene levels in liver biopsy.

Table (2) The Correlation between serum and biopsy levels of IFNL4 gene in CHCV patients.

Variables		serum Interferon Lambda 4
biopsy Interferon Lambda 4	Correlation Coefficient	*.054*
	P value	*.625*NS*
	N	*85*

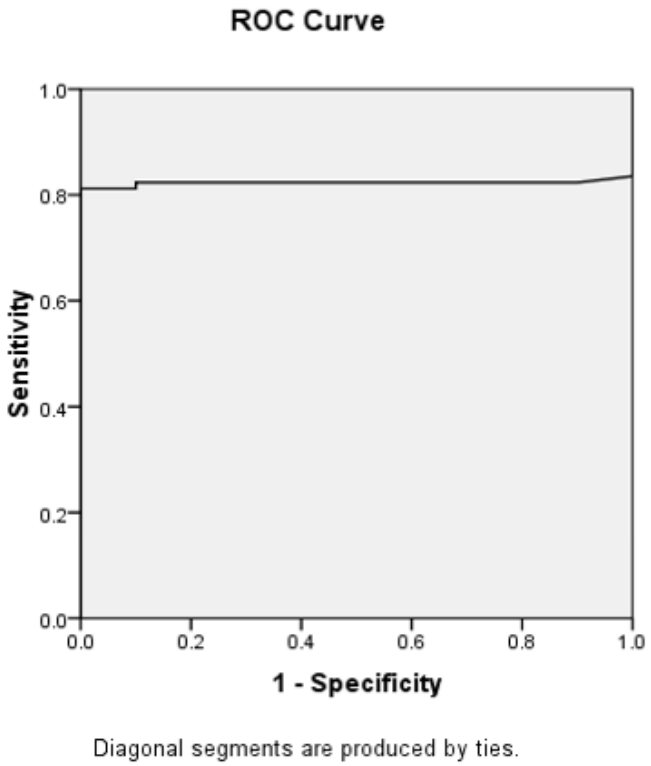

Figure (3): The Roc curve for detection of IFNL4 gene levels in CHCV patients liver biopsy.

Table (3): Shows the best cut off values, sensitivity %, specificity % with significant p value of IFNL4 gene in CHCV patient liver biopsy.

Area under curve	P value	95% Confidence Interval		Cutoff value	Sensitivity (%)	Specificity (%)
		Lower Bound	Upper Bound			
.823	*.001*S*	*.742*	*.904*	*1.14*	*82.4*	*90*

Table (4): Sequence of the primers used for real-time PCR.

Variables	Primer sequence	Gene bank accession number
IFN Lambda 4	Forward primer :5'-GAAGCCTCAGGTCCCAATTC3-3 Reverse primer :5'-AGTTCCGGGCCTGTATCCAG-3	NM_172138
β Actin	Forward primer :5'-TCATCACCATTGGCATGAG-3 Reverse primer :5'-AGCACTGTGTTGGCGTACAG-3	XM_172138

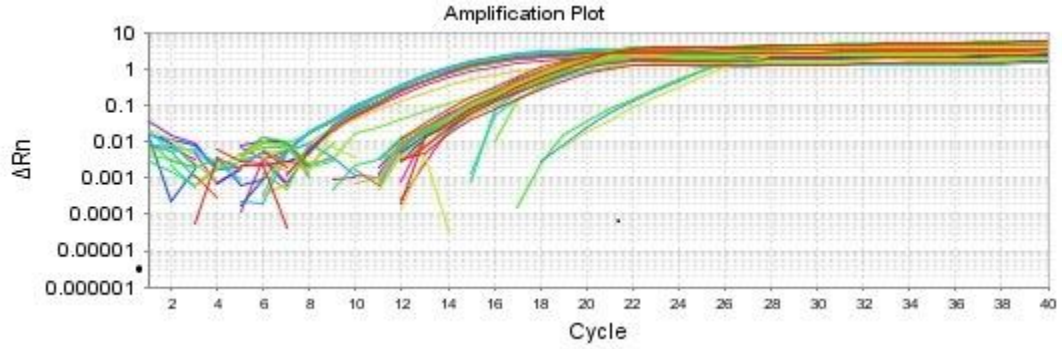

Figure (4): The Real-Time Amplification Plots quantitative PCR curve of Lambda 4 Gene in the liver biopsy of CHCV patients before treatment.

DISCUSSION

Egypt faces the largest burden of HCV infection in the world with a 10 % prevalence of chronic hepatitis C infection among persons aged 15-59 years, predominatly genotype 4 [13]. The primary goal of HCV therapy is to cure the infection. The infection is cured in more than 99% of patients who achieve an SVR. The sustained virological response is generally associated with resolution of liver disease in patients without cirrhosis. Patients with cirrhosis remain at risk of life-threatening complications; however hepatic fibrosis may regress and the risk of complications, such as hepatic failure and portal hypertension is reduced. Recent data suggest that the risk of HCC and all-cause mortality is significantly reduced, but not eliminated, in cirrhotic patients who clear HCV compared to untreated patients and non-sustained virological responders [14, 15]. HCV may also affect neurogonition and effective viral suppression is associated with reversal of cerebral magnetic response abnormalities [16]. IL28B gene polymorphism is the best baseline predictor of response to interferon alfa-based antiviral therapies in chronic hepatitis C. IFN-L4 polymorphism was identified as first potential functional variant for induction of IL28 expression. Individualization of interferon alfa-based therapies based on a combination of IL28B/IFN-L4 polymorphisms may help to

optimiz virologic outcome and economic resources [17].

In our study, we observed that IFNL4 gene have been shown to be expressed at higher levels in pretreatment liver biopsies from HCV-infected patients. Studies found that patients with chronic hepatitis C who carry rs12979860-T, which marks the ss469415590-ΔG allele, have somewhat higher hepatic expression of ISGs before treatment [18-22]. Prokunina-Olsson *et al.* discovered that IFNL4 gene have been shown to be expressed at higher levels in pretreatment liver biopsies from HCV-infected patients [7]. The mechanisms by which IFNL4 induces STAT1 and STAT2 phosphorylation, activates the individual interferon-stimulated response element (ISRE), Luc reporter and interferon stimulated genes (ISGs), and generates antiviral response in hepatoma cells. Thus, it is possible that IFNL4 activates JAK-STAT signaling through a unique receptor complex consisting of IFNLR1 and a currently undefined second receptor chain or that IFNL4 functions as a decoy cytokine competing with type-III IFNLs for binding of IFNLR1. We also found the IFNL4-caused pre-activation of interferon signaling prevents further activation by type-I and Type-III IFNLs [7].

In the present work, we found that there's significant difference between CHCV patients and healthy controls as regarding serum IFNL4 levels that the mean level in controls 49.85 is more than in the patients samples= 39,43. Langhans *et al.* [23] explained that IL-29 (IFN-lambda 1)serum levels exceeded IL-28 A/B (IFN-lambda 2/3) at least twofold, with IL-29 and IL-28 A/B levels being significantly higher in carriers of the rs 12979860 C allele than in TT homozygous individuals ($p<0.02$). IL-29 levels were substantially lower in patients with chronic hepatitis C than in healthy controls ($p=0.005$) and patients with spontaneously resolved hepatitis ($p=0.001$). And suggested that HCV proteins modify IFN-lambda production in DCs (dendritic cells) carriers of the rs 12979860 C allele associated with resolution of HCV infection exhibited increased IFN-lambda levels. Moreover, high IFN-lambda levels predisposed to spontaneous resolution of HCV infection. Thus, IFN-lambdas seem to play an important role in control of hepatitis C. DCs cells trigger adaptive immune responses and are an important source of antiviral cytokines. In hepatitis C virus DCs function is markedly impaired. Fischer *et al.* and Jo *et al.* [24, 25] found that by sequencing IFNL4 in 270 HapMap samples. In all populations, the unique favorable haplotype included the ss469415590- TT allele, which abrogates the IFNL4 protein. The unfavorable ss469415590-ΔG allele was found on a number of haplotypes, including two haplotypes that were reported as being neutral in Europeans despite carrying the unfavorable rs1297986-T allele, these two haplotypes include minor alleles of either non-synonymous variants rs73555604 or rs11764844. It is possible, therefore, that these variants modify the risk in carriers of the unfavorable ss469415590-ΔG allele and are the source of haplotype heterogeneity in Europeans [24, 25] howevere, data from Virahep C in African-American are too sparse to confirm this finding [7]. Genotyping for ss469415590 (IFNL4-ΔG) and rs12979860 was performed at the laboratory of Translational Genomics, National Cancer Institute with custom TaqMan allellic discrimination genotyping assays for quality control, blinded duplicate specimens were included in the panel; genotype concordance was 130 of 130 (100%) for IFNL4- ΔG and 129 of 130 (99.2%) for rs12979860 [7]. Bibert *et al.* [26] have attributed the effect of IFNL4-ΔG to decreased induction of IFNL3 and CXCL10 (IP-10), but it is unclear how that might explain the association of IFNL4-ΔG with lower HCV RNA levels in untreated patients. The proportion of women with cleared HCV infection varied markedly by race/ethnicity and by IFNL4-ΔG genotype [27].

Native human interferon lambda proteins are generated by the immune system in response to viral infection. This interferon family has been found to have antiviral activity against HCV. This interferon and its receptor are both expressed at high levels by hepatocytes but not all tissues suggesting that this reagent could have tissue specific effects, potentially equating to reduced toxicity compared to current experience with alpha interferon [28]. IFN-L4 seems to be the best single predictor of SVR in genotype 3 infected patients. For optimized prediction of SVR by treatment with dual

combination or first generation PI triple therapies, grouping of interferon-lambda haplotypes may be helpful with positive predictive values of 71-96% [17].

IFNs are part of a larger family of structurally related class-2 proteins that also includes six IL-10-related cytokines [28, 29]. This subset of cytokines can be grouped in the same family because they all signal via receptors that share certain common motifs in their extracellular domains [29-31]. These receptors comprise the class 2 cytokine receptor family. The IFN- λ proteins share common structural features with IL-10-related cytokines, particularly IL-22 [32, 33]; however, unlike IFN-λ, IL-22 does not induce antiviral activity in IL-22 receptor-positive target cells. This supports the functional classification of the IFN-λ proteins as a unique group of IFNs instead of a novel group of interleukins. The exon- intron structure of the IFN-λ, genes is similar to the organization of the genes encoding the IL-10-related cytokines [34, 35]. The high degree of sequence similarity between the IFN-λ, genes suggests that these genes evolved from a common predecessor relatively [35-37]. The sequence of IL29 is less similar to IL28A and IL28B than these genes are to each other. In addition, the segment that contained the duplicated IL29 was mutated significantly, which resulted in formation of a non-functional pseudogene, referred to as IFN-λ4ψ (or IFNL3) [34, 35]. Binding sites for several key transcription factors, including NF-kB and various IRF proteins, are present in the promoters of both the IFN-B gene (IFNB) and the IFN-λ genes [38-40]. The IFNB promoter contains several IRF-binding elements (IBE) (also known as "virus response elements" (VRE) or "positive regulatory domains" (PRD) that provide binding sites for phosphorylated IRF3 and/or IRF7 [41, 42]. Similar binding sites are also present in the promoters of the IFN-λ genes [38-40]. Therefore, it appears that the same set of transcription factors that regulate IFNB transcription also control expression of the IFN-λ, genes [43, 44]. The IFN-λs are usually co-expressed together with type-I IFNs by virus-infected cells virtually any cell type can express IFN-λ following viral infection [36, 37]. Furthermore, reports have shown that HCV infection also induces expression of IFN-λ [23, 45]. An inducible human protein-coding gene, IFNL4, which is related to known IFNs have been identified in genotype 4 CHCV patients.

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