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Genetic Variations in Interferon-Induced Genes and HCV Recurrence after Liver Transplantation

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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LIST OF ABBREVIATIONS

Full Name	Abbreviation
2',5'-Oligoadenylate Synthetase 1	OAS-1
2-5-oligoadenylate synthase	2,5A
Acute Rejection	AR
Beta-2-Microglobulin	B2M
Complementary DNA	cDNA
Cytomegalovirus Infection	CMV
Deoxyribonucleic Acid	DNA
Ethanol	EtOH
Hepatic Cellular Carcinoma	HCC
Hepatitis B Virus	HBV
Hepatitis C Virus	HCV
Human Immunodeficiency Virus	HIV
Interferon	IFN
Interferon Stimulated Gene-15	ISG-15
Interferon-Beta 1	IFNB-1
Liver Tissue Procurement and Distribution System	LTPADS
Non-Structural Proteins	NS
Open Reading Frame	ORF
Orthotopic Liver Transplantation	OLT
Phyenl:Chloroform:Isopropanol	PCI
Polymerase Chain Reaction	PCR
Quanitative Real Time-Polymerase Chain Reaction	Q-PCR
Reverse Transcription Polymerase Chain Reaction	RT-PCR
Ribonucleic Acid	RNA
Single Nucleotide Polymorphisms	SNPs
Threshold Cycle	
C_t	

ABSTRACT

GENETIC VARIANTS IN INTERFERON-INDUCED GENES AND HCV RECURRENCE AFTER LIVER TRANSPLANTATION

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

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Hepatitis C Virus (HCV) infection represents a worldwide pandemic and is currently the leading cause of cirrhosis and liver transplantation. After transplantation recurrence is almost universal with 96% of patients testing positive for viral RNA and exhibiting histological evidence of infection within the first year. Type I interferons (IFN) and interferon inducible genes are responsible for the innate antiviral state and single nucleotide polymorphisms (SNPs) within these genes may affect the patients ability to respond post-transplantation. We hypothesize the elucidation of associations between

SNPs in Type-I Interferon and Interferon inducible genes and HCV recurrence post-liver transplantation might help to identify HCV patients with different prognosis and improve liver transplant recipient selection. 100 HCV positive patients were genotyped using Allelic Discrimination on an ABI Prism 7700 sequence detector (Applied Biosystems) for SNPs in IFNB1, OAS-1, and ISG-15 to establish a relationship between SNPs and clinical complications post-transplantation. Quantitative real-time polymerase chain reaction (QPCR) was also run to determine the relationship between SNPs or disease state and the level of RNA expression. Results were collected and analyzed using Fishers exact test, Kaplan-Meir method, and the log-rank test. Results obtained indicated that SNPs in OAS-1 are associated with HCV recurrence within 12 months post-orthotopic liver transplantation (OLT) and OAS-1 SNP genotypes were significantly associated with the development of fibrosis within the first year. Additionally we observed an association between the SNP genotypes of OAS-1 and ISG-15 and CMV infection post-OLT. A significant distribution of ISG-15 genotypes was also found to correlate with acute rejection. These findings might help identify patients at high risk of developing complications within the first year.

INTRODUCTION

Hepatitis C Virus

Approximately 180 million people are infected with the hepatitis C virus (HCV) worldwide [1-3]. This represents a viral pandemic five times as widespread as human immunodeficiency virus (HIV) [4]. Although, the number of HCV cases over the last decade has declined due to improved blood screening methods, new cases continue to develop due to intravenous drug use. This important human pathogen causes chronic hepatitis in approximately 80% - 85% of infected individuals with 20% progressing to cirrhosis and 1-2% developing hepatocellular carcinoma (HCC) [5]. Interferon (IFN) alpha based therapies, the only therapies available for HCV patients, have been unable to eliminate viral replication in a significant percentage of the patients. Combination regimens of IFN alpha along with the nucleoside analogue Ribavirin are more successful than monotherapy, but overall response rates to treatment is 40-60% [6,7]. For those patients who fail to respond to antiviral drugs, there are currently no approved pharmacological therapies designed to delay or reverse the progression of fibrosis. In those cases, orthotopic liver transplantation (OLT) is the only viable option left for the patient. However, recurrence post-OLT is almost universal, leaving the majority of patients with only a mild form of the disease. As for the remaining patients they must deal with a more severe infection rapidly leading to cirrhosis and re-OLT. Several

studies have described the host genetic mechanisms behind recurrence and suggest single nucleotide polymorphisms (SNP) in host response genes may play a role.

Epidemiological Characteristics of HCV

HCV prevalence varies throughout the world depending upon region and risk factors in the society. In the United States there are an estimated 3.9 million people with HCV positive antibody and 2.7 million chronically infected [8]. HCV acute infection is usually characterized as mild or asymptomatic. Patients are able to achieve viral clearance in 15-20% of cases [9]. This is assumed to have occurred when viral ribonucleic acid (RNA) is no longer detectable in multiple blood samples of someone in whom acute infection was observed. Abnormal serum enzymes or the presence of HCV RNA persisting for more than six months indicates a chronic infection. Population data indicates that disease progression is slow and infected individuals can live 10-30 years with few symptoms. Yet, the risk of complications from chronic infection including end stage liver disease increases the longer a person lives. The rate of progression is highly variable and dependent upon a number of environmental and host factors [10].

Those most at risk are intravenous drug users and individuals receiving blood transfusions before 1992. Low income, lack of education, and numerous sexual partners are other risk factors associated with HCV positive patients [11]. Until recently the primary mode of transmission was through blood transfusions. Infections from blood transfusions are rare due to new screening assays developed in the 1980's and early 1990's, which significantly reduced transmission. Specifically the second-generation

antibody assay developed in 1992 reduced transfusion related transmission from 1.5% to nearly zero [12].

The debate as to whether or not HCV can be sexually transmitted results from conflicting evidence in different studies. HCV RNA has been found in seminal fluids, which makes transmission biologically plausible [13]. However, an American study of 599 black women found that the prevalence of HCV through sexual transmission was only 1.6% [14]. An increased risk of transmission often times results in combination with other risk factors such as unprotected sex, multiple partners, and anal intercourse. There does, however, appear to be a link between HCV and HIV transmission. Eyster et al. found that co-infected men were five times more likely to transmit both viruses than would be expected by coincidental transmission [15]. This suggests that HCV sexual transmission occurs but is inefficient.

Health care workers are another susceptible population as infection is an occupational risk when treating HCV positive patients. As a whole the health care community does not have an increased prevalence of HCV, but workers who treat such patients are at risk from needle stick injuries. Injuries caused by large bore needles and a high patient HCV RNA level carry the greatest risk [16].

Treatment

Early identification, treatment, and management of HCV generate the best results. Currently the main line of defense for chronic HCV is a combination of ribavirin and pegylated interferon alpha-2a or pegylated interferon alpha-2b. Each drug has a different

pharmacokinetic profile due to the variation in the polyethylene glycol molecule and is administered at different doses [20].

Patients who adhereto their prescribed treatment regimen 80% of the time experience a marked improvement, when compared to those who do not [21]. In spite of this, severe side effects may prevent total compliance and reduce the effectiveness of therapy. These effects can include psychiatric disorders, autoimmune syndromes, or hemolytic anemia. In the past, up to half of patients would discontinue therapy due to these types of symptoms; though, presently this number is closer to 20 – 30% [22]. Still, less than 50% of patients respond to medication leaving the majority in need of other options.

Liver Transplantation and HCV

At this time many of those infected are only beginning to reach the age at which they will experience liver complications. Over a period of 30 years, the risk cirrhosis increases substantially for both men and woman. Failure to effectively treat or manage HCV in patients' leads to decompensated liver disease along with an increased risk of developing HCC and the need for OLT. The primary indication for complications arising from HCV is OLT [17-19]. Unfortunately, this is not a cure in the majority of cases. The immunocomprimised state of the post-transplant patient allows for a high rate of HCV recurrence [23,24]. Recent data suggests that in approximately 20% of HCV-related OLT, allograft cirrhosis develops within 5 years [25-29]. Once cirrhosis has developed the risk of liver decompensation is 42% [30]. The current challenge for OLT remains

maintaining a sufficient donor pool and treating with medications before and after the transplant [30].

HCV related re-OLT increased significantly throughout the early 1990s, but there are no recent prevalence data to show if this trend will continue [31]. Patient survival after primary OLT for HCV infection throughout the 1990s was similar to that for most other indications for OLT [32-34]. However, Forman et al [35] showed an increased rate of death and allograft failure in HCV-positive compared with HCV-negative transplant recipients. In general, re-OLT has a lower survival rate than primary OLT [36,37].

Molecular Biology of The Hepatitis C Virus and the Host Response:

Poor outcomes following OLT for HCV disease have been associated with several host, viral, and non-host/non-viral factors. As is evident from the literature, there is confounding data in favor of and against these factors in the pathogenesis of severe recurrent HCV. Viral and host factors may contribute to the phenomenon that some patients with chronic HCV undergoing liver transplant progress recurrence and liver fibrosis post-OLT while others do not.

Upon infection pathogen associated molecular patterns presented by viral RNA induces numerous proteins, which contribute to the overall antiviral state of the cell. This includes transcribing IFN-beta 1 (IFNB-1), a potent cell messenger. IFNs, are the first-line defense against viral infections and propagation and key components of the host's innate antiviral immune response [38] [Figure 2]. The IFNs are a family of cytokines

produced by many host cell types in response to viral infection and other stimuli [39]. When cells bind these messengers, endogenous antiviral pathways are activated and prepare the cell to stop HCV from replicating. Many of these pathways have been shown to use the JAK/STAT signal transduction cascade. IFNB-1 binding its receptor induces phosphorylation of JAK, subsequently activating STAT via phosphorylation [40]. Once STAT is turned on it combines with other STATs to create homo or heterodimers (STAT 1 and/or 2) and translocates to the nucleus. Inside the nucleus STAT1/2 will bind with other proteins to become a functional transcription factor and stimulate interferon-stimulated genes (ISGs) including interferon stimulated gene-15 (ISG-15) and 2',5'-oligoadenylate synthetase 1 (OAS-1) [41].

Viral proteins interfere with the host signal transduction pathways thus creating a permissive environment. Similar to picornaviruses through their replication strategy, HCV translates one large open reading frame (ORF) [42-46]. A polyprotein of roughly 3,000 amino acids is encoded by viral polymerase, which with the aid of host cell proteases proteolytically cleaves the ORF resulting in ten distinct proteins [47]. These are grouped into structural proteins and non-structural proteins (NS). Structural proteins include core proteins and those that compose the envelope (E1 and E2). HCV utilizes these proteins to evade the innate immune response in a number of different ways. E2 contains hypervariable regions that mutate often in response to anti-HCV antibodies, which is potentiated by the high rate of mutation associated with RNA polymerase. The core proteins play a parallel role in evading host cellular responses by inducing protein inhibitors of the STAT making it unavailiable for deoxyribonucleic acid (DNA) binding

and transcription of essential ISGs [48]. Viral helicase, protease, and polymerase compose the NS grouping of proteins. They function in replication of the virus and propagation of the life cycle, but have also been shown to inhibit IFN-alpha and IFNB-1 expression [49].

Figure-1: Interferon Signaling Pathways

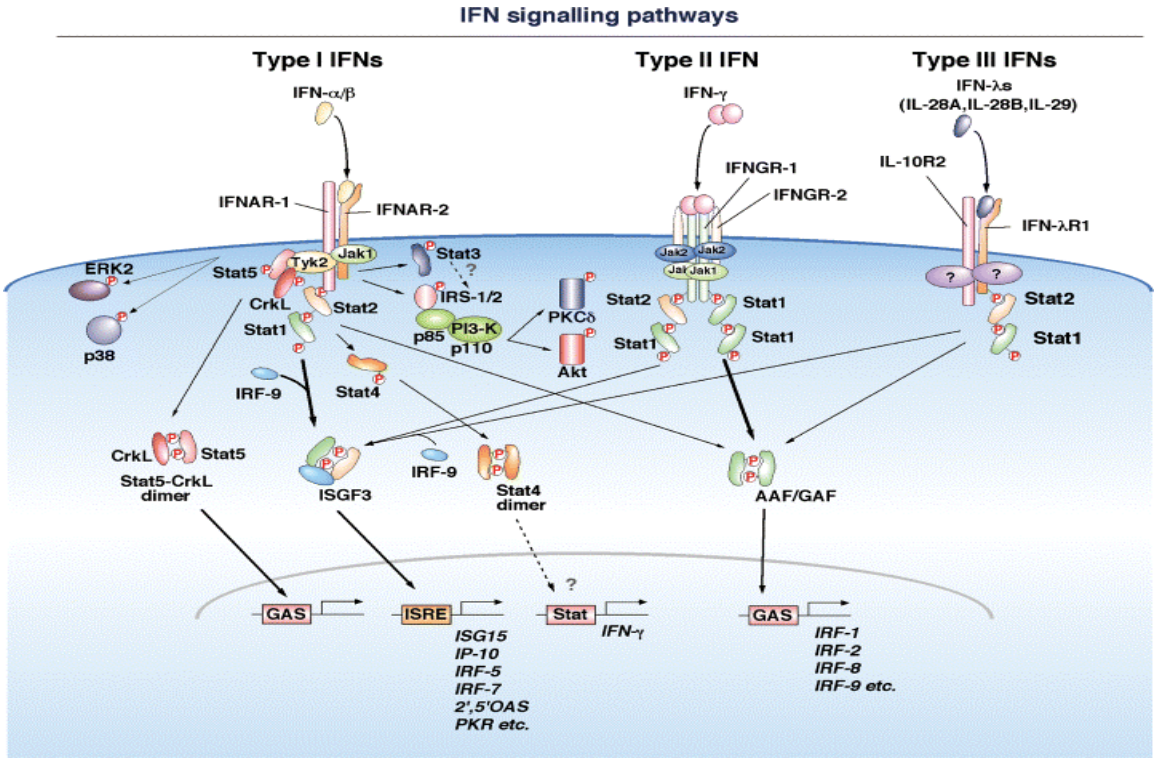
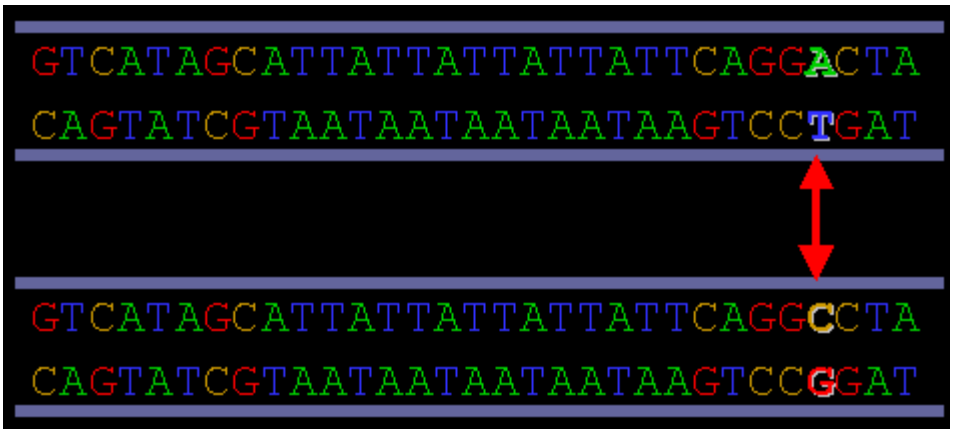

IFN signalling pathways
Type I IFNs
Type II IFN
Type III IFNs
IFN-α/β
IFN-γ
IFN-λs
(IL-28A,IL-28B,IL-29)
IFNAR-1
IFNAR-2
IFNGR-1
IFNGR-2
IL-10R2
IFN-λR1
ERK2
p38
Stat5
Tyk2
Jak1
Stat3
IRS-1/2
p85
PI3-K
p110
PKCδ
Akt
CrkL
Stat1
Stat2
Stat4
IRF-9
Jak2
Jak1
Stat5-CrkL
dimer
ISGF3
Stat4
dimer
AAF/GAF
GAS
ISRE
Stat
IFN-γ
ISG15
IP-10
IRF-5
IRF-7
2',5'OAS
PKR etc.
IRF-1
IRF-2
IRF-8
IRF-9 etc.

Figure-2: Single Nucleotide Polymorphisms


GTCATAGCATTATTATTATTATTCAGGACTA
CAGTATCGTAATAATAATAATAAGTCCTGAT
GTCATAGCATTATTATTATTATTCAGGCCTA
CAGTATCGTAATAATAATAATAAGTCCGGAT

Single Nucleotide Polymorphisms (SNPs)

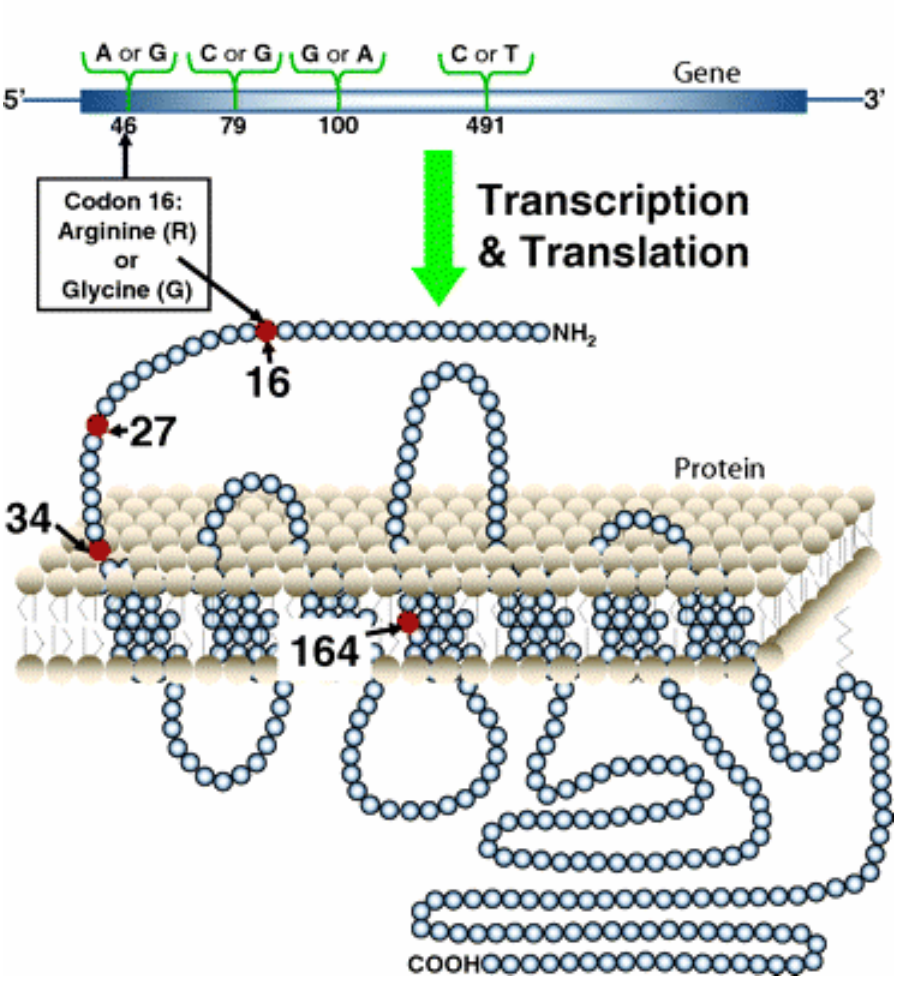
We are all the same! We are all different! Any two humans are approximately 99.9% identical at the DNA sequence level, yet substantial, often medically relevant phenotypic differences exist between individuals. A significant proportion of these phenotypic differences are caused by relatively small amounts of genetic variation interacting with environmental factors. A clinically important element of phenotypic variation relates to susceptibility to disease and response to therapy.

The variations in the DNA sequence that cause or contribute to disease are called either *mutations* or *polymorphisms*, based solely on their frequency in the population. By convention, DNA sequence variants that occur in > 1% of the population are termed *polymorphisms*, and those that occur in less than one percent of individuals are called *mutations*. Mutations are responsible for the relatively rare single-gene Mendelian disorders, while polymorphisms are associated with the more common complex genetic disorders. Mutations in DNA arise naturally or unnaturally (environmental exposure), and are not always disease causing (they are far more likely to occur in noncoding DNA than coding DNA because of the far greater number of base-pairs of noncoding DNA in the human genome). Variations in inherited DNA sequence between individuals can be due to the deletion or addition of bases, or to variable lengths of repetitive sequences within or between genes. However, the most common type of DNA sequence variants are SNPs in which a single base in the sequence is replaced by a different nucleotide. SNPs occur approximately every 200 to 300 base-pairs in the human genome. Since the genome

contains approximately 3 billion base-pairs, this means that there are between 10 to 15 million sites at which > 1% of the population differ from the majority. Although this seems like a large potential for diversity, simple arithmetic shows that even the most genetically diverse people are still at least 99.9% identical. If the density of SNPs was evenly spaced over the entire genome, this would mean that there are approximately 300,000 to 600,000 SNPs within the estimated 30,000 human genes. Many of these SNPs cause functional changes by affecting transcription factor binding sites, influencing splicing or stability of messenger RNA, or altering the amino acid sequence of the protein [Figure-3]. It is this variation that, in combination with environmental factors and epigenetic modification of DNA (epigenetic changes include methylation and demethylation of regulatory sequences and/or chemical modification on the histones that influence gene expression) that accounts for all of human phenotypic diversity, including disease susceptibility.

Figure-3: Relationship between SNPs and protein: ß$_2$-adrenergic receptor gene

Relationship between SNPs and protein: ß$_2$-adrenergic receptor gene. The ß$_2$-adrenergic receptor gene is shown from 5' to 3' with the coding nonsynonymous SNP sites indicated by their nucleotide position. At nucleotide position 46, there can be an adenine (A) or a guanine (G) that results in the 16th amino acid in the receptor being arginine or glycine, respectively (note: the genetic code is translated in "codons" of three nucleotide bases for every one amino acid). The site of the resulting amino acid in the extracellular portion of the receptor is indicated. Similarly, nucleotide substitutions of C to G, G to A, and C to T at nucleotide positions 79, 100, and 491 result in substitutions of glutamine to glutamate, valine to methionine, and threonine to isoleucine at amino acid positions 27, 34, and 164, respectively. Although these examples are for coding nonsynonymous SNPs that change the amino acid sequence, the vast majority of SNPs occur in non-amino acid coding regions of DNA where they have either no effect, or influence transcription factor binding sites, or messenger RNA splicing or stability.



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IFNB-1

A potent inflammatory cytokine, IFNB-1, is one the most well understood antiviral genes. An activator of the Jak-Stat pathway IFNB-1 regulates the transcription of thousands of effector genes [50]. Thus IFNB-1 represents a formidable barrier against viral infections. Machida et al. found that the HCV protein NS5A induces the expression of TLR4 leading to an enchance production of IFNB-1 and IL-6, thus HCV positive patients have shown an increased inflammatory and antiviral response [51].

At this point little is known about how polymorphisms affect IFNB-1. Morris et al. has looked at polymorphisms in IFNB-1 and its association with type I diabetes but found no correlation [52]. However, polymorphisms in other cytokines have been shown to have affects on the recurrence of HCV. Ben-Ari et al found that polymorphisms in IFN-gamma correlate with early recurrent hepatitis C after OLT [53]. The majority of patients without recurrence of HCV possess a polymorphism that leads to higher production of IFN-gamma [53]. While this is dealing with another cytokine entirely, it does suggest polymorphisms play a role in effecting immunity.

ISG-15

The antiviral effects of interferons are mediated through pathways that conclude with the production of interferon-stimulated genes. ISG-15 induction occurs early after interferon stimulation and functions to conjugate a large cohort of proteins. Constitutively present in higher eukaryotes it serves many functions including antiviral defense of the cell, as well as initiating the production of IFN-γ and augmenting the

proliferation and function of natural killer cells and lymphocytes [54]. ISG-15 can directly inhibit viral replication by arresting splicing of RNA in the nucleus [55]. Mainly, ISG-15 elicits cellular antiviral responses through conjugation of other proteins. Though one of the first interferon stimulated genes to be discovered little had been known about how ISG-15 conjugation affects target proteins. Current work on deciphering the crystal structure [56,57] has noted similarities between ubiquitin and ISG-15. Additionally, knowledge of its structure has given us insight in to how ISG-15 interacts with activating proteins. This information will be useful in the future when trying to understand how polymorphisms may affect ISG-15.

OAS-1

Another gene encoded from the interferon stimulated response element is the gene OAS-1. OAS-1 encodes the critical enzyme 2-5-oligoadenylate synthase (2,5A). This enzyme requires double stranded RNA structures, present in HCV, to become activated. ATP is polymerized to 2,5A in turn activating a ribonuclease, RNaseL. Ribonucleases are capable of degrading RNA thus hindering the virus's ability to replicate.

Several studies have demonstrated the effects of SNPs in OAS-1 and their effects on host's ability to respond to viral infections. Tessier et al. found an A/G polymorphism in the last nucleotide of intron 6 of OAS-1 to be associated with higher enzyme activity and was reported in subjects with type 1 diabetes when compared with healthy individuals [58]. Bonnevie-Nielson et al. found multiple polymorphisms within OAS-1 and studied their effects on viral pathogens [59]. They observed that an A/G

polymorphism on the splice site of exon 7 yields three different genotypes, AA, GA, and GG. These genotypes were shown to generate three different forms of the 2,5A protein. An association between the GG genotype and persistent HCV infection was found [60]. Moreover, this finding is in agreement with a recent publicaton showing that Japanese patients with the GG genotype are unable to respond to interferon treatment [61]. The frequency of G-alleles in exon 3 and exon 6 has also been shown to be significantly higher in patients infected with SARS [62,63].

Collectively the current literature has aided our understanding of how IFN-β, ISG15, and OAS-1 drive the innate immune response to viruses. SNPs have not been studied extensively in IFNB-1 and ISG-15, but the important roles these genes and their protein products play suggest that SNPs may have a significant impact in the host response to viruses.

Rationale

Identifying genetic risk factors that influence the incidence and severity of post-OLT complications is a priority for transplant biologists, as these may be used to develop algorithms to estimate individual patient risk and ultimately may result in the development of of new organ allocation systems. A study done in our lab aimed to elucidate the differences in gene expression between cirrhosis in the presence and absence of HCV infection by comparing the gene expression profile from HCV-cirrhotic livers with alcoholic cirrhotic livers using high-density oligonucleotide microarrays [64]. From this comparison 55 up-regulated and 68 down-regulated genes between HCV-

cirrhosis and alcoholic cirrhosis were observed. Transcripts that were more highly expressed in HCV-cirrhosis than in alcoholic-cirrhosis included IFN-inducible genes (i.e., interferon alpha-inducible protein 27 (*IFI27*); interferon gamma-inducible protein 30 (*IFI30*); ISG-15, OAS-1). IFNB-1 was another gene over expressed in HCV cirrhotic liver tissues. Based on these findings we decided to evaluate the presence of SNPs and their influence in the host response to HCV in ISG-15, OAS-1 and IFNB-1 genes.

We hypothesized that the elucidation of associations between SNPs in Type-I Interferon and Interferon inducible genes and HCV recurrence post-OLT might help to identify HCV patients with different prognosis and improve liver transplant recipient selection.

MATERIALS AND METHODS

Patients and Samples

One hundred and fifty-two liver transplant recipients were included in this retrospective study. OLT recipients were classified into three groups: HCV only (n=78), HCV and HCC (n=25, and livers from normal liver donors were used as a control group (n=48). The liver samples were collected between July 1999 and May 2006. Re-OLT and/or multiple organ transplantation, pre-operative diagnosis of liver complications (fulminate hepatitis, cryptogenic cirrhosis, primary billiary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, or metabolic liver diseases), recipient of a HCV or Hepatitis B virus positive organ, and HCV and HBV co-infection were exclusion criteria's for this study.

Our study included HCV positive patients with cirrhosis that underwent OLT. Liver tissues were provided by the IRB approved Tissue Bank (Study Protocol #00882 Liver Tissue Procurement and Distribution System (LTPADS)). The normal liver tissues were obtained from explanted liver donors chosen for isolated hepatocyte preparation for transplantation. Liver function and histopathology for these liver donors were shown to be normal. All these patients were seronegative for HCV antibodies. A sample was

submitted for histological examination. Liver histology evaluation was performed using Knodell score and Ishak grade.

OLT (86% from deceased donors) were performed at The Hume-Lee Transplant Center, Medical College of Virginia. For each patient, number of infections (classified as Cytomegalovirus infection (CMV), bacterial infections, and fungal infections) was evaluated post-transplant. Additionally, acute rejection (AR) episodes were evaluated.

Donor and recipient characteristics were included in the analysis (age, race, gender, ischemia time) as well pre and post-OLT HCV treatment and viral load.

DNA Isolation and Purification:

Genomic DNA was isolated from sections of frozen liver using protocols outlined by Hillis et al [64]. Phase Lock Gel Tubes light extraction kits (Eppendorf) were used to remove contaminates by mixing equal volumes of DNA and phyenl:chloroform:isopropanol (PCI). Samples were spun for 5 minutes and this process was repeated using the same volume of chloroform:isopropanol as PCI. DNA was precipitated with 100% ethanol (EtOH) and placed in a –20°C freezer for 20 min. Samples were spun down discarding the EtOH and the remaining pellet was dissolved with 40μL - 300 μL of 1x TE pH 7.6 depending upon size of pellet.

Allelic discrimination using Taqman analysis:

Assay Validation: Our first step in the study included the validation of the allelic discrimination reactions. We ran many different samples using real-time PCR conditions

(QPCR) to identify homozygous samples for the different SNP alleles. The identified samples were corroborated using sequencing reactions. We also checked the reproducibility of the assay by running the same samples in different runs. Because the more difficult situation for the system is the heterozygous samples, we ran a check by creating our own heterozygous controls by mixing equal concentrations of homozygous controls for each gene. Afterwards, these artificial samples were evaluated in the system as samples.

Reaction: QPCR analysis for SNPs in IFNB-1, OAS-1, and ISG-15 were performed using an ABI Prism 7700 Sequence Detector (Perkin Elmer). Predeveloped Taqman assay reagents C___2865711_1_ (dbSNP ID rs1051922) for IFNB1 +228 C/T SNP, C__11447121_10 (dbSNP ID rs1921) for ISG15 +323 A/G SNP, and C___2567429_10 (dbSNP ID rs2660) for OAS-1 +1295 A/G SNP along with Taqman Universal PCR master mix (Applied Biosystems) were used in each reaction. Custom TaqMan MGBT Probes (Applied Biosystems) were labeled with a 5' reporter dye (FAM, VIC) and a 3' non-florescent quencher (NFQ). Setup and operation was preformed using specified guidelines and protocols outlined in the ABI 7700 User Manual. PCR reactions were carried out under the following conditions: denaturation at 95°C for 10:00 min followed by a 15 sec annealing at 92°C and a 1 min extension at 60°C that was repeated for 40 cycles.

Allelic discrimination was run on the same plate using settings from the ABI 7700 User Manual. Each sample was characterized as either homozygote for FAM or VIC or

heterozygote. A minimum of fourteen positive controls (seven for FAM and seven for VIC) and seven negative controls were run for each dye.

RNA isolation and cleanup:

Isolation of RNA was done using prescribed protocol outlined by Haimon-Kochman et al [65]. Extraction by the phenol:chloroform method was utilized to remove contamination due to phase partitioning of RNA and proteins. Samples were stored for a minimum of 16hrs at -80°C, allowing the RNA time to precipitate. Ethanol at a concentration of 70% - 75% was used to wash the RNA pellet before cleanup. RNeasy mini-kits (Qiagen) were used for purification of the samples so as to eliminate any phenol contamination following the manufacturer's protocol. The final product was eluted in a mixture of DNase, RNase free water (Gibco) and RNasin (Ambion). Between 15µl and 25µl of the mixture was added to each sample and stored long term at -80°C.

Measurement of DNA and RNA quality and quantity:

The Spectronic Genesys 5 (Thermo) was used to assess quality and quantity of both DNA and RNA in accordance with the manufacturer's protocol. RNA samples were diluted at 1:10 before freezing so that spectrophotometer readings could be made without freezing and thawing the sample. DNA samples were kept on wet ice while spec readings were being taken, thus no dilution was necessary. Initially two measurements were taken at 260nm and 280nm from which the level of contamination could be measured from the 260/280 ratio (ideal samples having a ratio of 1.8-2.1) and RNA could

be quantitated from the 260nm reading (using long established equations). 270nm readings were taken on 32 RNA samples; the 260/270 ratios were an indicator for protein contamination.

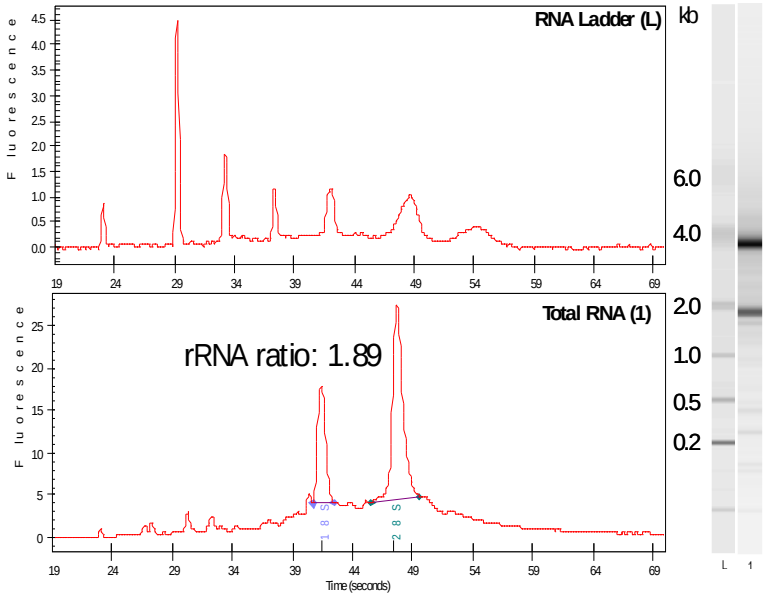
Evaluation of RNA integrity:

RNA integrity was checked using the Agilent 2100 Bioanalyzer, a capillary electrophoresis based system. To be considered optimal for microarray analysis, the samples needed to pass quality control criteria for sample preparation such as total RNA integrity and purity, as determined by 28S/18S ratios >1.5 and A260nm/A280nm ratios> 2.0 [Figure-4].

Figure-4: Evaluation of RNA integrity using Agilent 2100 Genetic Bionalazer.

TOTAL RNA SAMPLE

260/280 ➔ >2.0
260/270 ➔ >1.3
28S/18S ➔ >1.5



Gene Expression Assay:

Quantitation of gene expression levels for IFNB-1, ISG-15, and OAS-1 genes was performed in all the studied liver samples. After RNA isolation and evaluation of concentration, purity, and integrity, RNA samples (500ng) were converted into complementary DNA (cDNA) by reverse transcription polymerase chain reaction (RT-PCR) using the Gene Amp PCR System 9700 (PE Applied Biosystems). Total RNA from each sample was subjected to RT-PCR according to the manufacturer's protocol using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). cDNAs were used for the QPCR reactions. Each assay consisted of two unlabeled PCR primers and a FAM dye-labeled TaqMan MGB probe. The endogenous control, beta-2-microglobulin (B2M), was detected with a VIC dye-labeled TaqMan MGB probe (Human B2M (beta-2-microglobulin) Endogenous Control (VIC/TAMRA Probe, Primer Limited, Applied Biosystems). QPCR reactions were carried out in a 25μL reaction mixture using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). All amplifications were carried out in duplicate and threshold cycle (C_t) scores were averaged for calculations of relative expression values. The C_t scores for genes of interest were normalized against C_t scores for the corresponding B2M control. Relative expression was determined by the following calculation where the amount of target is normalized to an endogenous reference (B2M RNA) and relative to an arbitrary calibrator (the reference class of patients used in the comparison) [66]:

Relative Expression= $2^{-\Delta\Delta Ct}$, where$^{\Delta\Delta Ct}$

= ($^{\Delta Ct}$ of experimental group) - ($^{\Delta Ct}$ of calibrator group)

Determination of amplification efficiencies:

The $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes in the expression levels of the genes of interest compared with one of the normal urine samples. The $2^{-\Delta\Delta Ct}$ method assumes that the efficiencies for the endogenous control amplicon (B2M) and the gene of interest amplicon are the same. Efficiencies were determined for the amplicons B2M, on 1:5 dilution series. The slopes of Ct/log dilution plots for the reactions were calculated.

Data analysis:

Fisher's exact test was used to compare the frequencies for each genotype between cases and controls, along with the frequency of cases with presence versus absence of CMV infection, fungal infection, bacterial infection, and acute rejection. Survival was defined as the number of years from the date of transplant until date of death or last follow-up, censoring for patients still alive. Recurrence-free survival was defined as the number of years from the date of transplant until date of recurrence or date of last follow-up or death, censoring for patients who have either no recurred or who died before recurrence. Graft-survival will be defined as the number of years from the date of transplant until date of graft-survival or death, censoring for patients alive with graft function. For each genotype, the Kaplan-Meier method was used to estimate time to event for each genotype and the probability of survival at 1 and 3 years were reported along with their corresponding 95% confidence intervals. The log-rank test was used to assess whether there is a significant difference in overall, recurrence-free, and graft survival.

RESULTS

Patients:

Our retrospective study included 100 case and 48 control subjects. Among case group characteristics: Median age was 52 (range 30 – 65); 87% were male; 66% Caucasian, 20% Black/African American, 14% other. The median follow-up time among the 72 patients still alive was 2.4 years (range 0.6 - 7.3). Donor characteristics: Median donor age was 33 (range 9 – 76); 54% of donors were male; 73% Caucasian, 25% Black/African American, and 2% other; 86% of donors were deceased.

Liver tissues from normal liver donors with similar distribution in age, race, and gender were used as controls. Patient characteristics and histological evaluation of the cirrhotic tissues is shown in the [Table-1].

Table-1. Characteristics of the studied HCV patients

Patients (N)	100
Gender (Female/Male)	13/87
Race (Caucasian/African American/Other)	66/20/14
Age (Mean +/- SD)	52+/-17.8
Cirrhosis (Yes/No)	100/0
Knodell Score (6-8/>9)	17/83
Ishak grade (Stage 6)	100

Validation of allelic discrimination assays:

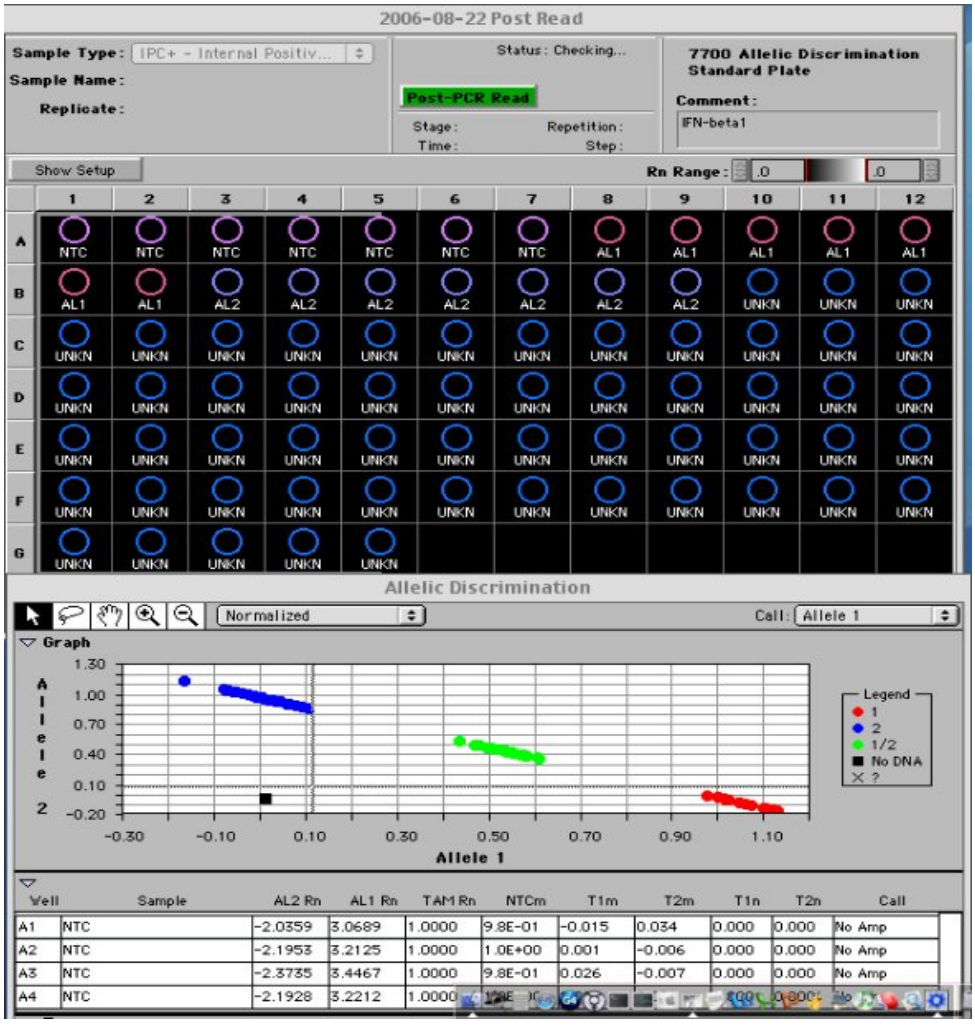
Allelic discrimination assays were initially validated for all studied SNPs. As was previously described, homozygous samples for the different studied genes were identified for the two alleles (Wt and mutant) by QPCR reactions. The intensity of fluorescence for each probe was separately analyzed representing the contribution of each individual allele. Checking the amplification plots assessed the presence and quantity of amplification. Non-template controls (NTC) (tube containing all the reagent components but no DNA) were analyzed in each run to ensure the absence of false positives. Finally, each positive control was confirmed using sequencing reactions.

Additionally, the Taqman software was checked to ensure it was properly distinguishing between homozygous and heterozygous calls. To accomplish this, heterozygous samples were created for each SNP by mixing different percentages of DNA from homozygous controls [Figure 5].

Using Allelic Discrimination the following SNP were genotyped: an A/G substitution in the 3'UTR of OAS-1 at position +1393, an A/G substitution of the ISG-15 gene at position +323, and a C/T substitution of IFNB1 at position +228. All polymorphisms were found in the NCBI database, which lists ISG-15 as a misense mutation and IFNB1 as a silent mutation. In each run, at least seven positive controls for each allele were included and known and unknown samples were added for testing the system calls. Seven NTC samples were included in each run.

Figure-5: Sample Post Read from SNP Genotyping

To accomplish this, heterozygous samples were created for each SNP by mixing different percentages of DNA from homozygous controls


2006-08-22 Post Read
Sample Type: IPC+ - Internal Positiv...
Sample Name:
Replicate:
Status: Checking...
Post-PCR Read
Stage:
Time:
Repetition:
Step:
7700 Allelic Discrimination
Standard Plate
Comment:
IFN-beta1
Show Setup
Rn Range: 0 0
1 2 3 4 5 6 7 8 9 10 11 12
A NTC NTC NTC NTC NTC NTC NTC AL1 AL1 AL1 AL1 AL1
B AL1 AL1 AL2 AL2 AL2 AL2 AL2 AL2 AL2 UNKN UNKN UNKN
C UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN
D UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN
E UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN
F UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN UNKN
G UNKN UNKN UNKN UNKN UNKN
Allelic Discrimination
Normalized
Call: Allele 1
Graph
Allele 2
1.30 1.00 0.70 0.40 0.10 -0.20
-0.30 -0.10 0.10 0.30 0.50 0.70 0.90 1.10
Allele 1
Legend
1
2
1/2
No DNA
?
Well Sample AL2 Rn AL1 Rn TAM Rn NTCm T1m T2m T1n T2n Call
A1 NTC -2.0359 3.0689 1.0000 9.8E-01 -0.015 0.034 0.000 0.000 No Amp
A2 NTC -2.1953 3.2125 1.0000 1.0E+00 0.001 -0.006 0.000 0.000 No Amp
A3 NTC -2.3735 3.4467 1.0000 9.8E-01 0.026 -0.007 0.000 0.000 No Amp
A4 NTC -2.1928 3.2212 1.0000

SNPs distribution between HCV patients and Controls:

The SNP in the 3'UTR of the OAS-1 gene showed a statistically significant difference in the distribution of the SNP genotypes in HCV positive patients and our control population (p=0.05). The homozygous genotype 1 was found in 46% (n=46) of HCV positive patients and the heterozygous genotype 1/2 was found in 49% (n=49) of patients. From the analysis of distribution of genotype between cases and controls, the homozygous genotype 1 for OAS-1 was also significantly higher in the control group when compared with HCV patients [Table 2].

The SNP at position +323 for ISG-15 and the SNP at position +228 for IFNB-1 did not appear to have any statistically significant distribution of genotypes in control and sample populations.

Allelic distribution and HCV recurrence post-liver transplantation:

In our study group, we observed evidence of histological recurrence of HCV within the first year post-OLT in 89% of the patients. Moreover, 33% of these patients presented bridging fibrosis during the first year post-OLT. The development of fibrosis during the first year post-OLT was a predictor of survival in this group (p=0.01) (data not shown). Three patients with aggressive HCV recurrence during the first year post-OLT required re-OLT.

Patients with histological evidence of HCV recurrence were dichotomized as HCV recurrence within 12 months (n=89) versus HCV recurrence after 12 months (n=11). An analysis showed the distribution of each genotype by time to recurrence

dichotomized in the same manner among patients followed at least 12 months or until time of recurrence.

In addition, statistical significance was observed for OAS-1 genotypes (p=0.04). Patients with the heterozygous genotype 1/2 of OAS-1 had the highest percentage of recurrence within 12 months [Table 3]. In univariable logistic regression models predicting fast HCV recurrence, defined as time to HCV recurrence less than 12 months, age of patient at transplant (p=0.21), age of donor at transplant (p=0.69), donor race (p=0.50), donor gender (p=0.41) were not significant predictors.

From the analysis of the different donor [Table-4A] and recipient [Table-4B] characteristics between OLT patients that developed fibrosis within the first year post-OLT versus the group of HCV transplant patients that did not, we observed that aged donors (defined as donor ≥60 years) was the only statistically significant parameter. We did not observe any association between race, gender, and ischemia time.

In addition, from the analysis of treatment for HCV (including interferon/ribavirin) and viral load at pre-OLT time between groups, we did not observe any statistically significant association [Table-5A]. From the analysis of the HCV treatment, viral load, and acute rejection episodes at post-OLT, we did not find any statistically significant difference. However, viral load and acute rejection episodes were higher in HCV OLT patients with HCV recurrence and fibrosis development within the first year post-OLT [Table-5B].

Moreover, from the analysis of the SNPs distribution between patients that developed fibrosis within the first year post-OLT versus the liver recipients that did not, we observed that the OAS-1 SNP distribution was statistically significant (p=0.05).

Finally, we did not observe any association between SNPs and graft and patient survival.

Table-2. SNPs distribution between HCV patients and Controls

	Controls	Cases	P values
ISG-15			
1	**28 (58.3%)**	**61 (61%)**	**0.21**
1 and 2	**8 (16.7%)**	**25 (25%)**	
2	**12 (25.0%)**	**14 (14%)**	
IFN-B1			
1	**6 (12.5%)**	**13 (13%)**	**0.86**
1 and 2	**24 (50.0%)**	**45 (45%)**	
2	**18 (37.5%)**	**42 (42%)**	
OAS-1			
1	**29 (60.4%)**	**46 (46%)**	**0.053***
1 and 2	**14 (29.2%)**	**49 (49%)**	
2	**5 (10.4%)**	**5 (5%)**	

Table-3. Allelic distribution and HCV recurrence post-liver transplantation*

	<12 months	>12 months	*P* values
ISG-15			**NS**
1	**53 (59.5%)**	**7 (63.6%)**	
1 and 2	**24 (27.0%)**	**3 (27.3%)**	
2	**12 (13.5%)**	**1 (9.1%)**	
IFNB-1			
1	**9 (10.1%)**	**2 (18.1%)**	**NS**
1 and 2	**45 (50.6%)**	**4 (36.4%)**	
2	**35 (39.3%)**	**5 (45.5%)**	
OAS-1			
1	**36 (40.4%)**	**6 (54.6%)**	**0.04***
1 and 2	**51 (57.3%)**	**4 (36.3%)**	
2	**2 (2.3%)**	**1 (9.1%)**	

*Patients that developed histological evidence of HCV recurrence within the first year post-OLT (n=89) versus the HCV patients that did not (n=11).

Table-4. Characteristics of donor (A) and recipients (B) between patients did or did not develop fibrosis post-liver transplantation.

A-

	Fibrosis	Non-fibrosis	*P* value
Aged donors (>60 yo.)	35.70%	6.70%	0.025*
Gender (Male)	50%	43.40%	0.25
Race (African-American)	28.50%	10%	0.153

B-

	Fibrosis	Non-fibrosis	*P* value
Aged recipients (>60 yo.)	7.10%	6.70%	0.46
Gender (Male)	21%	20.00%	0.3
Race (African-American)	21.40%	20%	0.305

Table-5. Characteristics pre-liver transplantation (A) and post-liver transplantation (B)

A-

	Fibrosis	Non-fibrosis	*P* value
Treatment pre-TX Interferon/ribavirin	21.40%	23.30%	0.299
Viral load pre-TX viral copies/10-E5	12.8+/-6.6	12.2+/-13.1	0.97
Ischemia Time (minutes)	345+/- 228	314+/-266	0.75

B-

	Fibrosis	Non-fibrosis	*P* value
Treatment post-Tx Interferon /ribavirin	28.50%	20%	0.24
Viral load post-Tx viral copies/E-10E mL	91.9+/-42.9	60.6+/-47.6	0.18
Rejection episodes	21.40%	16.70%	0.29

Associations of SNPs with clinical outcomes:

Correlations between the studied genes and post-OLT outcomes were noted. A multivariate analysis of SNP genotypes and clinical complications post-OLT found a statistically significant association with the genotypes of both OAS-1 and ISG-15 with CMV infection [Tables 6 and 7]. The analysis also found an association between the genotypes of ISG-15 and acute rejection. However, no significant association was found between the SNP genotypes of IFNB-1 and infection or rejection post-OLT [Table-8].

The homozygous genotype 1 (91.1%) and heterozygous genotype 1/2 (96%) of OAS-1 were found to be significantly higher in patients without CMV infection post-OLT (p=0.02). We also observed a significantly higher number of infections (60%) associated with the homozygous genotype 2.

As with OAS-1 the homozygous genotype 1 and heterozygous genotype 1/2 of ISG-15 were significantly higher in patients without CMV infection post-OLT (p=0.05). We also observed a higher percentage of patients with CMV infection with the homozygous genotype 2 of ISG-15. Additionally, the heterozygous genotype 1/2 (36%) correlated with an increase risk of acute rejection episodes post-OLT.

Table-6. ISG-15 SNP distribution and clinical outcomes

ISG15	1	1 and 2	2	P value
CMV				0.05*
No	55 (90.2%)	24 (96%)	9 (69.2%)	
Yes	6 (9.8%)	1 (4%)	1 (30.8%)	
Fungal infection				0.32
No	54 (90%)	19 (79.2%)	11 (78.6%)	
Yes	6 (10%)	5 (20.8%)	3 (21.4%)	
Bacterial infection				0.80
No	39 (63.9%)	14 (56%)	8 (57.1%)	
Yes	22 (36.1%)	11 (44%)	6 (42.9%)	
Acute Rejection				0.02*
No	54 (88.5%)	16 (64%)	10 (71.4%)	
Yes	7 (11.5%)	9 (36%)	4 (28.6%)	
Overall Survival (years)				0.25
N	61	25	14	
1 yr survival (95% CI)	0.885 (0.77, 0.94)	0.758 (0.54, 0.88)	0.851 (0.52, 0.96)	
3 yr survival (95% CI)	0.804 (0.67, 0.89)	0.648 (0.41, 0.81)	0.851 (0.52, 0.96)	
Recurrence-free Survival (years)				0.98
N	61	25	14	
1 yr survival (95% CI)	0.532 (0.39, 0.65)	0.631 (0.41, 0.79)	0.643 (0.34, 0.83)	
3 yr survival (95% CI)	0.287 (0.17, 0.42)	0.184 (0.04, 0.43)	0.143 (0.02, 0.37)	
Graft Survival (years)				0.28
N	61	25	14	
1 yr survival (95% CI)	0.869 (0.75, 0.93)	0.718 (0.50, 0.85)	0.851 (0.52, 0.96)	
3 yr survival (95% CI)	0.787 (0.65, 0.87)	0.667 (0.44, 0.82)	0.851 (0.52, 0.96)	

Table-7. OAS-1 SNP distribution and clinical outcomes

OAS-1	1	1 and 2	2	P value
CMV				0.02
No	41 (91.1%)	45 (91.8%)	2 (40%)	
Yes	4 (8.9%)	4 (8.2%)	3 (60%)	
Fungal infection				0.18
No	35 (79.55%)	45 (91.8%)	4 (80%)	
Yes	9 (20.45%)	4 (8.2%)	1 (20%)	
Bacterial infection				0.27
No	24 (52.2%)	33 (67.35%)	4 (80%)	
Yes	22 (47.8%)	16 (32.65%)	1 (20%)	
Acute Rejection				1.00
No	37 (80.4%)	39 (79.6%)	4 (80%)	
Yes	9 (19.6%)	10 (20.4%)	1 (20%)	
Overall Survival (years)				0.26
N	46	49	5	
1 yr survival (95% CI)	0.799 (0.65, 0.89)	0.898 (0.77, 0.96)	0.800 (0.20, 0.97)	
3 yr survival (95% CI)	0.704 (0.53, 0.82)	0.827 (0.68, 0.91)	0.800 (0.20, 0.97)	
Recurrence-free Survival (years)				0.09
N	46	49	5	
1 yr survival (95% CI)	0.480 (0.32, 0.62)	0.695 (0.53, 0.80)	0.200 (0.01, 0.58)	
3 yr survival (95% CI)	0.299 (0.15, 0.47)	0.193 (0.09, 0.33)	0	
Graft Survival (years)				0.59
N	46	49	5	
1 yr survival (95% CI)	0.777 (0.62, 0.87)	0.877 (0.75, 0.94)	0.800 (0.20, 0.97)	
3 yr survival (95% CI)	0.716 (0.55, 0.83)	0.807 (0.66, 0.89)	0.800 (0.20, 0.97)	

Table-8. IFNB1 SNP distribution and clinical outcomes

IFN-β1	1	1 and 2	2	P value
CMV				0.09
No	10 (76.9%)	43 (95.6%)	35 (85.4%)	
Yes	3 (23.1%)	2 (4.4%)	6 (14.6%)	
Fungal infection				0.09
No	11 (84.6%)	42 (93.3%)	31 (77.5%)	
Yes	2 (15.4%)	3 (6.7%)	9 (22.5%)	
Bacterial infection				0.72
No	7 (53.85%)	29 (64.4%)	25 (59.5%)	
Yes	6 (46.15%)	16 (35.6%)	17 (40.5%)	
Acute Rejection				0.54
No	9 (69.2%)	36 (80%)	35 (83.3%)	
Yes	4 (30.8%)	9 (20%)	7 (16.7%)	
Overall Survival (years)				0.53
N	13	45	42	
1 yr survival (95% CI)	0.666 (0.33, 0.86)	0.933 (0.81, 0.98)	0.810 (0.66, 0.90)	
3 yr survival (95% CI)	0.666 (0.33, 0.86)	0.784 (0.63, 0.88)	0.810 (0.66, 0.90)	
Recurrence-free Survival (years)				0.26
N	13	45	42	
1 yr survival (95% CI)	0.356 (0.09, 0.64)	0.664 (0.51, 0.78)	0.514 (0.35, 0.66)	
3 yr survival (95% CI)	0	0.290 (0.14, 0.46)	0.171 (0.06, 0.33)	
Graft Survival (years)				0.61
N	13	45	42	
1 yr survival (95% CI)	0.666 (0.33, 0.86)	0.888 (0.75, 0.95)	0.810 (0.66, 0.90)	
3 yr survival (95% CI)	0.666 (0.33, 0.86)	0.766 (0.61, 0.87)	0.810 (0.66, 0.90)	

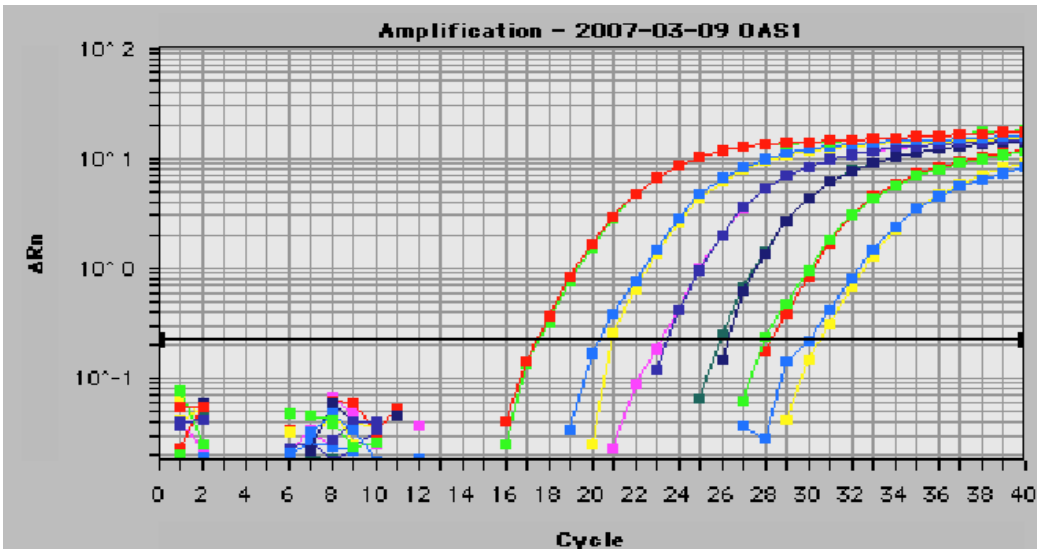
Effects of SNPs on gene expression:

The $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes in the expression levels of the genes of interest compared with one of the normal liver samples. The $2^{-\Delta\Delta Ct}$ method assumes that the efficiencies for the endogenous control amplicon (B2M) and the gene of interest amplicon are the same. Efficiencies were determined for the amplicons B2M, OAS-1, ISG-15, and IFNB-1 on 1:5 dilution series [Figure 6]. The slopes of Ct/log dilution plots for the reactions were -2.98, -3.02 [Figure-7], -3.18 [Figure-8], and -3.08, respectively; thus, all amplicons amplify with similar efficiencies

From the analysis of the gene expression levels and its relationship with the presence of the SNP, we did not identify associations for OAS-1 (p=NS). However, we observed that the HCV patients that presented HCV recurrence post-OLT with development of fibrosis expressed higher level of expression of OAS-1 that the HCV patients that did not, 5.9+/- 13.0 versus 12.4 +/-10.5 mRNA OAS/B2M fold change (p=0.05). In addition, the level of expression of OAS-1 in the liver from HCV patients was higher than in the normal livers, 13.9+/-12.9 vs. 4.23 +/- 3.26 mRNA OAS/B2M fold change (p=0.03). We did not observe any association between SNP distribution and gene expression for the other two studied genes.

Figure-6: Determining efficiencies for (A) endogenous control B2M and (B) target gene OAS-1

A


Amplification - 2007-03-09 OAS1
ΔRn
10^2
10^1
10^0
10^-1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

B

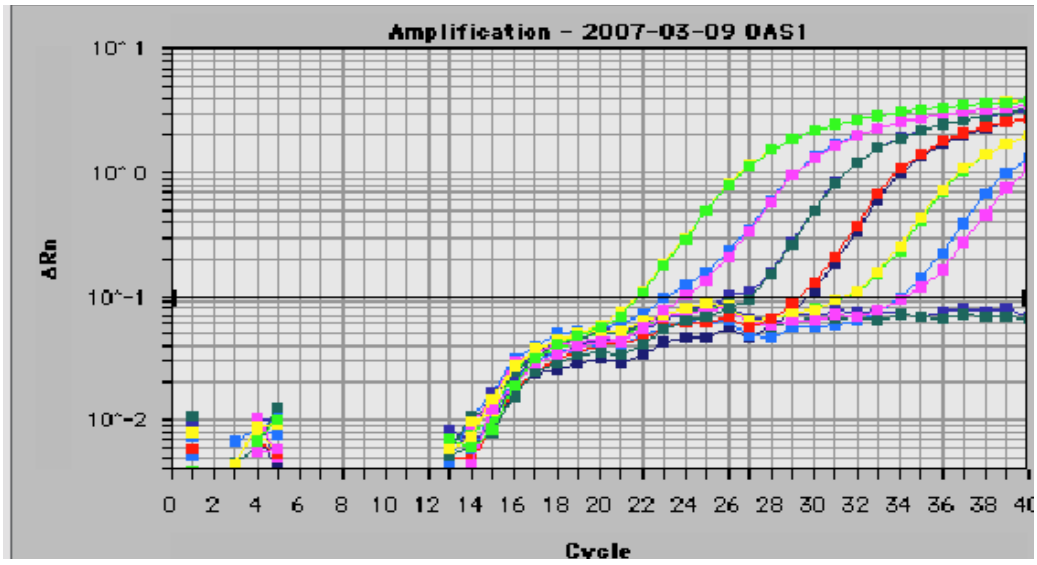
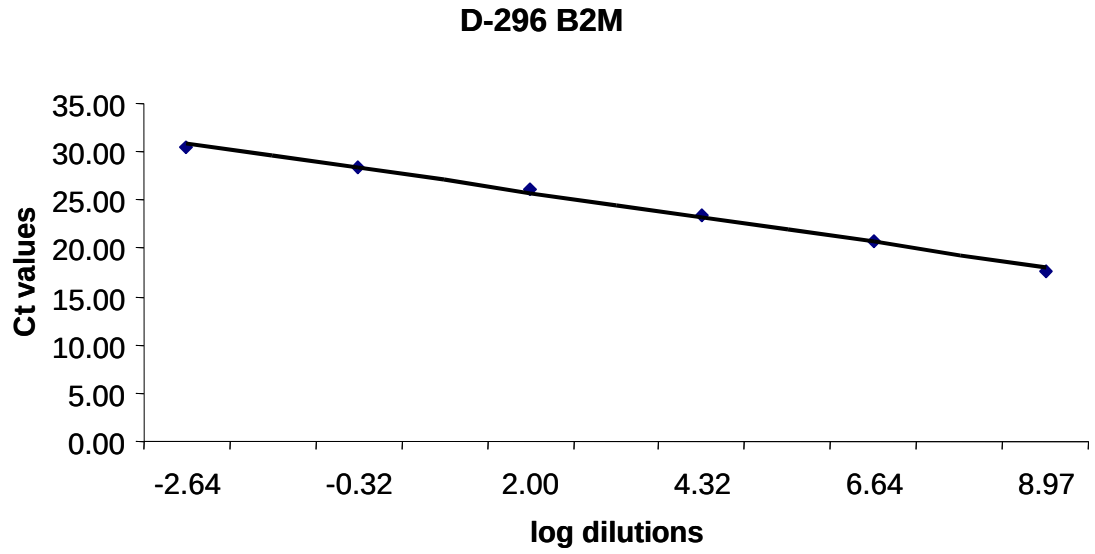

Amplification - 2007-03-09 OAS1
ΔRn
10^1
10^0
10^-1
10^-2
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

Figure-7 Dilution Slope for OAS-1


D-296 B2M
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct values
-2.64
-0.32
2.00
4.32
6.64
8.97
log dilutions

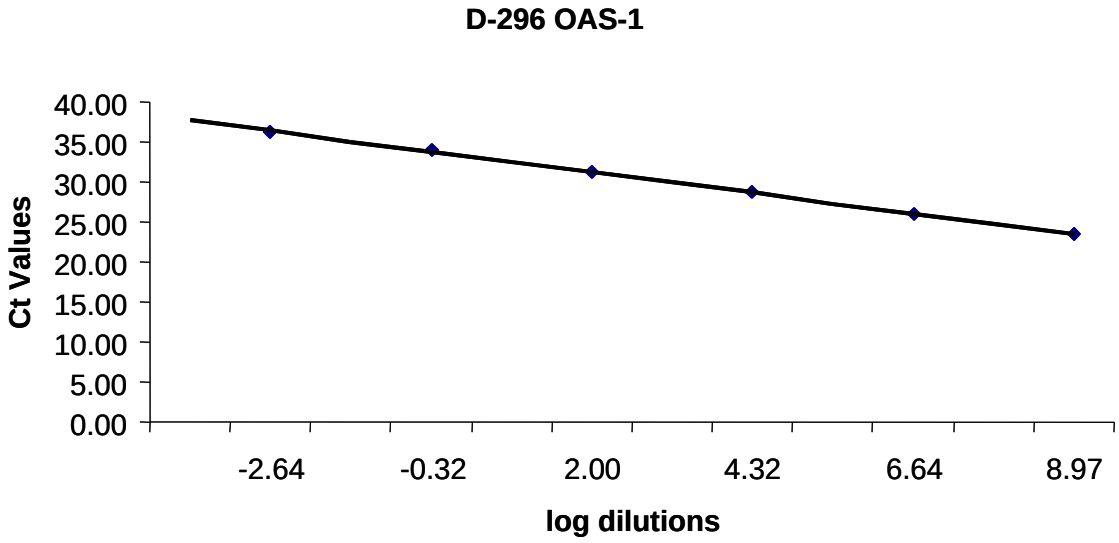
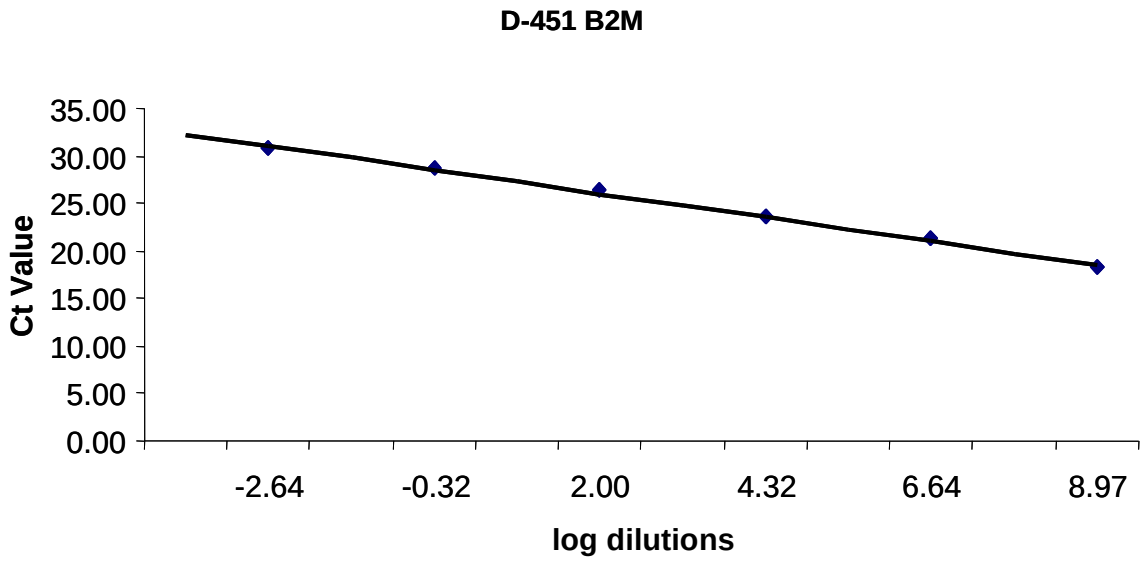
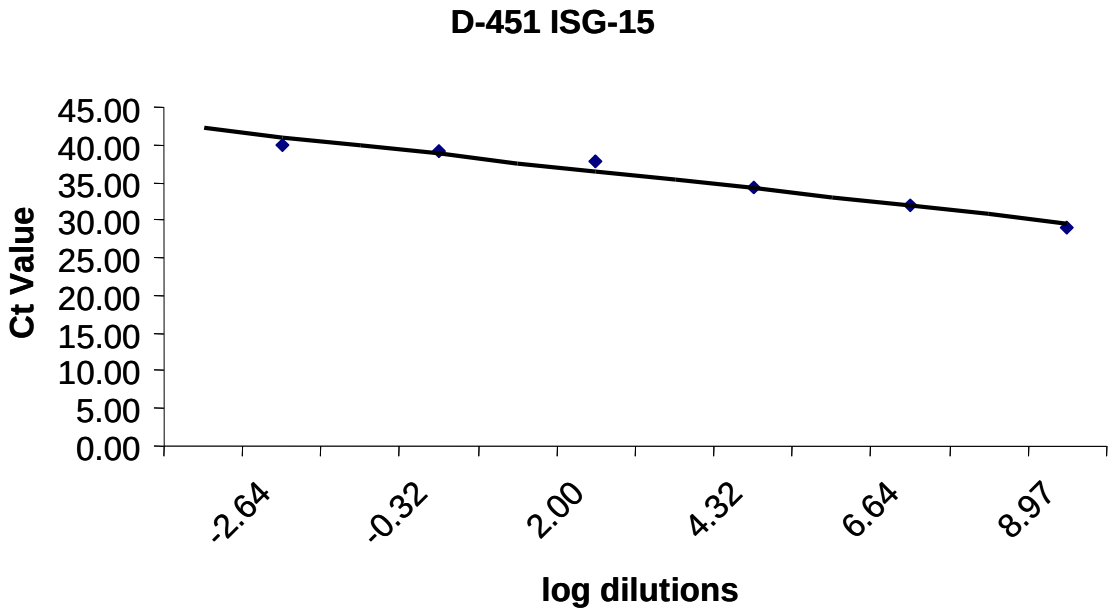

D-296 OAS-1
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct Values
-2.64
-0.32
2.00
4.32
6.64
8.97
log dilutions

Figure-8 Dilution Slope for ISG-15


D-451 B2M
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct Value
-2.64
-0.32
2.00
4.32
6.64
8.97
log dilutions


D-451 ISG-15
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct Value
-2.64
-0.32
2.00
4.32
6.64
8.97
log dilutions

DISCUSSION

Worldwide HCV is one of the most important pathogens causing liver-related morbidity and mortality. Infection is typically characterized as chronic, leading to the development of liver cirrhosis and hepatocellular carcinoma. Currently, HCV is the main cause of liver OLT. Recurrence post-OLT is almost universal, leaving the majority of patients with only a mild form of the disease. As for the remaining patients they must deal with a more serve infection rapidly leading to fibrosis and cirrhosis. Of the patients studied, 89% developed recurrence of HCV disease within 12 months after OLT. Moreover, thirty-three percent of patients with HCV recurrence progressed to histological fibrosis within 12 months post-OLT.

Hepatitis C recurrence

IFNs play a central role in eliminating viruses not only as therapeutic applications but also as natural cellular antiviral defense mechanisms [67]. They are naturally produced in response to viral infections, and to cellular exposure of IFNs themselves [68]. A number of ISGs have been reported to direct antiviral activity through distinct cellular pathways of translational control; including OAS-1, ISG-15, and IFNB-1. A previous study identified these genes as being differentially expressed in HCV positive liver tissues. This study was designed to evaluate the potential association between gene polymorphisms in these IFN and IFN inducible genes in liver allograft recipients and recurrence of HCV disease.

From the analysis of the data a significant association between fibrosis development and OAS-1 (A/G) genotype was observed. This agree with the findings of Knapp et al., whom observed an association between OAS-1 (A/G) SNP and HCV disease progression in immunocompetent individuals [60].

Moreover, the levels of OAS-1 RNA were lower in HCV patients without HCV recurrence within the first year post-OLT and comparable to the normal liver group. Interestingly, in a recent publication Gramenzi et al. observed the production of 2,5-OAS of subjects who were able to clear the virus was similar to that of healthy controls [69]. These results confirm the critical role that OAS-1 plays in the HCV infection.

Clinical Complications arising from Hepatitis C Virus

Associations between SNPs and clinical complications post-transplant were identified in the studied genes. Since these molecules are involved in the host immune response, alterations in their level of expression might affect the patients immune response already weakened by immunossupresive therapies. A higher number of infections have been previously described in HCV patients undergoing liver transplant when compared with non-HCV liver recipients. The presence of polymorphisms in molecules involved in the host immune reponse to the virus might explain these results.

A significant association was found between OAS-1 homozygous genotype 2 (60%) and Cytomegalovirus (CMV) infection post-OLT. An earlier study observed the OAS-1 SNP rs2660 is in virtually complete linkage disequalibrium to a splice site polymorphism and is associated with variation in OAS-1 enzyme activity. Skolova et al.

had also observe a direct relationship between the resistance of cells against CMV and the constitutive level of OAS-1 gene expression [70]. However, it remains to be determined why OAS-1 genotypes correlate with CMV infection.

It has been shown that viral infections including HCV result in the up-regulation of ISG-15 [56]. The G allele (which is the mutant allele) does correlate with an increased susceptibility to CMV infection and acute rejection. At this time it is unclear as to what direct effect this polymorphism has upon the protein and its functions. It is reasonable to assume that this change could disrupt ISG-15s ability to modify proteins during a viral infection thus leading to a decrease in the cells ability to elicit an appropriate response.

Conclusion

The role of interferon and interferon inducible genes has been shown to be critical in the innate antiviral response. This study showed that polymorphisms in OAS-1 and ISG-15 affect susceptibility to HCV recurrence and infections post-OLT. While IFNB1 did not show any association with recurrence or clinical state, this data would agree with the current positional data on the NCBI database.

We underlined that SNPs in the studied genes are likely implicated in different outcomes of HCV infection. In particular, we can speculate that genetic variants in OAS-1 gene might be associated with histological HCV recurrence within 12 months post-OLT. These genotypes may help to identify those patients at higher risk for developing complications during the first year post-OLT.

This project also demonstrated that the SNPs in OAS-1 and ISG-15 do affect HCV positive patients outcome post-OLT. While it is understood that polymorphisms do affect the antiviral response, it is unclear exactly how this occurs. More specific studies looking at the effects of polymorphism must be undertaken to answer these questions.

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