Aus der Abteilung Virologie
(Univ.-Prof. Dr. med. Frank T. Hufert)
im Zentrum Hygiene und Humangenetik

und

aus der Abteilung Gastroenterologie und Endokrinologie
(Prof. Dr. med. Dr. h.c. Giuliano Ramadori)
im Zentrum Innere Medizin

der Medizinischen Fakultät der Universität Göttingen

Chronic hepatitis C: Liver disease manifestations with regard to respective innate immunity receptors gene polymorphisms

INAUGURAL – DISSERTATION
zur Erlangung des Doktorgrades

der Medizinischen Fakultät
der Georg-August-Universität zu Göttingen

vorgelegt von
Eva Askar
aus
Fairoza-Homs/Syrien

Göttingen 2010

Dekan: Prof. Dr. med. C. Frömmel

I. Berichterstatter: Prof. Dr. med. G. Hunsmann
II. Berichterstatter/in: Prof. Dr. rer. nat. D. Kube
Tag der mündlichen Prüfung: 07.04.2011

Contents

1. Introduction

1.1 Hepatitis C virus

1.1.1 Structure

Hepatitis C virus (HCV) is a single-stranded plus-sense RNA virus which causes the previously socalled Non-A, Non-B- hepatitis (Choo et al. 1989). The virus is the only member of the *Hepacivirus* genus, which belongs to the *Flaviviridae* family (Brass et al. 2006).

HCV is a spherical enveloped particle, 40-70 nm in diameter. The virus genome encodes a long open reading frame flanked by two untranslated regions (UTRs) that contains signals for the synthesis of viral RNA (~9,600 nucleotides) and a single large polyprotein precursor of approximately 3,010 amino acid residues (Bartenschlager et al. 2004; Rehermann and Nascimbeni 2005). This polyprotein is processed by a combination of host and viral proteases into at least ten proteins in the following order: NH2- core- envelope 1 (E1)- envelope 2 (E2)- p7- nonstructural protein 2 (NS2)- NS3- NS4A- NS4B- NS5A- NS5B- COOH (Kato 2001).

The structural proteins, core, E2 and E1, are required for genome packing, virus attachment to its target cell, and the fusion process that delivers the genome into the cytoplasm. The hydrophobic p7 protein is thought to act as a viroporin (an ion channel) (Bartenschlager et al. 2004). The non-structural proteins have important roles in polyprotein processing and HCV replication. They may also modulate the response to interferon-alpha (IFN-α) therapy (NS5A) (Penin et al. 2004). An alternative reading frame to the core region encodes for the F protein, whose function is presently unknown (Bartenschlager et al. 2004).

1.1.2 Epidemiology

After applying stringent tests for detecting HCV-specific antibodies by enzyme-linked immunosorbent assay (ELISA) or the viral genome by reverse transcriptase polymerase chain reaction (RT-PCR) assays in screening blood donations, the risk of transfusion-associated HCV has been nearly eliminated (Bartenschlager et al. 2004). Nevertheless, the prevalence of infection keeps rising due to transfusion of untested samples and to

parenteral risk factors, mostly by needle sharing among intravenous drugs users (Bartenschlager et al. 2004), tattooing and body piercing. Sexual transmission and interauterine infections, on the other hand, seem to be rare (Bartenschlager et al. 2004). Currently, an estimated 3% of the world's population is infected with HCV and 3 to 4 million persons are newly infected each year (Shepard et al. 2005). Fig 1 shows the distribution of HCV infection worldwide. In Germany, the incidence of HCV decreased from about 11.0 to 6.6 cases per 100 000 German inhabitants between 2004 and 2009 (*Robert-Koch-Institut* 2010). Nearly 50% - 80% of the mostly asymptomatic acute infections with HCV develop to chronic hepatitis C (CHC) (Brass et al. 2006), which leads to liver cirrhosis in about 10% - 20% of patients within 20 years after infection and eventually hepatocellular carcinoma (Bartenschlager et al. 2004).

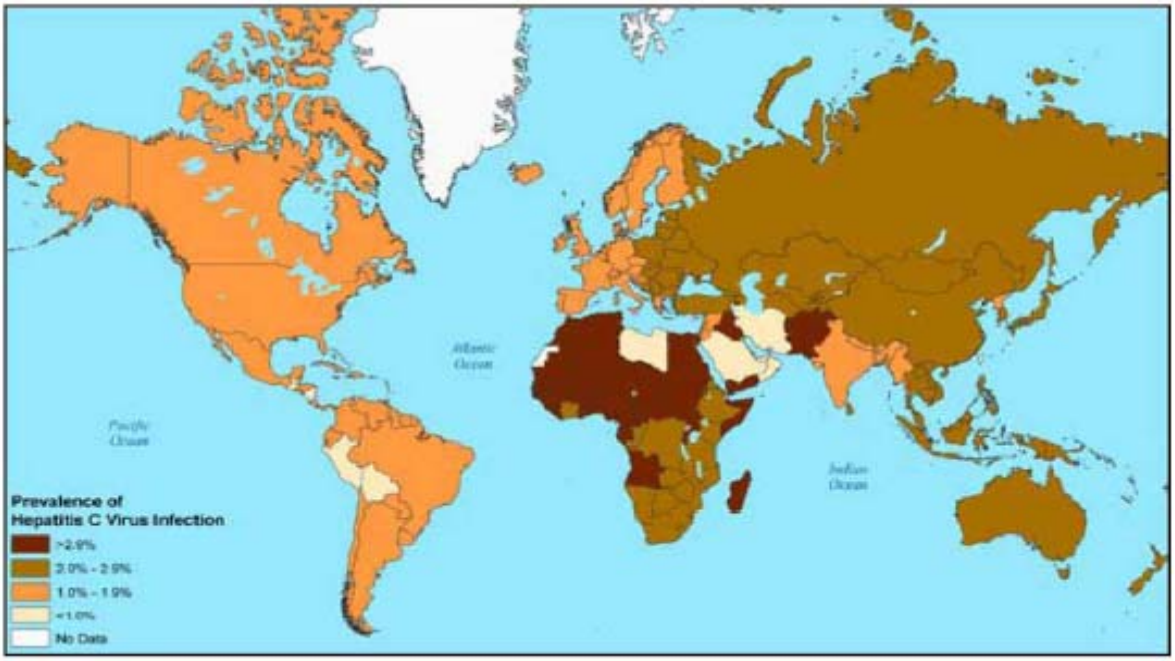

Fig. 1: Estimated global prevalence of HCV infection (adopted from Perz et al. (2004))

1.1.3 Viral life cycle

After entry to a host hepatocyte, HCV nucleocapsids are delivered to the cytoplasm, where the viral RNA acts directly as an mRNA. Translation is initiated through an internal ribosomal entry site (IRES) in the 5′ UTR. The translated polyprotein is co- and post-translationaly processed by cellular and viral proteases into ten viral proteins (Rehermann and Nascimbeni 2005). Following synthesis and maturation, non-structural proteins and viral RNA form membrane-associated replication complexes, which appear

as a perinuclear membranous web (MW) (Fig 2). These complexes then catalyse the transcription of complementary negative-strand RNA intermediates, from which progeny positive-strand RNA molecules are generated (Rehermann and Nascimbeni 2005). Capsid proteins and genomic RNA assemble into a nucleocapsid and bud through intracellular membranes into cytoplasmic vesicles, which then fuse with the plasma membrane (Rehermann and Nascimbeni 2005) (Fig. 2).

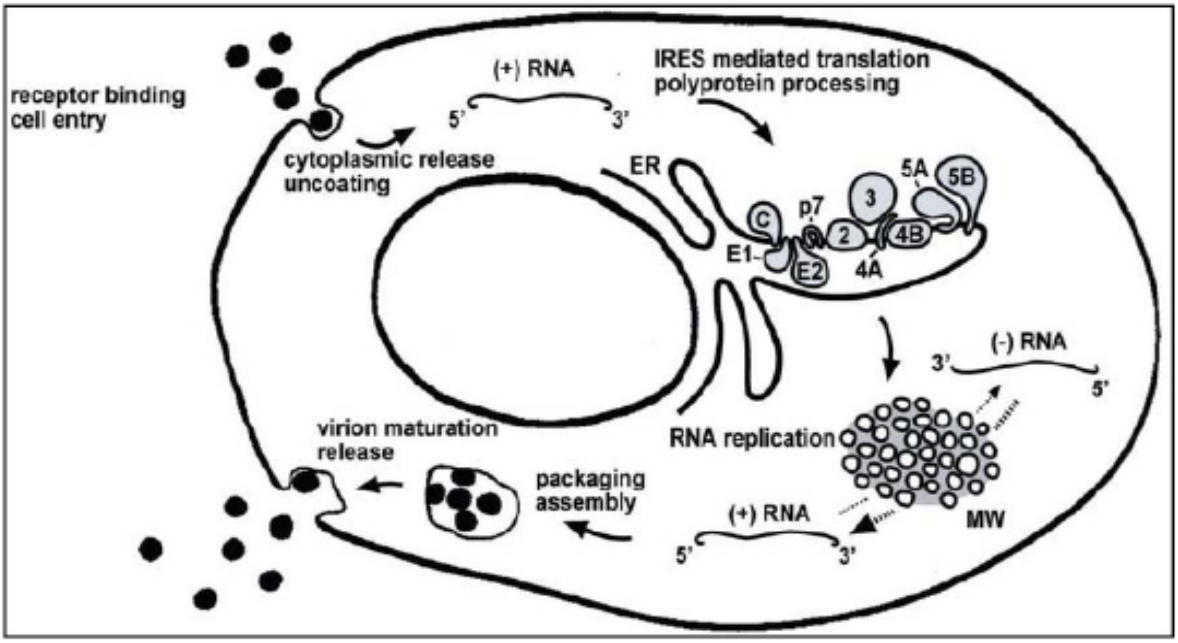

Fig. 2: A schematic illustration of the steps of HCV life cycle (adopted from Brass et al. (2006, p. 30))

1.1.4 HCV genotypes and subtypes

Due to the high error rate of the viral RNA-dependent RNA polymerase, HCV genomes can be grouped into at least 6 genotypes or 'clades' that differ in their nucleotide sequence by more than 30%. Furthermore, several subtypes differing in their nucleotide sequence by 20% - 25% can be defined within each HCV genotype (Bartenschlager et al. 2004; Simmonds et al. 2005). 70% - 80% of CHC in the United States and more than 60% in Western Europe and Asia are caused by genotype 1 (Pawlotsky et al. 2007) (especially subtypes 1b and 1a), followed by infections with the genotypes 2 and 3. Genotypes 4, 5 and 6 can only be found in distinct geographical regions like Egypt, South Africa and Southeast Asia, respectively (Bartenschlager et al. 2004).

1.1.5 Treatment

The current treatment for CHC involves a 48-week course of weekly injected polyethylene glycol (PEG)-conjugated IFN-α (PegIFN-α), which augments normal antiviral processes, combined with daily oral ribavirin, a DNA nucleoside analogue, the inhibitory mechanism of which on HCV replication is not completely understood (Iadonato and Katze 2009). This treatment is effective in about 80% of patients infected with HCV genotype 2 or 3 but in less than 50% of those with genotype 1 (Pawlotsky et al. 2007). Moreover, therapy is costly and often poorly tolerated due to its significant adverse effects that prevent some patients from completing the treatment cycle (Ge et al. 2009). Consequently, many studies have been intensively carried out to choose alternative therapies. Some approaches depended on the antiviral activity and the less side effects of interleukin-29 (IL-29)/interferon-lambda$_1$ (IFN-λ_1) (Doyle et al. 2006; Miller et al. 2009) currently being in phase 2b of clinical development; others relied on enhancing endogenous IFN-α production through Toll-like receptors (TLRs) ligands (e.g. the TLR7 agonist, isatoribine) (Horsmans et al. 2005).

1.2 TLRs and innate immunity

The essential role of the mammalian innate immune system to sense invading pathogens relies on pattern recognition receptors (PRRs) (Kawai and Akira 2006). PRRs are germline-encoded, constitutively expressed molecules that recognize pathogen-associated molecular patterns (PAMPs), which are necessary for the survival of microorganisms but not present in eukaryotes (Kawai and Akira 2006; Wagner and Bauer 2006). PRRs consist of membrane receptors, namely TLRs; and cytosolic receptors such as nucleotide-binding oligomerization domain (NOD)-like receptors and the RNA-helicase family, e.g. retinoic acid inducible gene-1 (RIG-1) (Akira et al. 2006; Seki and Brenner 2008; Takeuchi and Akira 2007).

TLRs were initially identified based on homology with the Toll receptor from Drosophila (Medzhitov et al. 1997). They are expressed predominantly on immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and, to a lesser extent, on a number of nonimmune cells such as fibroblasts, epithelial cells (Akira et al. 2006), and hepatocytes (Schwabe et al. 2006; Seki and Brenner 2008). All the ten TLRs discovered in humans contain a conserved intracellular signaling domain similar to that of the IL-1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Kawai and Akira 2006). The extracellular domain, however, is unique to the individual TLR as it

confers specificity for ligand recognition and contains varying numbers of leucine-rich-repeat (LRR) motifs (Akira et al. 2006).

While TLRs 1, 2, 4, 5, 6 and 10 are expressed on the cellular membrane, TLRs 3, 7, 8 and 9 are mainly found in the endosomal compartment allowing site-specific recognition of pathogens (Akira et al. 2006; Kawai and Akira 2006).

1.2.1 Main TLR ligands and signaling pathways

In general, viral PAMPS, namely double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and unmethylated CpG-containing DNA activate TLR3, TLR7/8, and TLR9, respectively. Bacterial PAMPS, however, are recognized by TLR1/TLR2 and TLR6/TLR2 heterodimers (lipoproteins), TLR4-CD14-MD2 complex (lipopolysaccharides (LPS)), and TLR5 (flagellin). TLR10 is an orphan receptor with currently unknown ligands (Akira et al. 2006; Schwabe et al. 2006) (Fig. 3).

Each TLR has its own intrinsic signaling pathway and induces specific biological responses against microorganisms such as dendritic cell maturation, cytokine production, and the development of adaptive immunity (Akira et al. 2006).

Generally, after ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules to the TIR domain of the TLR. Four adaptor molecules are involved, namely myeloid differentiation factor 88 (MyD88), which is critical for all TLRs' signaling except TLR3; TIR-associated protein (TIRAP); TIR-domain-containing adaptor protein- inducing interferon-ß (IFN-ß) (TRIF), which exclusively associate with TLR3 and TLR4; and TRIF-related adaptor molecule (TRAM) (Akira et al. 2006; Schwabe et al. 2006). The outcome of the signaling through MyD88 and TRIF is the activation of distinct signaling pathways, leading to the production of proinflammatory cytokines and type I IFNs (IFN-α/ß) (Akira et al. 2006) (Fig. 3).

1.2.2 The role of TLRs in HCV infection

HCV activates innate immune system but simultaneously can evade the host immunity through several strategies resulting in failure to eradicate the virus in most infected individuals (Schwabe et al. 2006; Seki and Brenner 2008). Some HCV nonstructural proteins like serine protease NS3/4A have been shown to interrupt TLR3, MyD88-dependent TLRs, and RIG-1 signaling in vitro through disrupting many downstream

molecules like inducing proteolytic degradation of TRIF, MyD88, and interferon promoter stimulator-1 (IPS-1), respectively (Seki and Brenner 2008). All these interactions might be responsible for the absence of the activation of type I IFN genes by HCV in the human liver (Mihm et al. 2004) and might contribute to HCV persistence (Gale and Foy 2005).

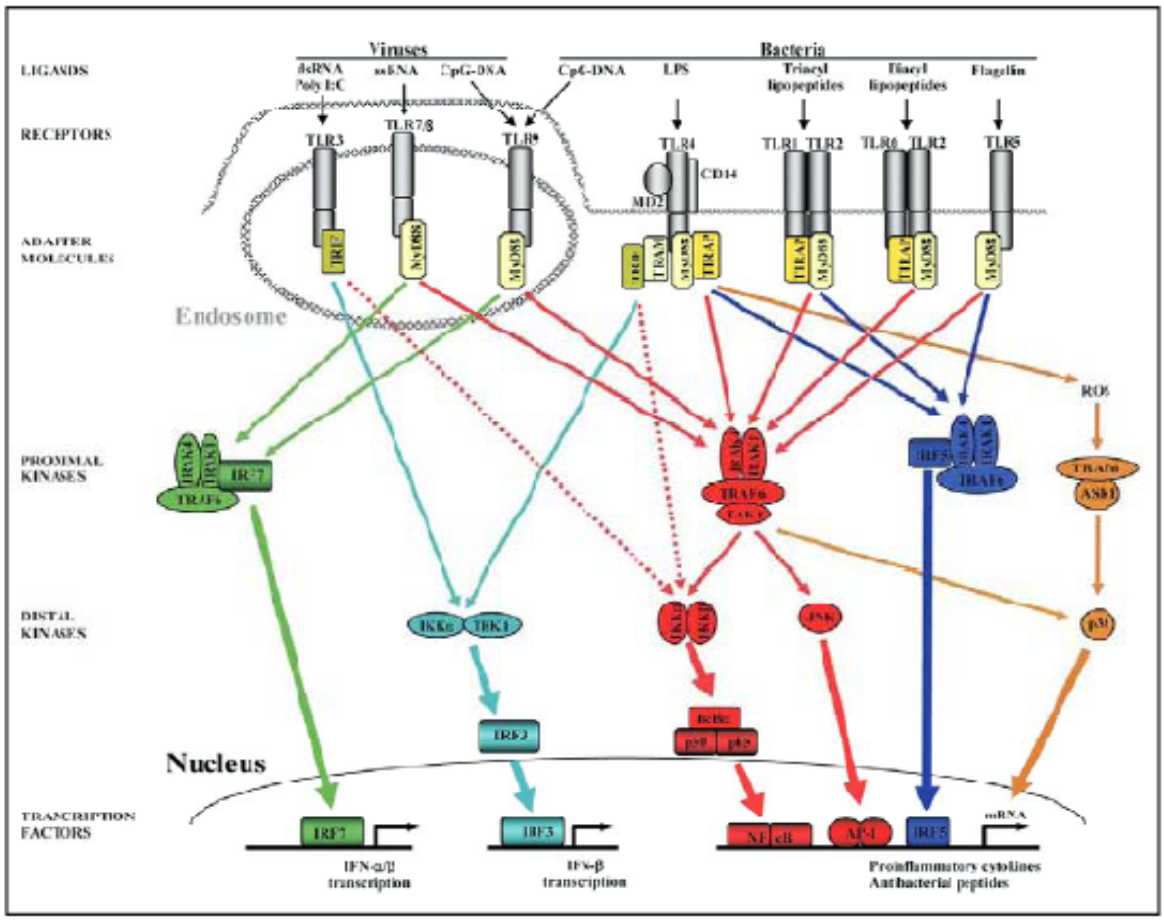

Fig. 3: Schematic overview of TLR ligands and signaling pathways (modified from Schwabe et al. (2006, p.1887)). Each TLR recognizes its specific ligand and interacts with one or several adapter molecules (MyD88, TRIF, TRAM, and TIRAP) to then induce activation of one or several downstream kinases and transcription factors, which up-regulate proinflammatory, antiviral, and antibacterial mediators.

1.3 Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) is just a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position, the least frequent allele should have a frequency of 1% or greater (Vignal et al. 2002). Although not all have

been identified so far, it is estimated that up to 10 million SNPs are probably present in the human genome (El-Omar et al. 2008).

Population-based association studies have a great benefit in estimating the risk of developing a certain multifactorial disease (Risch 2000; Schott et al. 2008; Meiler et al. 2005), or response to therapy in carriers and non-carriers of a particular genetic variant (Ge et al. 2009; Schott et al. 2008; Suppiah et al. 2009; Tanaka et al. 2009). The HapMap project (www.hapmap.org) is a valuable tool which facilitates the study of genetic polymorphisms relevant to human health and disease (El-Omar et al. 2008).

Recent studies have reported the association of TLRs genetic variations with several diseases such as susceptibility to sepsis, immunodefeciencies, atherosclerosis, asthma (Akira et al. 2006; Cook et al. 2004; Oh et al. 2009; Schott et al. 2008) and cancers (El-Omar et al. 2008; Fukata and Abreu 2008).

1.4 The aim of the study

The natural course of HCV infection is highly variable from one person to another (Seeff 2002), and the complete pattern of host responses to HCV is still not completely understood. However, roles of TLR7 and TLR3 are likely since the virus genome is ssRNA and encodes regions of extensive secondary dsRNA structure and since dsRNA is generated as an intermediate of the viral replication cycle (Gale and Foy 2005). Moreover, the membrane associated form of the endotoxin receptor component CD14 (mCD14) also colocalizes with TLR3 in intracellular compartments enhancing dsRNA sensing and TLR3 signaling (Lee et al. 2006; Vercammen et al. 2008). Further on, LPS translocated from the gut to the liver are suggested to exacerbate liver injury induced by toxin or ethanol via hepatic CD14 (Su 2002).

The study aimed to investigate whether specific SNPs with relevant minor allele frequencies (MAFs), according to information from Nucleotide Databank of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nhi.gov/SNP), within the genes encoding for the above-mentioned three receptors are correlated with relevant epidemiological and clinical manifestations of liver disease in CHC patients.

Two SNPs within the TLR3 gene (chromosome 4) were studied in the first article, namely, rs5743305 in the promoter region and rs3775291, a non-synonymous functional exon 4-located SNP that changes the amino acids from leucine to phenylalanine at position 412 of the TLR3 protein (Ranjith-Kumar et al. 2007). Both SNPs have been

reported to be associated with low antibody and lymphoproliferative responses to a measles vaccination (Dhiman et al. 2008).

The second article deals with rs2569190 within the CD14 gene (chromosome 5), the minor allele T of which has been shown to be correlated with the risk of developing liver cirrhosis in patients with alcohol-induced liver disease (ALD) (Campos et al. 2005; Jarvelainen et al. 2001; Meiler et al. 2005), but not in CHC patients (Meiler et al. 2005; Von Hahn et al. 2008). The study expanded the analysis to two further and independent cohorts and considered further histological parameters.

Taking the X-linked location of TLR7 gene into account, the third article studied the exon 3-located rs179008. The variant protein, encoded by the allele T which replaces A, has been described to be correlated with higher susceptibility to HCV infection and less chances of response to an IFN-α-based therapy in CHC females (Schott et al. 2008); and with higher viral loads, fast progression to advanced immune suppression in human immune deficiency virus (HIV) infection, and increased susceptibility to HIV-1 in women (Oh et al. 2009).

2. Summarised representation of the material and methods

2.1 Patients

A total of 137, 137 and 349, and 144 patients, mainly Caucasians, with CHC were enrolled into analyses in the three articles, respectively. The common studied group composed of consecutive out-patients who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center Goettingen (UMG), Germany, between 1993 and 2006. CHC was proved by the detection of HCV-specific antibodies and HCV RNA in patients sera using a highly sensitive nested RT-PCR over a period of at least 6 months (Mihm et al. 1996a). Patients with concomitant non-HCV infections and those with continued alcohol or other drug abuse were excluded.
Viral genotypes were determined using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium).
Routinely, before the start of therapy, liver biopsy procedures were performed to confirm liver disease histologically as described below. In parallel, biochemical liver disease parameters, i.e. serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γ-GT) were recorded.
Two additional subgroups were included into analysis in the third article. The first composed of 55 patients treated with IFN-α_{2a} (Roferon A; Hoffman-La Roche, Basel, Switzerland). The second contained 44 patients with self-limited HCV infection, which was detected by the presence of anti-HCV antibodies in the absence of detectable amounts of HCV RNA.
The 349 patients' cohort was studied in addition in the second article, where samples and hepatitis activity and fibrosis progression data were provided by the German Network of Competence for Hepatitis (Hep-Net).
The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

2.2 Histological evaluation

For histological evaluation, sections (5–10 μm) from formalin-fixed and paraffin-embedded liver tissue samples were stained with hematoxylin-eosin, trichrome, and

Prussian blue. Five lesions typical for hepatitis C were considered. Necroinflammatory activity (grading, score 1-3) and architectural alterations (staging, score 0-4), which were scored separately according to Desmet and colleagues (Desmet et al. 1994); steatosis degree (score 0-3); and the presence or absence of both portal lymphoid aggregates and bile duct damage (Mihm et al. 1997).

2.3 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from approximately 30 ml of heparinized peripheral blood samples were prepared by Ficoll density centrifugation using guanidinium isothiocyanate (Boyum 1984).

2.4 Isolation of genomic DNA

Genomic DNA (gDNA) was purified from PBMCs by means of QIAamp DNA Mini Kit following the blood and body fluid spin protocol (Qiagen, Hilden, Germany) or from 2 ml of serum using QIAamp DNA Blood Midi Kit (Qiagen). Both concentration and purity of PBMCs-derived gDNA were determined photometrically by reading the absorbance levels at 260 and 280 nm. To assure the integrity of gDNA, an electrophoresis using a 0.6% agarose gel was used.

2.5 SNP genotyping

gDNA was used for genotyping of the variant positions TLR3 rs5743305, TLR3 rs3775291, and TLR7 rs179008 using the commercially available TaqMan genotyping assays C_393058_10, C_1731425_10, and C_2259574_10, respectively (Applied Biosystems, Foster City, CA) in the sequence detection system Step One-Plus (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions.

For CD14 rs2569190, gDNA was amplified by real-time PCR using the TaqMan® Universal Master Mix (Applied Biosystems, Darmstadt, Germany) and 36 µmol/L of primers each (CD14: forward 5'-CTA GAT GCC CTG CAG AAT CCT T-3', reverse 5'-CCC TTC CTT TCC TGG AAA TAT TGC A-3'). Allelic discrimination was achieved by adding 8 µmol/L differentially fluorescence dye-labeled allele-specific minor groove binder probes (CD14: VIC 5'-CCT GTT ACG GTC CCC CTG-3', FAM 5'-CTG TTA CGG CCC CCC T-3'). Reactions and analyses were carried out in the sequence detection

system ABI prism 7000 (Applied Biosystems, Darmstadt, Germany) following the supplier's instructions.

2.6 Isolation of total cellular RNA and reverse transcription

Total cellular RNA was prepared from available freshly isolated PBMC and homogenized liver tissue samples by using CsCl density gradient centrifugation. To get complementary DNA (cDNA), an amount of 1 µg of total cellular RNA was reverse transcribed by using random hexamers (6 µM) for priming (Mihm et al. 1996b).

2.7 Quantification of gene expression

Quantification of gene expression in PBMC and liver tissue samples was performed by real-time RT-PCR in a Step One-Plus sequence detection system using commercially available TaqMan gene expression Assays on Demand for TLR3 (Hs00152933m1) and, as a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905m1) (Applied Biosystems, Darmstadt, Germany).

Competitive RT-PCR was applied to quantify hepatic HCV RNA and hepatic transcripts of the IFN-α/ß inducible antiviral myxovirus resistance protein-1 gene (MxA); IFN-α; albumin, as a reference gene, essentially as described (Mihm et al. 2004); the interferon-gamma (IFN-γ)-inducible protein 10 (IP-10); the gene encoding IFN-α/ß-inducible p44 (Patzwahl et al. 2001); and IFN-γ (Mihm et al. 1996b) . The relative amount of gene expression of IL-29/IFN-λ_1; the two subunits of IFN-λ heterodimeric receptor, IL-10 receptor beta (IL-10Rß) and IL-28 receptor alpha (IL28Rα); and IFN-α/ß receptor 2 (IFNAR2) was calculated by real-time RT-PCR using the sequence detection system ABI prism 7000 following the supplier's instructions (Doyle et al. 2006; Mihm et al. 2004).

2.8 Allele-specific transcript quantification (ASTQ) of TLR7 rs179008 variants

To quantify the relative proportion of A and T allele transcript variants of TLR7 rs179008 in RNA preparations from heterozygous females' fresh PBMCs, the commercially available TaqMan genotyping assay C_2259574_10 (Applied Biosystems, Foster City, CA) was applied. Heterozygote gDNA and homozygote gDNA and cDNA samples served as controls.

2.9 Statistical analysis

To compare the genetic variants' distribution with Hardy-Weinberg-equilibrium (HWE), a Log likelihood ratio χ^2-test was applied by means of an online statistical program (http://ihg.gsf.de).

χ^2-test and the parametric independent samples *t*-test were used where applicable, and the significance was set to a screening level of 0.05. All tests were performed by using PC STATISTIK software package version 4.0 (Hoffmann-Software, Giessen, Germany), SAS 9.2 through a cooperation with the Department of Genetic Epidemiology, UMG, and PASW 17 for Windows (SPSS Inc, Chicago, IL) with the assistance of Medistat GmbH, Kiel, Germany.

Parameters which showed significant correlations to the genotype in the univariate analysis were also analyzed using multivariate logistic regression or were stratified by age, sex, and HCV subtypes. To compare individual genotypes, linear or logistic regression models were applied. Cochran–Mantel Haenzel-test was performed to compare individual genotypes between HCV subtypes after stratification by sex and age.

Due to the TLR7 X-linked gene, females and males were analyzed both separately and combined due to the ASTQ results.

3. Summarised representation of the results

3.1 Genotyping for the respective SNPs under investigation

A total of 137, 137 and 349, and 144 CHC patients were genotyped for the SNPs: TLR3 rs5743305 (T/A) and rs3775291 (C/T), CD14 rs2569190 (C/T), and TLR7 rs179008 (A/T), respectively. Another group of 44 patients with self-limited HCV infection were genotyped for TLR7 rs179008. Genotype distribution and MAF were close to that given for Caucasians in NCBI and did not deviate from HWE**.**

3.2 ASTQ

Due to the X-linked location of TLR7, it is critical to assign the females heterozygous for rs179008 (A/T) to the wildtype, the variant genotype or to separate them in a true heterozygous group, therefore, ASTQ was performed. RNA preparations were found to contain either nearly equal numbers of both alleles' transcripts, or an excess of one of the two alleles (A or T) (i.e. an individual skewed mosaicism). Consequently, analyses had to be restricted to the comparison between TLR7 rs179008 A and T homo- and hemizygous patients.

3.3 Epidemiological characteristics

Demographic analysis revealed no significant relationship with TLR3 rs5743305, TLR3 rs3775291, or TLR7 rs179008 genotypes. The UMG patients homozygous for the variant allele T in CD14 rs2569190 were found to be on average 6.1 years younger than C carriers at the time of liver biopsy. This observation, however, was absent in Hep-Net patients.

As expected for a European cohort, HCV 1b was the predominant subtype that infected most of the patients, followed by subtypes 1a and 3a. An analysis of HCV genotype/subtype distribution revealed only a significant correlation with TLR3 rs3775291 in terms of an absence of the variant allele T homozygosity among patients with subtype 1a vs. 17.8% 1b-infected patients ($p=0.0167$).

3.4 Genotyping of individuals with self-limited HCV infection

The minor allele T of TLR7 rs179008 has been found to be significantly higher in CHC patients in comparison with healthy individuals (Schott et al. 2008). TLR7 rs179008 genotype distribution of the CHC patients was compared to a group of 44 patients with self-limited HCV infection. The proportion of T homo- and hemizygous patients or even of heterozygous females was not found to be significantly higher than their counterparts with self-limited infection, suggesting no altered capacity of resolving the infection spontaneously.

3.5 Biochemical parameters

None of the studied SNPs was found to be associated with serum transaminase (AST, ALT, or γ-GT) activities or with the number of patients who have normal or markedly elevated transaminase levels (i.e. greater than 2-fold of the upper normal limit).

3.6 Hepatitis C histological manifestations

Histological evaluation was performed on liver biopsy specimens taken from the naïve patients. While neither of the TLR3 studied SNPs was found to be associated with liver histological features, higher frequencies of the minor allele T of both CD14 rs2569190 and TLR7 rs179008 were found among males with portal lymphoid aggregates comparable to those who carry the wildtype alleles C ($p=0.004$) and A ($p=0.032$), respectively. The presence of portal lymphoid aggregates was not found to be correlated with gender, age, HCV subtypes, fibrosis or steatosis stage, but with hepatic inflammatory activity ($p=0.003$) and bile duct damage ($p<0.001$). A slight trend of TLR7 rs179008 variant allele carriers (TT females and T males) was also found to be higher among patients with bile duct damage ($p=0.051$).

Unlike the situation in ALD, CD14 -159T was not found to be related to fibrosis stage in CHC infection neither in UMG nor in Hep-Net studied groups, suggesting a gene-environment interaction.

3.7 Response to an IFN-α_{2a} monotherapy

TLR7 rs179008 T allele has been recently reported to be predictive of unfavourable outcome of IFN-α therapy in females with CHC (Schott et al. 2008). The initial virological response to a mono- IFN-α_{2a} therapy was analyzed with regard to the SNP

genotype in 55 patients. Response was achieved only in 30% of T carriers vs. 63% of A counterparts, yielding a slight trend of significance (p=0.069). Similar results were observed in a larger cohort of 145 patients treated with a combined IFN-α-based therapy (data not shown).

3.8 Quantification of gene expression

Expected to affect the transcriptional activity as a promoter SNP, TLR3 rs5743305 variants were found, however, to be unrelated to the number of TLR3 transcripts quantified neither in PBMC nor in liver tissue samples. This might explain the absence of association of this SNP with the above-mentioned characteristics. In contrast, patients homozygous for the variant allele T of TLR3 exon 4 SNP had a slightly higher hepatic TLR3 gene expression than C allele carriers (p=0.0191), which might have a relationship with the absence of infection with HCV subtype 1a in TT patients.

Innate immunity gene transcripts were quantified in freshly derived liver tissue samples and the numbers were related to TLR7 rs179008 genotype after correction for GAPDH or albumin as reference genes. Hepatic viral load and the number of gene transcripts shown to be raised in CHC when compared to healthy liver tissue such as IP-10, p44, MxA and IFN-γ (Mihm et al. 2004) did not reveal any significant association with the relative SNP. On the other hand, T homo- and hemizygotes were found to express significant lower amounts of IL-29/IFN-λ_1 (p=0.015), IL-10Rß (p=0.001), IL-28Rα (p=0.003) and relatively lower amounts of IFN-α and $IFNAR_2$ than A homo- and hemizygotes. This might be predictive for the probable future therapy with IFN-λ_1 rather than forecasting the outcome of the current IFN-α-based approaches.

Worth mentioning, total CD14 mRNA levels from freshly isolated PBMCs were not found to be related to CD14 rs2569190/C-159T genotypes in healthy blood donors (a co-authorship contribution (Mertens et al. 2009)).

3.9 Conclusions

The findings that were obtained in the course of this thesis contribute to the understanding of the relevance of genetic variants within PRRs in CHC disease (phenotypes).

TLR3 rs3775291 minor allele seems to confer a different susceptibility towards HCV subtype infection and a different hepatic gene expression of the receptor, but does not have any further effects on the clinical outcome of the liver disease.

CD14 rs2569190 minor allele, and to a lower extent TLR7 rs179008 variant allele, might have a role in the formation of portal lymphoid aggregates in males; the exact etiology and the importance of such a feature in disease development is still, however, unclear.

The only week correlation of TLR7 rs179008 with IFN-α-mediated or spontaneous eradication of HCV is in line with consentient results of five recent genome-wide association studies (GWASs). These GWASs have enrolled thousands of CHC patients from various ethnic populations and independently identified SNPs in the intergenic region between IFN-λ_2 and IFN-λ_3 genes on chromosome 19 to be the strongest predictable variants of sustained virological response to an IFN-α-based therapy (Ge et al. 2009; Rauch et al. 2010; Suppiah et al. 2009; Tanaka et al. 2009) and spontaneous clearance of HCV (Rauch et al. 2010; Thomas et al. 2009).
These recent convincing and comprehensive whole genome approaches might have sufficiently answered any role of single genetic variants at least with regard to IFN-α responsiveness and self-limited virus eradication in HCV infection.

Nevertheless, the finding of the significant association between TLR7 variants and distinct hepatic gene expression of IFN-λ_1 and IFN-λ receptor subunits might point to a role of TLR7 in HCV pattern recognition linked to endogenous IFN-λ_1 and IFN-λ receptor expression. The genetic constitution of its ligands has been shown to be determinant for responsiveness to endogenous or exogenous IFN-α.

IFN-λ_1 is currently being tested in a phase 2b clinical trial. Near future functional studies will elaborate whether the genetic variations in the intergenic region between the IFN-λ_2 and IFN-λ_3 genes might affect IFN-$\lambda_{2/3}$ gene expression and whether IFN-λ or its receptor expression affects IFN-α responsiveness.

4. References

Akira S, Uematsu S and Takeuchi O (2006): Pathogen recognition and innate immunity. Cell 124, 783-801.

Bartenschlager R, Frese M and Pietschmann T (2004): Novel insights into hepatitis C virus replication and persistence. Adv Virus Res 63, 71-180.

Boyum A (1984): Separation of lymphocytes, granulocytes, and monocytes from human blood using iodinated density gradient media. Methods Enzymol 108, 88-102.

Brass V, Moradpour D and Blum HE (2006): Molecular virology of hepatitis C virus (HCV): 2006 update. Int J Med Sci 3, 29-34.

Campos J, Gonzalez-Quintela A, Quinteiro C, Gude F, Perez LF, Torre JA and Vidal C (2005): The -159C/T polymorphism in the promoter region of the CD14 gene is associated with advanced liver disease and higher serum levels of acute-phase proteins in heavy drinkers. Alcohol Clin Exp Res 29, 1206-13.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW and Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359-62.

Cook DN, Pisetsky DS and Schwartz DA (2004): Toll-like receptors in the pathogenesis of human disease. Nat Immunol 5, 975-9.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M and Scheuer PJ (1994): Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 19, 1513-20.

Dhiman N, Ovsyannikova IG, Vierkant RA, Ryan JE, Pankratz VS, Jacobson RM and Poland GA (2008): Associations between SNPs in toll-like receptors and related intracellular signaling molecules and immune responses to measles vaccine: preliminary results. Vaccine 26, 1731-6.

Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S and Klucher KM (2006): Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 44, 896-906.

El-Omar EM, Ng MT and Hold GL (2008): Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 27, 244-52.

Fukata M and Abreu MT (2008): Role of Toll-like receptors in gastrointestinal malignancies. Oncogene 27, 234-43.

Gale M, Jr. and Foy EM (2005): Evasion of intracellular host defence by hepatitis C virus. Nature 436, 939-45.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG and Goldstein DB (2009): Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461, 399-401.

Horsmans Y, Berg T, Desager JP, Mueller T, Schott E, Fletcher SP, Steffy KR, Bauman LA, Kerr BM and Averett DR (2005): Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. Hepatology 42, 724-31.

Iadonato SP and Katze MG (2009): Genomics: Hepatitis C virus gets personal. Nature 461, 357-8.

Jarvelainen HA, Orpana A, Perola M, Savolainen VT, Karhunen PJ and Lindros KO (2001): Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease. Hepatology 33, 1148-53.

Kato N (2001): Molecular virology of hepatitis C virus. Acta Med Okayama 55, 133-59.

Kawai T and Akira S (2006): Innate immune recognition of viral infection. Nat Immunol 7, 131-7.

Lee HK, Dunzendorfer S, Soldau K and Tobias PS (2006): Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. Immunity 24, 153-63.

Medzhitov R, Preston-Hurlburt P and Janeway CA, Jr. (1997): A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394-7.

Meiler C, Muhlbauer M, Johann M, Hartmann A, Schnabl B, Wodarz N, Schmitz G, Scholmerich J and Hellerbrand C (2005): Different effects of a CD14 gene polymorphism on disease outcome in patients with alcoholic liver disease and chronic hepatitis C infection. World J Gastroenterol 11, 6031-7.

Mertens J, Bregadze R, Mansur A, Askar E, Bickeboller H, Ramadori G and Mihm S (2009): Functional impact of endotoxin receptor CD14 polymorphisms on transcriptional activity. J Mol Med 87, 815-24.

Mihm S, Hartmann H, Fayyazi A and Ramadori G (1996a): Preferential virological response to interferon-alpha 2a in patients with chronic hepatitis C infected by virus genotype 3a and exhibiting a low gamma-GT/ALT ratio. Dig Dis Sci 41, 1256-64.

Mihm S, Hutschenreiter A, Fayyazi A, Pingel S and Ramadori G (1996b): High inflammatory activity is associated with an increased amount of IFN-gamma transcripts in peripheral blood cells of patients with chronic hepatitis C virus infection. Med Microbiol Immunol 185, 95-102.

Mihm S, Fayyazi A, Hartmann H and Ramadori G (1997): Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology 25, 735-9.

Mihm S, Frese M, Meier V, Wietzke-Braun P, Scharf JG, Bartenschlager R and Ramadori G (2004): Interferon type I gene expression in chronic hepatitis C. Lab Invest 84, 1148-59.

Miller DM, Klucher KM, Freeman JA, Hausman DF, Fontana D and Williams DE (2009): Interferon lambda as a potential new therapeutic for hepatitis C. Ann N Y Acad Sci 1182, 80-7.

Oh DY, Baumann K, Hamouda O, Eckert JK, Neumann K, Kucherer C, Bartmeyer B, Poggensee G, Oh N, Pruss A, Jessen H and Schumann RR (2009): A frequent functional toll-like receptor 7 polymorphism is associated with accelerated HIV-1 disease progression. AIDS 23, 297-307.

Patzwahl R, Meier V, Ramadori G and Mihm S (2001): Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: detection by suppression-subtractive hybridization. J Virol 75, 1332-8.

Pawlotsky JM, Chevaliez S and McHutchison JG (2007): The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 132, 1979-98.

Penin F, Dubuisson J, Rey FA, Moradpour D and Pawlotsky JM (2004): Structural biology of hepatitis C virus. Hepatology 39, 5-19.

Perz JF, Farrington LA, Pecoraro C, Hutin YJF and Armstrong GL (2004): Estimated global prevalence of hepatitis C virus infection. 42nd Annual Meeting of the Infectious Diseases Society of America; Boston, MA, USA; Sept 30-Oct 3.

Ranjith-Kumar CT, Miller W, Sun J, Xiong J, Santos J, Yarbrough I, Lamb RJ, Mills J, Duffy KE, Hoose S, Cunningham M, Holzenburg A, Mbow ML, Sarisky RT and Kao CC (2007): Effects of single nucleotide polymorphisms on Toll-like receptor 3 activity and expression in cultured cells. J Biol Chem 282, 17696-705.

Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H, Gunthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Mullhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A and Bochud PY (2010): Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138, 1338-45, 1345 e1-7.

Rehermann B and Nascimbeni M (2005): Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 5, 215-29.

Risch NJ (2000): Searching for genetic determinants in the new millennium. Nature 405, 847-56.

Robert-Koch-Institut (2010): Zur Situation bei wichtigen Infektionskrankheiten in Deutschland: Virushepatitis B, C und D im Jahr 2009. Epid Bull 20, 177-190.

Schott E, Witt H, Neumann K, Bergk A, Halangk J, Weich V, Muller T, Puhl G, Wiedenmann B and Berg T (2008): Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. J Viral Hepat 15, 71-8.

Schwabe RF, Seki E and Brenner DA (2006): Toll-like receptor signaling in the liver. Gastroenterology 130, 1886-900.

Seeff LB (2002): Natural history of chronic hepatitis C. Hepatology 36, S35-46.

Seki E and Brenner DA (2008): Toll-like receptors and adaptor molecules in liver disease: update. Hepatology 48, 322-35.

Shepard CW, Finelli L and Alter MJ (2005): Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 5, 558-67.

Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ and Widell A (2005): Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42, 962-73.

Su GL (2002): Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 283, G256-65.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR and George J (2009): IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41, 1100-4.

Takeuchi O and Akira S (2007): Recognition of viruses by innate immunity. Immunol Rev 220, 214-24.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K and Mizokami M (2009): Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41, 1105-9.

Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB and Carrington M (2009): Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461, 798-801.

Vercammen E, Staal J and Beyaert R (2008): Sensing of viral infection and activation of innate immunity by toll-like receptor 3. Clin Microbiol Rev 21, 13-25.

Vignal A, Milan D, SanCristobal M and Eggen A (2002): A review on SNP and other types of molecular markers and their use in animal genetics. Genet Sel Evol 34, 275-305.

Von Hahn T, Halangk J, Witt H, Neumann K, Muller T, Puhl G, Neuhaus P, Nickel R, Beuers U, Wiedenmann B and Berg T (2008): Relevance of endotoxin receptor CD14 and TLR4 gene variants in chronic liver disease. Scand J Gastroenterol 43, 584-92.

Wagner H and Bauer S (2006): All is not Toll: new pathways in DNA recognition. J Exp Med 203, 265-8.

5. Copies of the publications

5.1 The first publication: TLR3 gene polymorphisms and liver disease manifestations in chronic hepatitis C

Askar E., Bregadze R, Mertens J, Schweyer S, Rosenberger A, Ramadori G, Mihm S. (2009): TLR3 gene polymorphisms and liver disease manifestations in chronic hepatitis C. J Med Virol 81, 1204-11.

Journal of Medical Virology 81:1204–1211 (2009)

TLR3 Gene Polymorphisms and Liver Disease Manifestations in Chronic Hepatitis C

Eva Askar,[1] Rusudan Bregadze,[1] Jasmin Mertens,[1] Stefan Schweyer,[2] Albert Rosenberger,[3] Giuliano Ramadori,[1] and Sabine Mihm[1]*

[1]*Department of Gastroenterology and Endocrinology, University Medical Center, Georg-August-Universität, Göttingen, Germany*
[2]*Department of Pathology, University Medical Center, Georg-August-Universität, Göttingen, Germany*
[3]*Department of Genetic Epidemiology, University Medical Center, Georg-August-Universität, Göttingen, Germany*

Phenotypes of liver disease due to chronic hepatitis C virus (HCV) infection show a wide range of variations in terms of histological manifestations and the clinical outcome. Sensing of viral double-stranded RNA (dsRNA) by Toll-like receptor 3 (TLR3) is likely involved in early pathogen detection and the host response to viral infection. This study analyzed epidemiological and clinical data from a total of 137 patients with chronic HCV infection with regard to two polymorphic positions within the TLR3 gene: rs5743305 (T/A) is located within the promoter region and might affect transcriptional activity, rs3775291 (C/T) is a non-synonymous single nucleotide polymorphism (SNP) located within exon 4 and the variant receptor has been shown to be functionally impaired. TLR3 promoter and the exon 4 variations were not found to be associated with TLR3 gene expression in peripheral blood mononuclear cells (PBMCs). In the liver, however, a tendency of higher TLR3 gene expression was found for exon 4 TT genotypes. Both variations were not found to be associated with clinical parameters of chronic disease. On the other hand, an analysis of the TLR3 exon 4 genotype distribution with respect to HCV subtype revealed an absence of TT genotype among HCV subtype 1a infected individuals. This study thus failed to reveal any association of the two SNPs under investigation with clinical parameters of chronic hepatitis C. However, data argue for a functional relevance of the exon 4 SNP in terms of conferring a different susceptibility towards HCV subtype infection. ***J. Med. Virol. 81:1204–1211, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: single nucleotide polymorphism (SNP); hepatitis C virus (HCV); Toll-like receptor (TLR); HCV genotypes; T-976A; L412F

© 2009 WILEY-LISS, INC.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped single-stranded plus-sense RNA virus that causes acute and often chronic hepatitis [Choo et al., 1989]. Currently, an estimated 3% of the world's population—about 210 million people—is infected with HCV [Shepard et al., 2005]. The natural outcome of infection ranges from silent to self-limited to chronic [Micallef et al., 2006]. The natural course of chronic disease is also highly variable and ranges from asymptomatic to mild disease to cirrhosis and hepatocellular carcinoma (HCC) and HCV-related co-diseases [Seeff, 2002]. The complete pattern of host responses to HCV infection is still unclear. However, the HCV genome encodes regions of extensive secondary double-stranded RNA (dsRNA) structure, and the viral replication cycle includes dsRNA as an intermediate; both entities are assumed to be sensed by pattern recognition receptors (PRRs) during infection [Gale and Foy, 2005].

PRRs comprise membrane Toll-like receptors (TLRs), which are either expressed on the cell surface or on endosomal–lysosomal membranes, and cytosolic receptors such as nucleotide-binding oligomerization domain (NOD)-like receptors and the RNA-helicase family [reviewed in Akira et al., 2006; Takeuchi and Akira, 2007; Seki and Brenner, 2008]. Viral dsRNA is a ligand of TLR3 which is expressed within the endosomal compartment of conventional dendritic cells (cDCs) [Alexopoulou et al., 2001; Matsumoto et al., 2004]. Upon phagocytosis of infectious material and signaling through TLR3, DCs become activated. In the liver,

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: MI 474/1-1.

*Correspondence to: Sabine Mihm, Division of Gastroenterology and Endocrinology, Department of Internal Medicine, Georg-August-Universität, Robert-Koch-Straße 40, Göttingen 37075, Germany. E-mail: smihm@med.uni-goettingen.de

Accepted 3 March 2009

DOI 10.1002/jmv.21491

Published online in Wiley InterScience (www.interscience.wiley.com)

TLR3 is expressed by Kupffer cells, natural killer (NK) cells, and by hepatocytes [Seki and Brenner, 2008]. TLR3 is also expressed on a variety of epithelial cells, for example, biliary or intestinal epithelium; unlike DCs, epithelial cells appear to express TLR3 on the cell surface [Akira et al., 2006]. More recent studies point, in addition, to a co-stimulatory role of TLR3 on human T lymphocytes [Tabiasco et al., 2006; Wesch et al., 2006].

TLR3 ligand-binding leads to the activation of the transcription factors interferon-regulatory factor-3 (IRF-3) and nuclear factor-κB (NF-κB) and, finally, to the induction of interferon-β (IFN-β) and proinflammatory cytokines, respectively [Santoro et al., 2003; Matsumoto et al., 2004]. In contrast to other members of TLR which associate with a common adapter molecule, myeloid differentiation factor 88 (MyD88), the TLR3 pathway depends on another adaptor protein, the TIR-domain containing adaptor inducing IFN-β (TRIF) [reviewed in Akira et al., 2006; Seki and Brenner, 2008; Vercammen et al., 2008]. IFN-β, in turn, activates several other genes, including 2′-5′-oligoadenylate synthetases, protein kinase R, Mx GTPase, and P56, which contribute to an antiviral effect via the inhibition of protein synthesis or viral replication [Vercammen et al., 2008].

The completion of the human genome project opened up the opportunity to dissect complex human traits and to understand basic pathways of health and disease. Population-based association studies are very useful for examining genes with a role in common multifactorial diseases that have a strong environmental component [Risch, 2000]. These studies often estimate the risk of developing a certain disease in carriers and non-carriers of particular genetic variations especially single nucleotide polymorphisms (SNPs). Genetic variations in the TLRs and their signaling molecules have been correlated with susceptibility to various diseases including sepsis or malignancies [Cook et al., 2004; Akira et al., 2006; El-Omar et al., 2008; Fukata and Abreu, 2008] and to vaccination efficiency [Dhiman et al., 2008].

The human TLR3 gene contains SNPs with relevant minor allele frequencies (MAFs) for the Caucasian population. Whereas SNPs within the promoter region might affect transcriptional activity (e.g., rs5743305 (T-976A)), an amino acid exchange from leucine to phenylalanine at position 412 (rs3775291 (L412F)) has been shown to be associated with a functional impairment of the molecule in terms of mediating signaling [Ranjith-Kumar et al., 2007]. This study aimed at investigating whether genetic variations within the TLR3 promoter and the TLR3 protein are associated with severity of HCV infection.

MATERIALS AND METHODS

Patients

A total of 137 consecutive out-patients, mainly Caucasians, with chronic hepatitis C who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center, Göttingen between 1993 and 2006 were enrolled. Chronic HCV infection was diagnosed by the detection of HCV-specific antibodies and by HCV RNA in patients sera using a highly sensitive nested RT-PCR over a period of at least 6 months as described [Mihm et al., 1996a]. Viral genotypes were determined using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium). As part of a routine clinical evaluation, liver biopsy procedures were performed. Liver disease was confirmed histopathologically as described elsewhere [Mihm et al., 1997]. Patients with hepatitis B virus (HBV) or hepatitis A virus (HAV) co-infections and those with continued alcohol or other drug abuse were excluded. The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

Histopathological Evaluation

Liver biopsies were taken from patients before beginning of therapy for histopathological evaluation. In brief, sections (5–10 μm) from formalin-fixed and paraffin-embedded liver tissue samples were stained with hematoxylin-eosin, trichrome, and Prussian blue reaction. According to Desmet et al. [1994], necroinflammatory changes (grading) and architectural alterations (staging) were scored separately. Other lesions typical for hepatitis C such as the steatosis degree, portal lymphoid aggregates, or bile duct damage were studied additionally, the definition of each is given elsewhere [Mihm et al., 1997].

Isolation of Genomic DNA and SNP Genotyping

Genomic DNA (gDNA) was purified from peripheral blood mononuclear cell (PBMC) samples by means of QIAamp DNA Mini Kit following the blood and body fluid spin protocol (Qiagen, Hilden, Germany) or, when PBMCs were not available, from 2 ml of serum using QIAamp DNA Blood Midi Kit (Qiagen). The concentration and the purity of gDNA isolated from PBMCs, but not from serum due to the little amount of cells, were determined photometrically by reading the absorbance levels at 260 and 280 nm. An electrophoresis using a 0.6% agarose gel was used to assure the integrity of gDNA.

Genotyping of the variant positions rs5743305 and rs3775291 was performed in the sequence detection system Step One-Plus (Applied Biosystems, Darmstadt, Germany) using the commercially available TaqMan genotyping assays C_393058_10 and C_1731425_10, (Applied Biosystems, Foster City, CA), respectively, with 4 ng PBMCs-derived gDNA or an aliquot corresponding to 6.7 μl serum in 10 μl reactions according to the supplier's instructions.

Isolation of Total Cellular RNA and Quantitation of Gene Expression

Where available, total cellular RNA was prepared by CsCl density gradient centrifugation from freshly

TABLE I. TLR3 rs5743305 Genotype Distribution in Chronic Hepatitis C Patients

		TLR3 rs5743305 genotype					
	n	TT	TA	AA	*P*-value	MAF	*P*-value
Total number (%)	137	50 (36.5)	62 (45.3)	25 (18.2)	0.4806[a]	0.409	
Gender							
Female/male	60/77	25/25	23/39	12/13	0.3514	0.392/0.422	0.6114
Age							
Mean ± SD		44.2 ± 12.6	43.2 ± 11.1	45.9 ± 13.1	0.9452		

MAF, minor allele frequency.
[a]Exact test for Hardy–Weinberg equilibrium.

isolated PBMC and homogenized liver tissue samples from a total of 34 and 48 hepatitis C patients, respectively. PBMCs had been isolated from approximately 30 ml of heparinized peripheral blood samples by Ficoll density centrifugation and from homogenized liver tissue essentially as described [Mihm et al., 1996b].

An amount of 1 μg of total cellular RNA was reverse transcribed by using random hexamers (6 μM) for priming. Quantitation of gene expression was performed in a total volume of 10 μl by real-time RT-PCR in a Step One-Plus sequence detection system (Applied Biosystems, Darmstadt, Germany) using commercially available TaqMan gene expression assays for the quantitation of TLR3 (Hs00152933m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905m1) transcripts (Applied Biosystems, Darmstadt, Germany).

Statistical Analysis

To avoid bias, data were stratified by age (≤40 years, >40 years), sex, and HCV subtypes. To compare individual genotypes, linear, or logistic regression models were applied. To compare individual genotypes between HCV subtypes Cochran–Mantel Haenzel-test was performed after stratification for sex and age. The local level of significance was set to a screening level of $P = 0.05$. All calculations were performed with SAS 9.2 or PC STATISTIK software package version 4.0 (Hoffmann-Software, Giessen, Germany).

RESULTS

A total of 137 patients with a diagnosis of chronic HCV infection were included. Representative for a European sample, most of the patients were infected with HCV subtype 1b, followed by those who are infected with virus subtypes 1a and 3a. Before the start of a therapy, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transferase (γ-GT) activities were recorded. At the same time, liver biopsy specimens were taken and evaluated histopathologically. Five criteria were examined, including the activity of hepatitis, the degree of fibrosis, the degree of steatosis, the presence of portal lymphoid aggregates, and presence of bile duct damage.

All patients were genotyped for two bi-allelic SNPs within the TLR3 gene: rs5743305 (T/A), which is located at position 976 within the promoter region (relative to the transcriptional start site), and rs3775291 (C/T), which is a non-synonymous SNP within exon 4 and causes an amino acid exchange from leucine to phenylalanine at position 412 of the protein molecule. For both SNPs, genotype distribution and MAF were close to that given for Caucasians in public databases (Tables I and II). For the patients as a whole, no deviation from Hardy–Weinberg equilibrium was found (Tables I and II). Epidemiological analysis revealed no significant relationship between genotypes and both gender and age (Tables I and II).

To investigate whether the TLR3 promoter variants are associated with differences in TLR3 gene expression, the number of TLR3 transcripts was quantified both in PBMC and in liver tissue samples from a total of 34 and 48 hepatitis C patients, respectively (Fig. 1). Data were related to TLR3 promoter genotypes and, as a control, to TLR3 exon 4 genotypes, for which effects on transcriptional activity were expected less probable. TLR3 promoter variants were not found to be associated

TABLE II. TLR3 rs3775291 Genotype Distribution in Chronic Hepatitis C Patients

		TLR3 rs3775291 genotype					
	n	CC	CT	TT	*P*-value	MAF	*P*-value
Total number (%)	137	63 (46.0)	58 (42.3)	16 (11.7)	0.6983[a]	0.328	
Gender							
Female/male	60/77	29/34	25/33	6/10	0.8200	0.308/0.344	0.5311
Age							
Mean ± SD		44.0 ± 12.3	43.2 ± 11.6	47.3 ± 12.4	0.4909		

MAF, minor allele frequency.
[a]Exact test for Hardy–Weinberg equilibrium.

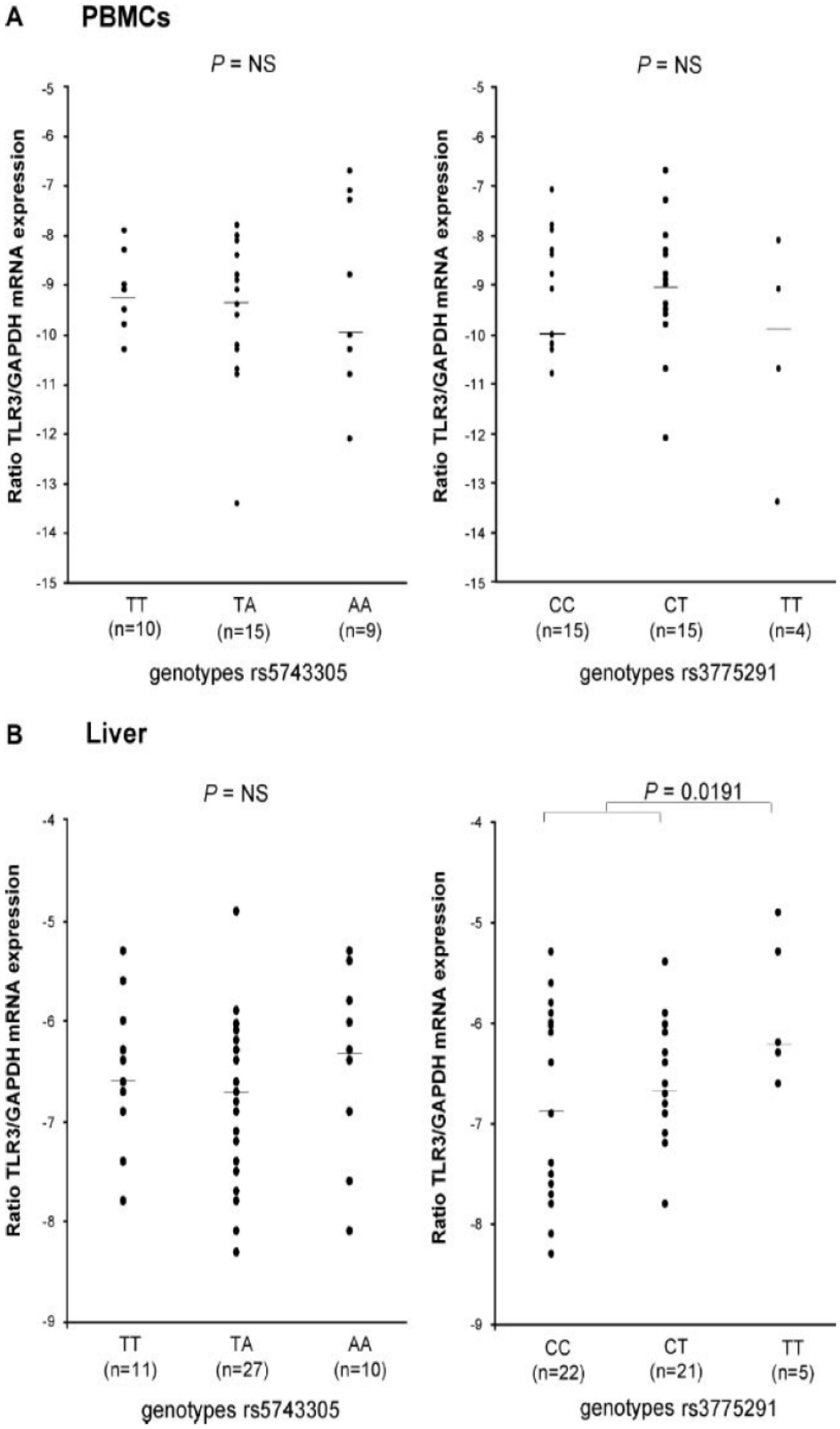

Fig. 1. TLR3 mRNA expression in PBMC and liver tissue samples from patients with chronic hepatitis C with regard to two TLR3 SNPs. Total cellular RNA from freshly isolated PBMCs (**A**) and liver tissue specimens (**B**) was analyzed for the expression of TLR3 mRNA in relation to GAPDH mRNA as a reference by commercially available real-time PCR gene expression assays as described in Materials and Methods Section. Data were related to the TLR3 promoter SNP rs5743305 under study and to the TLR3 exon 4 SNP rs3775291. Medians and *P* values are given. NS, non-significant.

with significant differences in PBMCs or in hepatic TLR3 mRNA expression (Fig. 1). Neither in healthy individuals, TLR3 promoter SNP genotype appeared to be related to the amount of TLR3 transcripts in PBMCs (data not shown).

In line with the lack of an association between TLR3 promoter genotypes and TLR3 gene expression, TLR3 promoter genotypes were not found to be significantly related to clinical data as serum transaminase activities (Table III) or histopathological manifestations (Table IV).

The polymorphism within TLR3 exon 4, which causes an amino acid exchange in the protein molecule, has been demonstrated to be functional in terms of mediating signaling [Ranjith-Kumar et al., 2007]. In chronic hepatitis C patients, this SNP was not found to be related to PBMCs but to hepatic TLR3 gene expression (Fig. 1). TT homozygous patients seemed to have higher hepatic TLR3 gene expression than C allele carriers ($P = 0.0191$). On the other hand, this SNP was not found to be associated with clinical data related to a chronic liver disease due to HCV infection: no significant relationship of TLR3 exon 4 variants was observed with serum transaminase activities (Table III) or with histological manifestations (Table IV).

An analysis of genotype distribution with regard to TLR3 promoter SNP revealed no significant difference among patients with HCV subtypes 1a, 1b, or 3a (Table V). In contrast, the TLR3 exon 4 genotype distribution with respect to HCV subtype, was not found to yield different distributions of TLR3 genotypes among patients with HCV subtypes 1a, 1b, or 3a infection. The proportion of patients with TT genotype and HCV subtype 1b infection amounts to 17.8%, only 9.1% of patients with HCV subtype 3a infection, and none of the patients with HCV subtype 1a infection were found to be homozygous for the variant allele T. A statistical comparison yielded a significant difference when patients with subtype 1a were compared to those with subtype 1b infection ($P = 0.0167$; Table VI).

DISCUSSION

Data revealed a lack of an association between hepatitis C patient's TLR3 promoter genotype and TLR3 mRNA expression, which was assessed both in freshly prepared PBMC and liver tissue specimens. In line with these findings, no evidence was found for an association of TLR3 promoter genotypes and clinical parameters of the chronic disease.

The second SNP under study is located within the coding region of the TLR3 gene. The variant allele causes an amino acid exchange and the variant protein was shown to be impaired in its signaling function [Ranjith-Kumar et al., 2007]. This SNP was not found to be related to TLR3 gene expression in PBMCs, as expected, nor was it to clinical parameters of chronic HCV infection. However, a relationship with TLR3 mRNA expression in liver tissue in terms of slightly enhanced transcripts in patients who carry the TT

TABLE III. Biochemical Serum Parameters and the Number of Patients With Elevated Parameters in Chronic Hepatitis C With Regard to TLR3 rs5743305 and rs3775291 Genotype Distribution

	rs5743305 genotype				rs3775291 genotype			
	TT (n = 50)	TA (n = 62)	AA (n = 25)	*P*-value	CC (n = 63)	CT (n = 58)	TT (n = 16)	*P*-value
AST[a] (mean ± SD)	44.2 ± 40.3	40.9 ± 41.0	48.0 ± 49.5	0.5367	43.5 ± 41.4	43.1 ± 45.7	43.9 ± 32.9	0.9833
No. of patients with elevated AST (%)	21 (42.0)	27 (43.5)	10 (40.0)	0.9974	25 (39.7)	24 (41.4)	9 (56.3)	0.6208
ALT[b] (mean ± SD)	85.8 ± 102.6	84.5 ± 115.7	78.4 ± 75.6	0.6404	76.9 ± 79.0	90.8 ± 130.2	85.9 ± 86.3	0.8958
No. of patients with elevated ALT (%)	29 (58.0)	32 (51.6)	13 (52.0)	0.8213	31 (49.2)	34 (58.6)	9 (56.3)	0.5417
γ-GT[c] (mean ± SD)	44.3 ± 45.6	48.6 ± 62.5	41.2 ± 28.1	0.6869	49.8 ± 55.6	37.5 ± 42.4	58.9 ± 62.3	0.2861
No. of patients with elevated γ-GT (%)	16 (32.0)	17 (27.4)	8 (32.0)	0.0992	22 (34.9)	13 (22.4)	6 (37.5)	0.3456

P values were calculated after stratifying for sex, age, and HCV subtypes.
The number of patients with significantly elevated serum activities of transaminases (>2-fold of the upper normal limit) was considered.
[a]AST, aspartate aminotransferase. Upper normal limit is 19 U/ml for males, and 15 U/ml for females.
[b]ALT, alanine aminotransferase. Upper normal limit is 23 U/ml for males, and 19 U/ml for females.
[c]γ-GT, γ-glutamytransferase. Upper normal limit is 28 U/ml for males, and 18 U/ml for females.

TABLE IV. Histological Manifestations in Chronic Hepatitis C Infected Patients With Regard to rs5743305 and rs3775291 Genotype Distribution

	rs5743305 genotype				rs3775291 genotype			
Histological manifestations	TT	TA	AA	*P*-value[a]	CC	CT	TT	*P*-value[a]
Hepatitis activity								
Mild	30 (39.5)	34 (44.7)	12 (15.8)	0.4184	38 (50.0)	30 (39.5)	8 (10.5)	0.2893
Moderate or severe	20 (32.8)	28 (45.9)	13 (21.3)		25 (41.0)	28 (45.9)	8 (13.1)	
Fibrosis								
Absent or mild	34 (39.5)	37 (43.0)	15 (17.4)	0.8594	42 (48.8)	36 (41.9)	8 (9.3)	0.1972
Moderate, marked, cirrhosis	16 (31.4)	25 (49.0)	10 (19.6)		21 (41.2)	22 (43.1)	8 (15.7)	
Steatosis								
Absent or mild	38 (35.2)	50 (46.3)	20 (18.5)	0.5929	50 (46.3)	48 (44.4)	10 (9.3)	0.5581
Moderate or marked	12 (41.4)	12 (41.4)	5 (17.2)		13 (44.8)	10 (34.5)	6 (20.7)	
Portal lymphoid aggregates								
Absent	30 (35.7)	43 (51.2)	11 (13.1)	0.0949	40 (47.6)	33 (39.3)	11 (13.1)	0.5176
Present	20 (37.7)	19 (35.9)	14 (26.4)		23 (43.4)	25 (47.2)	5 (9.4)	
Bile duct damage								
Absent	35 (39.3)	40 (44.9)	14 (15.7)	0.5650	40 (44.9)	40 (44.9)	9 (10.1)	0.6828
Present	15 (31.3)	22 (45.8)	11 (22.9)		23 (47.9)	18 (37.5)	7 (14.6)	

The number of patients is followed by their percentage in brackets.
[a]*P* values were calculated after stratifying for sex, age, and HCV types.

genotype was found. Moreover, another association between TLR3 exon 4 SNP and the epidemiological parameter of HCV subtype infection became evident, in that patients who are homozygous for the wild-type allele or who are heterozygous are more or less equally infected by the predominant viral subtypes 1a, 1b, and 3a but none of the patients who are homozygous for the variant T allele were infected by HCV subtype 1a.

TLR3 is one of several PRRs sensing ss or ds viral RNA in man [Takeuchi and Akira, 2007]. Viral RNA is also recognized by TLR7, the expression of which is restricted to non-myeloid plasmacytoid DCs (pDCs), the main type I IFN producing cells that are activated in particular in response to systemic RNA virus infections [Takeuchi and Akira, 2007] and by cytosolic receptors in infected cells.

HCV has been demonstrated to target adapter molecules that link viral RNA sensing and downstream effector events as type I IFN induction [Gale and Foy, 2005]. HCV serine protease NS3/4A has been shown to induce proteolytic degradation of TRIF, MyD88, and interferon promoter stimulator-1 (IPS-1) leading to disruption of TLR3, MyD88-dependent TLRs, and retinoic acid inducible gene-1 (RIG-1) signaling in vitro [reviewed in Seki and Brenner, 2008]. All these interactions might be responsible for the absence of the activation of type I IFN genes by HCV in the human liver [Mihm et al., 2004] and might contribute to HCV persistence [Gale and Foy, 2005].

The lack of an association between the two TLR3 promoter and exon 4 variants and liver disease manifestations argues against a role of TLR3 in the outcome of liver disease due to chronic HCV infection. An involvement of TLR3 gene expression on mesangial renal cells, however, has been suggested for HCV-associated glomerulonephritis on the basis of the presence of immune complexes containing viral RNA, enhanced TLR3 mRNA expression, and an enhanced expression of chemokines [Wornle et al., 2006].

The finding of a different susceptibility to HCV subtype 1a infection in individuals who are homozygous for the variant allele of the TLR3 exon 4 SNP is in line with findings by us and by others on different susceptibilities to viral infections in relation to polymorphisms in PRRs or in molecules mediating type I IFN responsiveness. TLR7 polymorphisms have been shown to be

TABLE V. HCV Subtype Distribution in Chronic Hepatitis C Infected Patients With Regard to rs5743305 Genotypes

		TLR3 rs5743305 genotype				*P*-value[a]	
HCV subtype	n	TT	TA	AA	MAF	TT/TA/AA	C-carrier/AA
1a	31	16 (51.6)	12 (38.7)	3 (9.7)	0.290	0.1726[b]	0.2466[b]
1b	73	23 (31.5)	37 (50.7)	13 (17.8)	0.432		
1a+1b	6	1 (16.7)	3 (50.0)	2 (33.3)			
2a	1	0	1 (100.0)	0			
2b	4	0	2 (50.0)	2 (50.0)			
3a	22	10 (45.5)	7 (31.8)	5 (22.7)	0.386	0.3636[c]	0.4031[c]

MAF, minor allele frequency.
[a]Cochran–Mantel Haenzel-test with age–sex-strata.
[b]Comparing HCV subtype 1a with 1b.
[c]Comparing HCV subtypes 1a, 1b, and 3a.

TABLE VI. HCV Subtype Distribution in Chronic Hepatitis C Infected Patients With Regard to rs3775291 Genotypes

		TLR3 rs3775291 genotype				*P*-value[a]	
HCV subtype	n	CC	CT	TT	MAF	CC/CT/TT	T-carrier/TT
1a	31	15 (48.4)	16 (51.6)	0 (0.0)	0.258	0.0551[b]	0.0167[b]
1b	73	30 (41.1)	30 (41.1)	13 (17.8)	0.384		
1a + 1b	6	3 (50.0)	2 (33.3)	1 (16.7)			
2a	1	0	1 (100.0)	0			
2b	4	3 (75.0)	1 (25.0)	0			
3a	22	12 (54.5)	8 (36.4)	2 (9.1)	0.273	0.1653[c]	0.0689[c]

MAF, minor allele frequency.
[a]Cochran–Mantel Haenzel-test with age–sex-strata.
[b]Comparing HCV subtype 1a with 1b.
[c]Comparing HCV subtypes 1a, 1b, and 3a.

associated with the incidence of chronic HCV infection, severity, and with responsiveness to an IFN-α-based therapy [Schott et al., 2007, 2008]. Polymorphisms within the IRF-1 gene have been found to be related to resistance to HCV subtype 3a [Wietzke-Braun et al., 2006] or to human immunodeficiency virus-1 (HIV-1) infections [Ball et al., 2007]. In line with these findings, the two TLR3 SNP variants under investigation have been shown to be associated with low humoral and cellular immunity in response to measles vaccination [Dhiman et al., 2008]. Although animal models of virus infection argue against a role of TLR3 in the initial, cell-autonomous recognition of viral infection that induces a first wave of type I IFN production [reviewed in Akira et al., 2006], TLR3 has been shown to promote cross-priming of cytotoxic T lymphocytes against viruses that do not directly infect DCs after phagocytosis of infected material [Schulz et al., 2005].

Taken together, the recent observation of an association of TLR3 exon 4 genotype and HCV subtype infection supports the concept that genetic variations in viral RNA sensing molecules and/or molecules mediating type I IFN signaling do determine susceptibility to viral infections. A higher hepatic TLR3 expression might be related to the absence of HCV 1a infection in patients homozygous for TLR3 exon 4 variant T allele. Nevertheless, once a chronic infection is established, this polymorphism appears not to affect the clinical outcome of hepatitis C disease in general.

ACKNOWLEDGMENTS

We thank all physicians of the Department of Gastroenterology and Endocrinology who were involved in patients' care and control for their kind cooperation. We also thank Waltraut Kopp for expert technical assistance. E.A. is grateful for her scholarship from Damascus University, Syria. R.B. was supported by a research scholarship by the German academic exchange service (DAAD).

REFERENCES

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124:783–801.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732–738.

Ball TB, Ji H, Kimani J, McLaren P, Marlin C, Hill AV, Plummer FA. 2007. Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed uninfected Kenyan sex workers. AIDS 21:1091–1101.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.

Cook DN, Pisetsky DS, Schwartz DA. 2004. Toll-like receptors in the pathogenesis of human disease. Nat Immunol 5:975–979.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Dhiman N, Ovsyannikova IG, Vierkant RA, Ryan JE, Pankratz VS, Jacobson RM, Poland GA. 2008. Associations between SNPs in toll-like receptors and related intracellular signaling molecules and immune responses to measles vaccine: Preliminary results. Vaccine 26:1731–1736.

El-Omar EM, Ng MT, Hold GL. 2008. Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 27:244–252.

Fukata M, Abreu MT. 2008. Role of Toll-like receptors in gastrointestinal malignancies. Oncogene 27:234–243.

Gale M, Jr., Foy EM. 2005. Evasion of intracellular host defence by hepatitis C virus. Nature 436:939–945.

Matsumoto M, Funami K, Oshiumi H, Seya T. 2004. Toll-like receptor 3: A link between toll-like receptor, interferon and viruses. Microbiol Immunol 48:147–154.

Micallef JM, Kaldor JM, Dore GJ. 2006. Spontaneous viral clearance following acute hepatitis C infection: A systematic review of longitudinal studies. J Viral Hepat 13:34–41.

Mihm S, Hartmann H, Fayyazi A, Ramadori G. 1996a. Preferential virological response to interferon-alpha 2a in patients with chronic hepatitis C infected by virus genotype 3a and exhibiting a low gamma-GT/ALT ratio. Dig Dis Sci 41:1256–1264.

Mihm S, Hutschenreiter A, Fayyazi A, Pingel S, Ramadori G. 1996b. High inflammatory activity is associated with an increased amount of IFN-gamma transcripts in peripheral blood cells of patients with chronic hepatitis C virus infection. Med Microbiol Immunol 185: 95–102.

Mihm S, Fayyazi A, Hartmann H, Ramadori G. 1997. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology 25:735–739.

Mihm S, Frese M, Meier V, Wietzke-Braun P, Scharf JG, Bartenschlager R, Ramadori G. 2004. Interferon type I gene expression in chronic hepatitis C. Lab Invest 84:1148–1159.

Ranjith-Kumar CT, Miller W, Sun J, Xiong J, Santos J, Yarbrough I, Lamb RJ, Mills J, Duffy KE, Hoose S, Cunningham M, Holzenburg A, Mbow ML, Sarisky RT, Kao CC. 2007. Effects of single nucleotide polymorphisms on Toll-like receptor 3 activity and expression in cultured cells. J Biol Chem 282:17696–17705.

Risch NJ. 2000. Searching for genetic determinants in the new millennium. Nature 405:847–856.

Santoro MG, Rossi A, Amici C. 2003. NF-kappaB and virus infection: Who controls whom. EMBO J 22:2552–2560.

Schott E, Witt H, Neumann K, Taube S, Oh DY, Schreier E, Vierich S, Puhl G, Bergk A, Halangk J, Weich V, Wiedenmann B, Berg T. 2007. A Toll-like receptor 7 single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection. J Hepatol 47:203–211.

Schott E, Witt H, Neumann K, Bergk A, Halangk J, Weich V, Muller T, Puhl G, Wiedenmann B, Berg T. 2008. Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. J Viral Hepat 15:71–78.

Schulz O, Diebold SS, Chen M, Naslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljestrom P, Reis e Sousa C. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 433:887–892.

Seeff LB. 2002. Natural history of chronic hepatitis C. Hepatology 36:S35–S46.

Seki E, Brenner DA. 2008. Toll-like receptors and adaptor molecules in liver disease: Update. Hepatology 48:322–335.

Shepard CW, Finelli L, Alter MJ. 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 5:558–567.

Tabiasco J, Devevre E, Rufer N, Salaun B, Cerottini JC, Speiser D, Romero P. 2006. Human effector CD8+ T lymphocytes express TLR3 as a functional coreceptor. J Immunol 177:8708–8713.

Takeuchi O, Akira S. 2007. Recognition of viruses by innate immunity. Immunol Rev 220:214–224.

Vercammen E, Staal J, Beyaert R. 2008. Sensing of viral infection and activation of innate immunity by toll-like receptor 3. Clin Microbiol Rev 21:13–25.

Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D. 2006. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. J Immunol 176:1348–1354.

Wietzke-Braun P, Maouzi AB, Manhardt LB, Bickeboller H, Ramadori G, Mihm S. 2006. Interferon regulatory factor-1 promoter polymorphism and the outcome of hepatitis C virus infection. Eur J Gastroenterol Hepatol 18:991–997.

Wornle M, Schmid H, Banas B, Merkle M, Henger A, Roeder M, Blattner S, Bock E, Kretzler M, Grone HJ, Schlondorff D. 2006. Novel role of toll-like receptor 3 in hepatitis C-associated glomerulonephritis. Am J Pathol 168:370–385.

5.2 The second publication: Endotoxin receptor CD14 gene variants and histological features in chronic HCV infection

Askar E, Ramadori G, Mihm S (2009): Endotoxin receptor CD14 gene variants and histological features in chronic HCV infection. World J Gastroenterol 15, 3884-90.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.3884

World J Gastroenterol 2009 August 21; 15(31): 3884-3890
World Journal of Gastroenterology ISSN 1007-9327
© 2009 The WJG Press and Baishideng. All rights reserved.

BRIEF ARTICLES

Endotoxin receptor *CD14* gene variants and histological features in chronic HCV infection

Eva Askar, Giuliano Ramadori, Sabine Mihm

Eva Askar, Giuliano Ramadori, Sabine Mihm, Department of Gastroenterology and Endocrinology, University Medical Center, Georg-August-University, D-37075 Goettingen, Germany
Author contributions: Askar E performed genotype analyses, collected and analyzed the data and wrote the manuscript; Ramadori G and Mihm S supervised the study and edited the manuscript.
Supported by A grant of the Deutsche Forschungsgemeinschaft MI 474/1-1; Askar E was supported by a scholarship from Damascus University, Syria
Correspondence to: Sabine Mihm, Professor, Dr. rer. nat., Department of Gastroenterology and Endocrinology, University Medical Center, Georg-August-University, Robert-Koch-Strasse 40, D-37075 Goettingen, Germany. smihm@med.uni-goettingen.de
Telephone: +49-551-398946 Fax: +49-551-397855
Received: May 14, 2009 Revised: July 17, 2009
Accepted: July 24, 2009
Published online: August 21, 2009

Abstract

AIM: To analyze the correlation between CD14 rs2569190/C-159T single nucleotide polymorphism (SNP) and disease progression in chronic hepatitis C.

METHODS: Liver biopsy specimens from a total of 137 and 349 patients with chronic hepatitis C were separately evaluated with respect to necroinflammatory activity (grading) and architectural changes (staging). In one group, further histological lesions characteristic for hepatitis C, hepatitis C virus subtypes, and biochemical parameters of liver disease were also investigated. Samples of genomic DNA were genotyped for the respective SNP by 5'-nuclease assays using fluorescent dye-labeled allele-specific probes.

RESULTS: Genotype distribution did not deviate from the Hardy-Weinberg equilibrium. In the first group, patients homozygous for the variant allele T were found to be younger than C allele carriers (39.6 ± 12.5 *vs* 45.7 ± 11.5, $P = 0.008$). Among the histological lesions studied, portal lymphoid aggregates were more frequently observed among TT homozygotes than among C carriers (21/37 *vs* 32/100, $P = 0.008$). The presence of portal lymphoid aggregates was closely correlated with hepatic inflammation ($P = 0.003$) and with bile duct damage ($P < 0.001$). The degree of fibrosis, in contrast, was not found to be related to the *CD14* gene C-159T polymorphism.

CONCLUSION: The data suggest a possible relationship between CD14 C-159T polymorphism and the formation of portal lymphoid aggregates, but not liver fibrosis progression in chronic hepatitis C.

© 2009 The WJG Press and Baishideng. All rights reserved.

Key words: CD14; Endotoxins; Hepatitis C virus; Inflammation; Lipopolysaccharides; Liver fibrosis; Portal system; Single nucleotide polymorphism

Peer reviewer: Christian P Strassburg, MD, Professor, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

Askar E, Ramadori G, Mihm S. Endotoxin receptor *CD14* gene variants and histological features in chronic HCV infection. *World J Gastroenterol* 2009; 15(31): 3884-3890 Available from: URL: http://www.wjgnet.com/1007-9327/15/3884.asp DOI: http://dx.doi.org/10.3748/wjg.15.3884

INTRODUCTION

Hepatitis C virus (HCV), which currently infects about 3% of the world's population (an estimated 210 million people), is a major cause of chronic viral liver disease[1]. Chronic hepatitis C is characterized by mostly mild hepatic inflammatory activity which does, however, hold a significant risk of proceeding to liver cirrhosis and hepatocellular carcinoma[2]. Further characteristic histological alterations may include steatosis, bile duct lesions, and portal lymphoid aggregates[3-5]. While steatosis, for instance, has been shown to be associated with HCV subtype infection and is suggested to be modulated by HCV proteins[4,6], progression of fibrosis in chronic hepatitis C has been attributed to age, gender, or alcohol consumption[7], and to host genetic factors[8]. Different genetic backgrounds have also been found to be associated with the susceptibility to HCV subtype infection[9,10].

As a result of its anatomic links to the gut, the liver is constantly exposed to gut-derived bacterial products, e.g. lipopolysaccharides (LPS), which are suggested to be important cofactors in toxin- or ethanol-induced liver disease by exacerbating ongoing injury[11]. Endotoxemia arises from increased translocation of endotoxins from the gut lumen because of altered intestinal permeability and decreased hepatic clearance capacity[12].

Hepatocytes and Kupffer cells, the resident liver macrophages which play a major role in the clearance of systemic bacterial infection, express the membrane-associated form of the endotoxin receptor CD14 (mCD14) at low levels in comparison to peripheral blood monocytes (reviewed by Schwabe *et al*[12]). Moreover, sinusoidal endothelial cells and activated hepatic stellate cells, the main fibrogenic cell type in the injured liver, also express mCD14[12,13]. mCD14 is anchored by glycophosphatidyl inositol, being part of a cell surface receptor complex which additionally contains the dimerized Toll-like receptor 4 and MD-2 (reviewed by Pålsson-McDermott & O'Neill[14]). Furthermore, it also localizes with TLR3, the double-stranded RNA (dsRNA) receptor, in intracellular compartments enhancing dsRNA sensing and TLR3 signalling[15,16]. In addition to the membranous form, a soluble form of CD14 lacking the glycophosphatidyl inositol anchor is thought to modulate LPS responses *via* local stimulatory (promoting) and systemic anti-inflammatory (competing) mechanisms[17].

Recently, a single nucleotide polymorphism (SNP) within the *CD14* gene, rs2569190/C-159T, has been demonstrated to be associated with the risk of developing liver cirrhosis, but not steatosis or less advanced stages of fibrosis, in patients with alcohol-induced liver disease (ALD)[18-20]. These results were attributed to the finding that, *in vitro*, the T allele is more actively transcribed than the C allele[21], leading consequently to the assumption that the TT carriers' hepatic cells may be prone to enhanced inflammatory reactions after endotoxin exposure. The relationship between rs2569190 TT genotype and liver disease progression, however, was not observed in a study by von Hahn and colleagues, in which the variant allele T was alternatively shown to be solely associated with cryptogenic chronic liver disease[22].

The current study aimed to investigate, in 2 different cohorts, whether the variant position rs2569190/C-159T within the *CD14* gene is associated with hepatitis C liver disease manifestations, and allowed replicated analysis as is demanded for genetic association studies[23].

MATERIALS AND METHODS

Ethics

The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

Patients

A total of 137 mainly Caucasian chronic hepatitis C patients (mean age 44.0 ± 12.0 years, median 42 years) who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center Goettingen (UMG), Germany, between 1993 and 2006 were enrolled. The chronic nature of infection was proven by detection of HCV-specific antibodies and HCV RNA in the patients' sera using a highly sensitive nested reverse transcription polymerase chain reaction (RT-PCR) over a period of at least 6 mo as described[24]. As part of routine clinical evaluation, liver biopsy procedures were performed and liver disease was confirmed in the course of a defined histological evaluation as described below. Biochemical liver disease parameters, i.e. serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transferase (γ-GT) were recorded in parallel. Patients with concomitant non-hepatitis C viral infections and those with continued alcohol or other drug abuse were excluded.

Samples and data from another 349 chronic hepatitis C patients (mean age 45.8 ± 13.5 years, median 45 years) were kindly provided by the German Network of Competence for Hepatitis (Hep-Net)[25].

Determination of HCV genotype

HCV genotyping was performed for the 137 patients (UMG group) using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium).

Histological evaluation

Before the start of therapy, liver biopsies were taken from patients for histological evaluation. In brief, sections (5-10 μm) from formalin-fixed and paraffin-embedded liver biopsies were stained with hematoxylin-eosin, trichrome, and Prussian blue. According to Desmet and colleagues, necroinflammatory activity (grading, score 1 to 3), and structural alterations (staging, score 0 to 4) were scored separately[26]. Other lesions typical of hepatitis C such as degree of steatosis (score 0 to 3), the presence or absence of portal lymphoid aggregates, and the presence or absence of bile duct damage were studied additionally as previously described[4]. Hep-Net samples were independently scored by two experienced pathologists according to the German guidelines with regard to inflammation activity and fibrosis progression[25,26].

Isolation of genomic DNA

Genomic DNA (gDNA) was purified from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Mini Kit following the blood and body fluid spin protocol (Qiagen, Hilden, Germany). The concentration and the purity of the DNA isolated from PBMCs were determined spectrophotometrically by reading the absorbance levels at 260 and 280 nm. The integrity of gDNA was ascertained through electrophoresis using a 0.6% agarose gel. Alternatively, when PBMCs were not available, gDNA was purified from a 2 mL sample of serum by means of the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

SNP genotyping by 5'nuclease assay

gDNA (10 ng derived from PBMCs or an aliquot corresponding to 12.5 μL serum) was amplified in a total volume of 20 μL by real-time PCR using the TaqMan® Universal Master Mix (Applied Biosystems, Darmstadt, Germany) and 36 μmol/L of primers each (CD14: forward 5'-CTAGATGCCCTGCAGAATCCTT-3', reverse 5'-CCCTTCCTTTCCTGGAAATATTGCA-3'). Allelic discrimination was achieved by adding 8 μmol/L differentially fluorescence dye-labeled allele-specific minor

3886 ISSN 1007-9327 CN 14-1219/R World J Gastroenterol August 21, 2009 Volume 15 Number 31

Table 1 Epidemiological characteristics of chronic hepatitis C patients with regard to CD14 rs2569190 genotype (UMG group)

	n	CD14 rs2569190 genotype				MAF	*P*
		CC	CT	TT	*P*		
Total number (%)	137	30 (21.9)	70 (51.1)	37 (27)	0.865[1]	0.526	
Gender (Female/male)	60/77	11/19	36/34	13/24	0.214[2]	0.517/0.532	0.795[3]
Age (mean ± SD)		46.5 ± 13.1	45.3 ± 10.8	39.6 ± 12.5	0.008[a]		
HCV subtype							
1a	31	7 (22.6)	17 (54.8)	7 (22.6)			
1b	73	18 (24.7)	38 (52.0)	17 (23.3)		0.500	
1a + 1b	6	1 (16.7)	3 (50.0)	2 (33.3)			
					0.073[b]		0.087[3]
2a	1	0	0	1 (100.0)			
2b	4	2 (50.0)	1 (25.0)	1 (25.0)		0.630	
3a	22	2 (16.7)	11 (50.0)	9 (33.3)			

MAF: Minor allele frequency. [1]Exact test for the Hardy-Weinberg equilibrium; [2]C carriers *vs* TT (χ^2 test); [3]χ^2 test was applied; [a]C carriers *vs* TT (independent samples *t*-test); [b]C carriers *vs* TT, HCV type 1 *vs* non-type 1 infections (χ^2 test).

groove binder probes (CD14: VIC 5'-CCTGTTACGG TCCCCCTG-3', FAM 5'-CTGTTACGGCCCCCCT-3'). Reactions and analyses were carried out in the sequence detection system ABI prism 7000 (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions.

Statistical analysis

Quantitative parameters were described by mean and standard deviation or median and inter-quartile range, and the Kolmogorov-Smirnov test was applied to investigate whether these distributions were Gaussian. The UMG group and Hep-Net group were compared regarding age using the parametric independent samples *t*-test.

For describing the ordinal and nominal scaled parameters such as gender, HCV subtype, hepatitis activity, fibrosis, steatosis, portal lymphatic aggregates, and bile duct damage, absolute frequencies and percentages were determined. χ^2 tests were applied to investigate the association of these parameters with the genotype or minor allele frequency (MAF).

To avoid bias, data were also stratified by age (< 44 years, and $\geqslant 44$ years), and parameters which showed significant correlations to the genotype in the univariate analysis were also analyzed using multivariate logistic regression. The results of stratifying and logistic regression were noted in the text and/or the tables where necessary.

All tests were performed two-sided and the level of significance was set at 0.05. The test results were interpreted in an exploratory way because no alpha adjustment for multiple testing was carried out. Statistical analyses were performed with the assistance of Medistat GmbH, Kiel, Germany, using PASW 17 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Epidemiological characteristics

A total of 137 and 349 patients with chronic hepatitis C (UMG and Hep-Net groups, respectively) were genotyped for the bi-allelic SNP within the *CD14* gene, rs2569190/C-159T. The variant allele T in the first group was found to be about as frequent as the wild-type C allele leading to a CC:CT:TT genotype distribution of 30:70:37 and a T allele frequency of 0.526 (Table 1). Hep-Net patients followed a distribution of 109:170:70 leading to a lower MAF of 0.444 (Table 2). MAFs were close to that given for Caucasians in public databases. The genotype distribution in both groups followed the Hardy-Weinberg equilibrium (Tables 1 and 2, respectively).

No significant difference was found between UMG and Hep-Net patients' regarding gender and age (χ^2 test, $P = 0.781$, independent samples *t*-test, $P = 0.177$, respectively). Epidemiological analysis revealed no significant relationships between the patients' CD14 rs2569190 genotype and gender (Tables 1 and 2). However, when analyzing patients' age with regard to the studied SNP genotypes, the UMG patients homozygous for the variant allele T were found to be on average 6.1 years younger than C carrier patients (mean age, 45.7 ± 11.5 years) at the time of liver biopsy taken before the start of therapy (Table 1). This observation, however, was absent in Hep-Net patients (Table 2).

Similar to the European population, most UMG patients were infected with HCV subtype 1b, followed by 1a and 3a. No significant difference was found between the distribution of HCV type 1 and non-type 1 infections among the three SNP genotypes (Table 1).

Biochemical parameters

Before the start of therapy, AST, ALT, and γ-GT serum activities were recorded for UMG patients as indicators of liver injury in chronic hepatitis C. The median levels of AST and ALT showed an increase from the wild-type to the variant type (Table 3). The number of TT patients with markedly elevated serum ALT activities, i.e. greater than two-fold the upper normal limit, was found to be markedly higher than the number of TT patients with normal ALT activities yielding a slightly increased T allele frequency among patients with markedly elevated ALT (χ^2 test, $P = 0.044$) (Table 3). However, after stratification by age, this was no longer significant (data not shown).

Table 2 Epidemiological characteristics of chronic hepatitis C patients with regard to CD14 rs2569190 genotype (Hep-Net group)

	n	CD14 rs2569190 genotype				MAF	*P*
		CC	CT	TT	*P*		
Total number (%)	349	109 (31.2)	170 (48.7)	70 (20.1)	0.828[1]	0.444	
Gender (Female/male)	148/201	47/62	66/104	35/35	0.151[a]	0.459/0.433	0.484[2]
Age (mean ± SD)		47.2 ± 13.5	44.6 ± 13.6	46.5 ± 13.3	0.616[b]		

[1]Exact test for the Hardy-Weinberg equilibrium; [2]χ^2 test was applied; [a]C carriers *vs* TT (χ^2 test); [b]C carriers *vs* TT (independent samples *t*-test).

Table 3 Biochemical serum parameters and the number of patients with elevated parameters in chronic hepatitis C with regard to CD14 rs2569190 genotype (UMG group)

	CD14 rs2569190 genotype				MAF	[2]*P*
	CC	CT	TT	[2]*P*		
AST (median, IQR)	21.0, 15.8-50.5	28.5, 17.0-52.3	32.0, 16.5-76.5			
[1]Number of patients with elevated/normal AST	9/21	29/41	19/18	0.159[a]	0.588/0.481	0.082
ALT (median, IQR)	36.0, 25.8-85.5	46.5, 26.8-87.3	50.0, 34.5-153.5			
[1]Number of patients with elevated/normal ALT	11/19	38/32	23/14	0.171[a]	0.583/0.462	0.044
γ-GT (median, IQR)	35.5, 13.8-58.8	27.0, 14.0-56.3	32.0, 15.5-53.5			
[1]Number of patients with elevated/normal γ-GT	10/20	19/51	11/26	0.934[a]	0.513/0.531	0.781

AST: Aspartate aminotransferase. Upper normal limit is 19 U/mL for males, and 15 U/mL for females; ALT: Alanine aminotransferase. Upper normal limit is 23 U/mL for males, and 19 U/mL for females; γ-GT: γ-glutamyltransferase. Upper normal limit is 28 U/mL for males, and 18 U/mL for females; IQR: Inter-quartile range. [1]Markedly elevated serum activities of transaminases (> two-fold the upper normal limit) were considered; [2]χ^2 test was applied; [a]C carriers *vs* TT. After stratification by age, all *P*-values were non-significant.

Hepatitis C disease activity and progression

Liver biopsy specimens were taken before the start of an interferon-based therapy and evaluated histologically. Hep-Net patients had higher frequencies of advanced degrees of hepatitis activity and fibrosis progression (Tables 4 and 5) (χ^2 test, $P < 0.001$ for both parameters). Both UMG and Hep-Net patients showed no correlation between their CD14 C-159T genotype and hepatitis activity or fibrosis (Tables 4 and 5, respectively). Other lesions typical and more characteristic of hepatitis C, namely the degree of hepatic steatosis, the presence or absence of lymphoid aggregates and bile duct damage, were additionally studied in UMG patients. With regard to the degree of steatosis and bile duct damage, no significant association with CD14 C-159T genotype distribution could be found (Table 4). T allele homozygous patients, however, were found to have portal lymphoid aggregates more frequently than C carriers (21/37 *vs* 32/100, respectively, χ^2 test, $P = 0.008$) (Table 4).

To avoid spurious findings, a separate analysis was carried out to identify other factors which might underlie the formation of portal lymphoid aggregates. No relationship was found between the presence or absence of portal lymphoid aggregates and sex, age, HCV subtype, biochemical parameters, the stage of fibrosis, or the degree of steatosis (logistic regression analysis, data not shown). In accordance with previous studies, however, a significant relationship between the presence of portal lymphoid aggregates and hepatic inflammatory activity (χ^2 test, $P = 0.003$) and bile duct damage (χ^2 test, $P < 0.001$) was found[3,5,27]. Nevertheless, even in the subgroups which had portal lymphoid aggregates with other lesions, a shift towards the T allele was always observed: 15 TT and 13 CT among the 32 patients who had both a high grade of hepatitis activity and portal lymphoid aggregates: (MAF = 0.672 compared to 0.481 for the remaining patients); 13 TT and 17 CT among the 35 patients who presented with both bile duct damage and portal lymphoid aggregates: (MAF = 0.614 compared to 0.495 for the others) (data not shown).

DISCUSSION

Among chronic hepatitis C patients, no evidence was found for a relationship between the endotoxin receptor CD14 rs2569190/C-159T genotype and the progression of liver fibrosis. This finding was obtained by analyzing two different patient cohorts, one derived from the UMG ($n = 137$) (Table 4), the other from the Hep-Net, a Germany-wide collection of samples ($n = 349$) (Table 5). It is in line with previous findings on Caucasian patients[20,22].

By considering the sum of the evidence, the situation of chronic liver disease resulting from HCV infection appears to be different from the situation of chronic liver disease resulting from alcohol consumption: whereas in ALD, progression of fibrosis was shown to be associated with TT genotypes[18-20], in chronic hepatitis C it did not appear to be related to this genetic variation.

Of note, a recent report found the influence of an environmental factor on the association of rs2569190/C-159T and total serum IgE levels in Russian children[28]. Depending on the *Helicobacter pylori* (*H pylori*) infection status, seronegative or seropositive, the T allele was associated with a decreased or an increased IgE serum concentration, respectively[28]. The observation of the lack of an association of rs2569190/C-159T genotype with chronic hepatitis C disease progression, but an

3888 ISSN 1007-9327 CN 14-1219/R World J Gastroenterol August 21, 2009 Volume 15 Number 31

Table 4 Histological features in chronic hepatitis C-infected patients with regard to CD14 rs2569190 genotype (UMG group) *n* (%)

Histological features	CD14 rs2569190 genotype			*P*	MAF	*P*
	CC	CT	TT			
Hepatitis activity						
Mild	19 (25.0)	40 (52.6)	17 (22.4)		0.487	
Moderate	10 (19.2)	24 (46.2)	18 (34.6)	0.172[b]	0.574	0.152
Severe	1 (11.1)	6 (66.7)	2 (22.2)			
Fibrosis						
Absent	7 (38.9)	6 (33.3)	5 (27.8)		0.517	
Mild	14 (20.6)	35 (51.5)	19 (27.9)			
Moderate	5 (19.2)	15 (57.7)	6 (23.1)	0.758[b]		0.727
Marked	3 (21.4)	7 (50.0)	4 (28.6)		0.539	
Cirrhosis	1 (9.1)	7 (63.6)	3 (27.3)			
Steatosis						
Absent	14 (23.3)	28 (46.7)	18 (30.0)		0.528	
Mild	10 (20.8)	26 (54.2)	12 (25.0)	0.695[b]		0.887
Moderate	5 (26.3)	10 (52.6)	4 (21.1)		0.517	
Marked	1 (10.0)	6 (60.0)	3 (30.0)			
Portal lymphoid aggregates						
Absent	22 (26.2)	46 (54.8)	16 (19.1)	0.008[b]	0.464	0.011
Present	8 (15.1)	24 (45.3)	21 (39.6)		0.623	
Bile duct damage						
Absent	21 (23.6)	46 (51.7)	22 (24.7)	0.411[b]	0.506	0.368
Present	9 (18.8)	24 (50.0)	15 (31.3)		0.563	

[a]χ^2 test was applied to compare mild *vs* moderate and severe hepatitis activity, absent, mild *vs* moderate and marked fibrosis and cirrhosis, and absent, mild *vs* moderate and marked steatosis; [b]C carriers *vs* TT.

Table 5 Hepatitis activity (grading) and fibrosis (staging) in chronic hepatitis C-infected patients with regard to CD14 rs2569190 genotype (Hep-Net group) *n* (%)

	CD14 rs2569190 genotype			*P*	MAF	*P*
	CC	CT	TT			
Hepatitis activity						
Mild	19 (27.1)	35 (50.0)	16 (22.9)		0.479	
Moderate	73 (32.2)	113 (49.8)	41 (18.1)	0.513[b]	0.435	0.359
Severe	17 (32.7)	22 (42.3)	13 (25.0)			
Fibrosis						
Absent	4 (57.1)	2 (28.6)	1 (14.3)		0.449	
Mild	35 (28.9)	61 (50.4)	25 (20.7)			
Moderate	44 (32.8)	63 (47.0)	27 (20.2)	0.928[b]		0.837
Marked	18 (27.7)	37 (56.9)	10 (15.4)		0.441	
Cirrhosis	8 (36.4)	7 (31.8)	7 (31.8)			

[a]χ^2 test was applied to compare mild *vs* moderate and severe hepatitis activity, absent, mild *vs* moderate and marked fibrosis and cirrhosis; [b]C carriers *vs* TT.

association with ALD and the dependency of the association on *H pylori* infection status for an atopy-related parameter, suggests that LPS sensitivity depends on both genetic and environmental factors (gene-environment interaction).

The lack of an association between this CD14 SNP and liver disease progression in chronic hepatitis C patients, is also in accordance with a finding by Huang *et al*[29] who, by functional genomic scanning, identified seven SNPs within seven genes which carry the most relevant risk for developing cirrhosis in Caucasian hepatitis C patients, with CD14 not being among them.

In contrast, a significantly higher frequency of rs2569190 T homozygote hepatitis C patients was found among patients with portal lymphoid aggregates (Table 4). As with lymphocyte infiltrations, primary follicles and secondary follicles in the lymph nodes, portal lymphoid aggregates have been described to occur in various patterns ranging from vague lymphoid aggregation to round defined follicles to well-formed follicles with clearly identifiable germinal centers[30]. Their presence has been attributed to the participation of the host's immune system in liver disease pathogenesis[3,5]. To our knowledge, even today, the etiology and relevance of this manifestation for disease development and/or progression remains unclear. The presence of portal lymphoid aggregates was the only histological manifestation found to be related to CD14 rs2569190/C-159T genotypes (Table 4). As reported in other studies, the presence of portal lymphoid aggregates was found to be closely correlated with bile duct damage and the degree of inflammatory activity[3,5,27], but not with sex, age[3,4], or HCV subtype[4]. The finding of a correlation between portal lymphoid aggregates and HCV subtype 1b infection in Chinese[3] but not European patients is obviously related to a different

HCV subtype distribution. The comparison between subtype 1b- and type 2-infected patients, as is possible among Chinese patients, cannot be carried out among European patients because of the low proportion of type 2 infections. Portal lymphoid aggregates have also usually been found in the early stages of liver disease and to have disappeared in cirrhosis[27,31]. Thus, our finding of a positive association of TT status and the presence of portal lymphoid aggregates, on the one hand, and the absence of an association with disease progression, on the other hand, are concordant with that observation.

The relationship between TT genotype and younger age in the UMG group (Table 1) was neither observed among the patients from the Hep-Net group (Table 2) nor among patients from another German study[20]. In contrast to what might have been expected, these three cohorts do differ in demographic and clinical features. For instance, as outlined, the Hep-Net group comprised a higher proportion of patients with greater degrees of hepatic inflammatory activity and also more advanced stages of fibrosis than the UMG group (Tables 4 and 5). The cohort studied by Meiler *et al*[20] was found to differ significantly in age from the Hep-Net group and both in age and gender distribution from the UMG group (data not shown). Von Hahn's cohort was similar to the UMG and Hep-Net groups with regard to gender distribution, however, further demographic analysis, i.e. association of genotypes and age, was not given[22].

In conclusion, in contrast to what was reported for ALD, our analyses did not reveal an increased risk for chronic hepatitis C patients, homozygous for the CD14 C-159T T allele, to develop more pronounced fibrosis but suggested a relationship of this variation in the formation of portal lymphoid aggregates.

ACKNOWLEDGMENTS

The authors thank all physicians of the Department of Gastroenterology and Endocrinology, UMG, who were involved in the patients' care for their kind cooperation, PD Dr. Stefan Schweyer for his help in histological evaluation of patients' liver biopsies, and Waltraut Kopp for expert technical assistance. Sincere thanks are given to PD Dr. Margarete Odenthal, Institute of Pathology, University of Cologne, for valuable discussions on the manuscript and also to the German Network of Competence for Hepatitis (Hep-Net) for kindly facilitating the use of hepatitis C patients' material and data. Thanks are also given to Ulrike Schulz, Medistat, for professional statistical analyses.

COMMENTS

Background

Progressive hepatic fibrosis develops in patients with chronic liver diseases irrespective of etiology and with a marked inter-individual variability. Different host genetic backgrounds have been shown to act as co-factors in promoting ongoing liver disease progression exacerbated by gut-derived bacterial lipopolysaccharides (endotoxins).

Research frontiers

The variant allele T of a well-known single nucleotide polymorphism (SNP) in the endotoxin receptor *CD14* gene has been reported to be associated with increased alcohol-related liver cirrhosis. In this study, the authors show that the T allele is correlated with the presence of portal lymphoid aggregates rather than being associated with fibrosis progression in chronic hepatitis C virus (HCV) infection.

Innovations and breakthroughs

Recent reports have shown a lack of an association between the T allele and liver fibrosis progression in the context of chronic HCV infection. Genetic association studies, however, require to be replicated. Apart from performing an analysis in two different cohorts, this study is the first to expand the analysis to further histological lesions typical of HCV infection with regard to CD14 rs2569190/C-159T.

Applications

Understanding the mechanisms underlying chronic hepatocellular injury in hepatitis C is important for therapy applications. This study argues for a possible relationship of CD14 rs2569190 T allele in the formation of portal lymphoid aggregates, the presence of which has been attributed to the host's immunological participation in liver disease pathogenesis. Moreover, it excludes a possible role of the variation in promoting fibrosis progression.

Terminology

Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen. Cirrhosis is the last stage of fibrosis. A SNP is a variation of one base in the DNA; the nucleotide observed is different from the norm at this position. It occurs with a frequency of > 1% in the normal population. Portal lymphoid aggregates are defined as a densely packed collection of small lymphocytes within the portal tract without or with the formation of a germinal center. Lipopolysaccharide is the main component of the outer cell wall of gram-negative bacteria.

Peer review

This is a valuable population-based association study, which is useful for examining a well-known genetic variation with a role in a common multifactorial disease that may have a strong environmental component. In contrast to the situation in alcoholic liver cirrhosis, the SNP rs2569190/C-159T is not related to chronic HCV-induced fibrosis progression, but to another histological feature, namely, the presence of portal lymphoid aggregates.

REFERENCES

1 **Shepard CW**, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 2005; **5**: 558-567
2 **Seeff LB**. Natural history of chronic hepatitis C. *Hepatology* 2002; **36**: S35-S46
3 **Luo JC**, Hwang SJ, Lai CR, Lu CL, Li CP, Tsay SH, Wu JC, Chang FY, Lee SD. Clinical significance of portal lymphoid aggregates/follicles in Chinese patients with chronic hepatitis C. *Am J Gastroenterol* 1999; **94**: 1006-1011
4 **Mihm S**, Fayyazi A, Hartmann H, Ramadori G. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. *Hepatology* 1997; **25**: 735-739
5 **Wong VS**, Wight DG, Palmer CR, Alexander GJ. Fibrosis and other histological features in chronic hepatitis C virus infection: a statistical model. *J Clin Pathol* 1996; **49**: 465-469
6 **Negro F**. Mechanisms and significance of liver steatosis in hepatitis C virus infection. *World J Gastroenterol* 2006; **12**: 6756-6765
7 **Poynard T**, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997; **349**: 825-832
8 **Bataller R**, North KE, Brenner DA. Genetic polymorphisms and the progression of liver fibrosis: a critical appraisal. *Hepatology* 2003; **37**: 493-503
9 **Askar E**, Bregadze R, Mertens J, Schweyer S, Rosenberger A, Ramadori G, Mihm S. TLR3 gene polymorphisms and liver disease manifestations in chronic hepatitis C. *J Med Virol* 2009; **81**: 1204-1211
10 **Wietzke-Braun P**, Maouzi AB, Mänhardt LB, Bickeböller H, Ramadori G, Mihm S. Interferon regulatory factor-1 promoter polymorphism and the outcome of hepatitis C

virus infection. *Eur J Gastroenterol Hepatol* 2006; **18**: 991-997
11 **Su GL**. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G256-G265
12 **Schwabe RF**, Seki E, Brenner DA. Toll-like receptor signaling in the liver. *Gastroenterology* 2006; **130**: 1886-1900
13 **Paik YH**, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 2003; **37**: 1043-1055
14 **Pålsson-McDermott EM**, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 2004; **113**: 153-162
15 **Lee HK**, Dunzendorfer S, Soldau K, Tobias PS. Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. *Immunity* 2006; **24**: 153-163
16 **Vercammen E**, Staal J, Beyaert R. Sensing of viral infection and activation of innate immunity by toll-like receptor 3. *Clin Microbiol Rev* 2008; **21**: 13-25
17 **Kitchens RL**, Thompson PA. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. *J Endotoxin Res* 2005; **11**: 225-229
18 **Campos J**, Gonzalez-Quintela A, Quinteiro C, Gude F, Perez LF, Torre JA, Vidal C. The -159C/T polymorphism in the promoter region of the CD14 gene is associated with advanced liver disease and higher serum levels of acute-phase proteins in heavy drinkers. *Alcohol Clin Exp Res* 2005; **29**: 1206-1213
19 **Järveläinen HA**, Orpana A, Perola M, Savolainen VT, Karhunen PJ, Lindros KO. Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease. *Hepatology* 2001; **33**: 1148-1153
20 **Meiler C**, Muhlbauer M, Johann M, Hartmann A, Schnabl B, Wodarz N, Schmitz G, Scholmerich J, Hellerbrand C. Different effects of a CD14 gene polymorphism on disease outcome in patients with alcoholic liver disease and chronic hepatitis C infection. *World J Gastroenterol* 2005; **11**: 6031-6037
21 **LeVan TD**, Bloom JW, Bailey TJ, Karp CL, Halonen M, Martinez FD, Vercelli D. A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. *J Immunol* 2001; **167**: 5838-5844
22 **Von Hahn T**, Halangk J, Witt H, Neumann K, Müller T, Puhl G, Neuhaus P, Nickel R, Beuers U, Wiedenmann B, Berg T. Relevance of endotoxin receptor CD14 and TLR4 gene variants in chronic liver disease. *Scand J Gastroenterol* 2008; **43**: 584-592
23 **Lohmueller KE**, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003; **33**: 177-182
24 **Mihm S**, Hartmann H, Fayyazi A, Ramadori G. Preferential virological response to interferon-alpha 2a in patients with chronic hepatitis C infected by virus genotype 3a and exhibiting a low gamma-GT/ALT ratio. *Dig Dis Sci* 1996; **41**: 1256-1264
25 **Manns MP**, Meyer S, Wedemeyer H. The German network of excellence for viral hepatitis (Hep-Net). *Hepatology* 2003; **38**: 543-544
26 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520
27 **Freni MA**, Artuso D, Gerken G, Spanti C, Marafioti T, Alessi N, Spadaro A, Ajello A, Ferraù O. Focal lymphocytic aggregates in chronic hepatitis C: occurrence, immunohistochemical characterization, and relation to markers of autoimmunity. *Hepatology* 1995; **22**: 389-394
28 **Virta M**, Pessi T, Helminen M, Seiskari T, Kondrashova A, Knip M, Hyöty H, Hurme M. Interaction between CD14-159C>T polymorphism and Helicobacter pylori is associated with serum total immunoglobulin E. *Clin Exp Allergy* 2008; **38**: 1929-1934
29 **Huang H**, Shiffman ML, Friedman S, Venkatesh R, Bzowej N, Abar OT, Rowland CM, Catanese JJ, Leong DU, Sninsky JJ, Layden TJ, Wright TL, White T, Cheung RC. A 7 gene signature identifies the risk of developing cirrhosis in patients with chronic hepatitis C. *Hepatology* 2007; **46**: 297-306
30 **Murakami J**, Shimizu Y, Kashii Y, Kato T, Minemura M, Okada K, Nambu S, Takahara T, Higuchi K, Maeda Y, Kumada T, Watanabe A. Functional B-cell response in intrahepatic lymphoid follicles in chronic hepatitis C. *Hepatology* 1999; **30**: 143-150
31 **Monteverde A**, Ballarè M, Pileri S. Hepatic lymphoid aggregates in chronic hepatitis C and mixed cryoglobulinemia. *Springer Semin Immunopathol* 1997; **19**: 99-110

S- Editor Li LF **L- Editor** Cant MR **E- Editor** Zheng XM

5.3. The third publication: Toll-like receptor 7 rs179008/Gln11Leu gene variants in chronic hepatitis C virus infection

Askar E, Ramadori G, Mihm S. (2010): Toll-like receptor 7 rs179008/Gln11Leu gene variants in chronic hepatitis C virus infection. J Med Virol 82, 1859-68.

Journal of Medical Virology 82:1859–1868 (2010)

Toll-Like Receptor 7 rs179008/Gln11Leu Gene Variants in Chronic Hepatitis C Virus Infection

Eva Askar, Giuliano Ramadori, and Sabine Mihm*

Department of Gastroenterology and Endocrinology, University Medical Center, Georg-August-University, Goettingen, Germany

Hepatitis C virus (HCV) infection affects an estimated 3% of the world's population. The natural outcome of infection and the natural course of disease are highly variable. Sensing of viral single-stranded RNA (ssRNA) by Toll-like receptor 7 (TLR7) is likely involved in early pathogen detection and host response to viral infections. This study analyzed epidemiological and clinical data from 136 patients with HCV infection with regard to rs179008/Gln11Leu, a non-synonymous polymorphism within exon 3 of the X-linked TLR7 gene, the variant allele of which is suggested to code for a functionally impaired protein. Allele-specific transcript quantification (ASTQ) analyses in heterozygous females revealed individual skewed mosaicism in peripheral blood mononuclear cells (PBMCs). Thus, analyses were restricted to homo- and hemizygous individuals. Among the clinical and histological parameters studied, the variant allele T was found to be solely associated with the presence of portal lymphoid aggregates. Whereas hepatic viral load and expression of genes known to be induced in chronic HCV infection were not found to differ in patients with wild-type or variant TLR7 rs179008 genotype, significant lower gene expression of interleukin-29 (IL-29)/lambda$_1$ interferon (IFN-λ_1) and both of its receptor subunits was found for T homo- and hemizygous patients. Irrespective of the minor differences in disease phenotype including hepatic viral load, natural, and alpha interferon (IFN-α)-mediated outcome of infection, and disease activity and progression, the significant differences in hepatic IL-29/IFN-λ_1 and IFN-λ receptor gene expression between TLR7 rs179008 T and A allele patients might have implications for responsiveness to future IFN-λ-based approaches. ***J. Med. Virol. 82:1859–1868, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: toll-like receptor7 (TLR7); single-stranded RNA (ssRNA); hepatitis C virus (HCV); single nucleotide polymorphism (SNP); portal lymphoid aggregates

© 2010 WILEY-LISS, INC.

INTRODUCTION

Chronic infection caused by hepatitis C virus (HCV), an enveloped single-stranded RNA (ssRNA) virus [Choo et al., 1989], develops in 70–80% of patients [Schwabe et al., 2006]. Patients are at a high risk of developing severe disease as liver cirrhosis and hepatocellular carcinoma [Schwabe et al., 2006]. Toll-like receptors (TLRs) play a critical role in the innate immune sensing of the invasion of pathogenic microorganisms [Akira and Takeda, 2004]. Alpha interferon (IFN-α) is an important antiviral cytokine produced principally by plasmacytoid dentritic cells (pDCs), which circulate in the blood at low frequency and even lower in chronic hepatitis C [Kanto et al., 2004], through the stimulation of TLR7 and TLR9 [Hornung et al., 2005; Ito et al., 2005]. TLR7 senses unmethylated viral ssRNA [Diebold et al., 2004; Heil et al., 2004]. The expression of TLR7 in humans is mainly confined to the endosome–lysosome membrane of pDCs (including hepatic pDCs), hepatic natural killer cells [Seki and Brenner, 2008], and B lymphocytes [Hornung et al., 2002]. When the virus or virus-infected apoptotic cells are taken up by phagocytes, viral RNA is released in the highly acidified phagolysosome by degradation enzymes, leading to ssRNA release and recognition by TLR7. Upon TLR7 stimulation, a complex cascade is formed, starting with myeloid differentiation factor 88 (MyD88) and ending with the production of

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: MI 474/1-1.

*Correspondence to: Sabine Mihm, Department of Gastroenterology and Endocrinology, University Medical Center Goettingen, Robert-Koch-Straße 40, Goettingen 37075, Germany.
E-mail: smihm@med.uni-goettingen.de

Accepted 7 June 2010

DOI 10.1002/jmv.21893

Published online in Wiley Online Library (wileyonlinelibrary.com)

IFN-α/IFN-inducible genes and proinflammatory cytokines through the phosphorylation of interferon regulatory factor 7 and the liberation of nuclear factor-κB, respectively [reviewed in Akira et al., 2006; Schwabe et al., 2006; Seki and Brenner, 2008].

Macrophages overexpressing HCV non-structural proteins NS3, NS3/4A, NS4B, or NS5A show a strong suppression of TLR-MyD88-dependent signaling pathway. NS5A interacts with MyD88 to prevent cytokine production, such as interleukin-1 (IL-1), IL-6, and beta interferon (IFN-β) in response to TLR7 ligands [Abe et al., 2007]. In addition, pDCs from HCV patients have reduced deviation marker (HLA-DR) and IFN-α expression in response to TLR7 ligand, which is associated with an impaired activation of naive CD4 T cells [Yonkers et al., 2007]. TLR7 has been recently of particular medicinal chemistry interest because its small molecule ligands may serve as immune stimulants by enhancing endogenous IFN-α production and thus, they may complement IFN-α therapy of chronic HCV infection, especially in IFN-α-resistant patients. Horsmans and co-workers have applied a well-tolerated intravenous isatoribine treatment with only few mild to moderate adverse events for 1 week to chronic hepatitis C patients. It has resulted in viral load reduction regardless of the patients' HCV genotype, an induction of the antiviral immunity marker 2′,5′-oligoadenylate synthetase, and an increase in the levels of the gamma interferon (IFN-γ)-inducible protein 10 (IP-10) and neopterin, a marker of macrophage activation [Horsmans et al., 2005]. Moreover, a high-affinity ligand of TLR7, namely SM360320, has been found to inhibit HCV replication both through type I IFN production by leukocytes, and direct activation of antiviral mechanisms in infected hepatocytes [Lee et al., 2006].

TLR7 gene is located on the X-chromosome and contains three exons [Du et al., 2000]. Recently, the leucine (Leu) variant encoded by the T allele of the non-synonymous single nucleotide polymorphism (SNP) rs179008, which is located within TLR7 exon 3 and leads to the replacement of the wild allele A-encoded glutamine (Gln) at codon 11 in the protein (Gln11Leu), has been correlated with higher susceptibility to HCV infection and less chances of response to an IFN-α-based therapy in chronic HCV-infected females [Schott et al., 2008]. Moreover, this variant has been associated with higher viral loads, accelerated progression to advanced immune suppression in human immune deficiency virus (HIV) infection, increased susceptibility to HIV-1 in women, and decreased IFN-α production after stimulation of healthy peripheral blood mononuclear cells (PBMCs) with the TLR7 ligand imiquimod [Oh et al., 2009].

Taking the X-linked location into account, the present study aimed to investigate the correlation between TLR7 rs179008 genotype and disease parameters in chronic hepatitis C, including the natural outcome of infection, that is, chronic versus self-limited course, histological features, and the initial virological response to an IFN-α-based treatment on the one hand, and hepatic expression of innate immunity genes on the other hand.

PATIENTS AND METHODS

Patients

From a total of 144 mainly Caucasian chronic hepatitis C patients who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center Goettingen (UMG), Germany, between 1993 and 2006, 136 with complete data sets (mean age 45.0 ± 12.3, median 44 years, 60 females) were enrolled in epidemiological, biochemical, and histological analyses. Chronic infection was proven by detection of HCV-specific antibodies and HCV RNA in the patients' sera using a highly sensitive nested RT-PCR over a period of at least 6 months as described [Mihm et al., 1996a]. Before the start of therapy, liver biopsy procedures were performed and liver disease was confirmed in the course of a defined histological evaluation as described below. Biochemical liver disease parameters, that is, serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γ-GT) were recorded in parallel. Patients with concomitant non-C viral infections and those with continued alcohol or other drug abuse were excluded.

A total of 55 patients (mean age 46.1 ± 12.3, median 45 years, 25 females) were treated with IFN-α_{2a} (Roferon A; Hoffman-La Roche, Basel, Switzerland) at an initial dose of 6×10^6 IU 3× per week for at least 4 months (mean, 7.7 months; range 4–12 months). Depending on well-being and response parameters, both dose and duration were adapted individually. Initial virological response to therapy, which is defined as the elimination of HCV RNA below the limit of detectability during the first 4 months for a period of at least three consecutive months, was analyzed with regard to TLR7 rs179008 genotype.

Another group of 44 patients with self-limited HCV infection (mean age 37.0 ± 10.3, median 36 years, 14 females) was studied in addition. Spontaneous elimination was assured by the presence of anti-HCV antibodies in the absence of detectable amounts of HCV RNA (for detailed epidemiological and serological description of this cohort please refer to Wietzke-Braun et al. [2007]).

The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

Determination of HCV Genotype

HCV genotyping was performed using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium).

Histological Evaluation

Before the start of therapy, liver biopsies were taken from patients for histological evaluation. In

brief, sections (5–10 μm) from formalin-fixed and paraffin-embedded liver biopsies were stained with hematoxylin–eosin, trichrome, and Prussian blue. According to Desmet et al. [1994], necroinflammatory activity (grading, score 1–3) and architectural alterations (staging, score 0–4) were scored separately. Other lesions typical of hepatitis C such as the degree of steatosis (score 0–3), the presence or absence of portal lymphoid aggregates, and the presence or absence of bile duct damage were studied additionally as previously described [Mihm et al., 1997].

Preparation of PBMCs

PBMCs from ~30 ml of heparinized peripheral blood samples were prepared by Ficoll density centrifugation using guanidinium isothiocyanate as described [Boyum, 1984].

Isolation of Genomic DNA (gDNA) and Total Cellular RNA

gDNA was purified from PBMCs or from 2 ml samples of serum using the QIAamp DNA Mini or Midi Kits, respectively, following the blood and body fluid spin protocol (Qiagen, Hilden, Germany). The concentration and the purity of the DNA isolated from PBMCs were determined photometrically by reading the absorbance levels at 260 and 280 nm. The integrity of gDNA was ascertained through electrophoresis using a 0.6% agarose gel.

Total cellular RNA was prepared from available freshly isolated PBMCs and homogenized liver tissue samples by CsCl density gradient ultracentrifugation essentially as described [Mihm et al., 1996b].

Reverse Transcription

To get complementary DNA (cDNA), an amount of 1 μg of total cellular RNA was reverse transcribed by using random hexamers (6 μM) for priming as described previously [Mihm et al., 1996b].

Genotyping for the Variant Position rs179008/Gln11Leu

Allelic discrimination of the TLR7 exon 3-located SNP was performed by the commercially available TaqMan genotyping assay C_2259574_10 (Applied Biosystems, Foster City, CA). Reactions of 10 μl containing 4 ng of PBMCs-derived gDNA—or an aliquot corresponding to 6.7 μl serum—were performed in the sequence detection system StepOne-Plus (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions.

Allele-Specific Transcript Quantitation (ASTQ) of TLR7 rs179008 Variants

Discrimination and quantitation of TLR7 rs179008 transcript variants (A and T) was achieved by applying the commercially available TaqMan genotyping assay C_2259574_10 (Applied Biosystems, CA) on cDNA samples (3.2 ng). Heterozygote gDNA and homozygote gDNA and cDNA samples served as controls.

Quantification of Hepatic Gene Expression

Competitive RT-PCR was applied to quantify mRNA transcripts of HCV, the IFN-α/β inducible antiviral myxovirus resistance protein-1 gene (MxA), IFN-α and, as a reference gene, albumin, essentially as described [Mihm et al., 2004], and transcripts of IP-10, the gene encoding IFN-α/β-inducible p44 [Patzwahl et al., 2001], IFN-γ [Mihm et al., 1996b]. The relative number of interleukin-29 (IL-29)/lamda$_1$ interferon (IFN-λ_1), IFN-λ receptor subunits (IL-10Rβ and IL28Rα), and IFN-α/β receptor 2 (IFNAR2) mRNA transcripts was calculated by real-time RT-PCR using the sequence detection system ABI prism 7000 following the supplier's instructions (Applied Biosystems, Darmstadt) as described [Mihm et al., 2004; Doyle et al., 2006]. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) transcripts served as a housekeeping gene, using a commercially available TaqMan gene expression Assay on Demand (Hs 99999905 ml) (Applied Biosystems). Comparable results were found when relating the targets to β-actin transcripts (data not shown).

Statistical Analysis

Females and males were analyzed both separately (data shown in the text where necessary), and combined due to the ASTQ results. Quantitative parameters were described by mean and standard deviation if the data are normally distributed, or median and inter-quartile range (IQR) if the distribution is not normal. χ^2-test and independent samples t-test were applied where applicable. The level of significance was set to a screening level of 0.05. All tests were performed by using PC STATISTIK software package version 4.0 (Hoffmann-Software, Giessen, Germany).

RESULTS

Genotyping of HCV With Regard to TLR7 rs179008

A total of 136 patients with chronic hepatitis C (60 females/76 males) were genotyped for the bi-allelic SNP rs179008/Gln11Leu within exon 3 of the X-linked TLR7 gene (Table I). Genotype distribution in women followed Hardy–Weinberg equilibrium ($P = 0.318$). The frequency of the minor allele (MAF) was found to be close to that given for Caucasians in public databases and to be similar in females and males ($P = 0.175$).

ASTQ

Due to the X-linked location of TLR7, in females, the random inactivation of one X chromosome leads to cellular mosaicism with two populations of cells differing in the parental origin of the active X [Fish, 2008]. In heterozygous females, the presence of two kinds of cells might set up a competition between them [Migeon,

TABLE I. TLR7 rs179008 Genotype Distribution in Patients With Chronic or Self-Limited HCV Infection With Regard to Epidemiological and Biochemical Parameters

		TLR7 rs179008 genotype						
		Females			Males			
	n	AA	AT	TT	A	T	*P*	MAF
Patients with chronic HCV infection								
Number (%)	136	34 (56.7)	20 (33.3)	6 (10.0)	61 (80.3)	15 (19.7)	0.175[b]	0.267/0.197[g]
Age (mean ± SD)		45.8 ± 10.7	46.6 ± 11.5	50.0 ± 13.6	40.7 ± 11.2	46.9 ± 15.9	0.069[c]	
HCV types								
HCV type-1 n (%)	109	28 (53.9)	19 (36.5)	5 (9.6)	47 (82.5)	10 (17.5)	0.449[d]	0.279/0.175[g]
HCV non-1 n (%)	27	6 (75.0)	2 (25.0)	0 (0)	14 (73.7)	5 (26.3)		0.143/0.263[g]
Biochemical serum parameters								
AST (median, IQR)		23.0, 15–38	18.5, 13.5–31.5	45, 16–80	29, 18–67	36, 18–52		
No. patients with elevated/normal AST[a]	136	12/22	6/14	3/3	28/33	7/8	0.411[d]	
ALT (median, IQR)		32.5, 22–60	31.5, 20.5–44	58, 22.90	58, 36.5–129	78, 47–92		
No. patients with elevated/normal ALT[a]	136	13/21	7/13	3/3	36/25	12/3	0.078[d]	
γ-GT (median, IQR)		19.5, 10–45	19.5, 13.5–41	17, 5–34	39, 23.5–62.5	24, 14–72		
No. patients with elevated/normal γ-GT[a]	136	10/24	5/15	2/4	17/44	5/10	0.419[d]	
Patients with self-limited HCV infection								
Number (%)	44	7 (50.0)	6 (42.9)	1 (7.1)	26 (86.7)	4 (13.3)	0.766[e]	0.286/0.133[g]
Age (mean ± SD)		39.3 ± 10.4	38.0 ± 13.2	30	36.0 ± 9.9	40.5 ± 9.7	0.722[f]	

[a]Markedly elevated serum activities of transaminases (>2-fold of the upper normal limit) were considered. Upper normal limits for females/males, respectively, are: 15 U/L/19 U/L for AST; 19 U/L/23 U/L for ALT; and 18 U/L/28 U/L for γ-GT.
[b]χ^2-test was applied to compare MAF between females and males.
[c]Independent samples *t*-test was applied, the trend was valid only in males ($P = 0.083$).
[d]χ^2-test was applied to compare A with T homo- and hemizygous patients (combined females males).
[e]χ^2-test was applied to compare TLR7 rs179008 genotype distribution in patients with chronic- and patients with self-limited HCV infection.
[f]Independent samples *t*-test was applied.
[g]MAF is given for females/males, respectively.

2006]. Non-random inactivation (skewing) has been implicated for discrete cell populations, for example, dendritic cells [Fish, 2008; Migeon, 2006]. In order to find out whether heterozygous females should be assigned either to the wild-type, the variant genotype or to be considered as a separate, that is, true heterozygous group, ASTQ was performed to quantify the relative proportion of A and T allele transcript variants in RNA preparation from freshly isolated PBMCs (Fig. 1). gDNA, a natural source of equal amounts of A and T sequences served as a control. RNA preparations from three heterozygous women were found to contain nearly equal numbers of both alleles' transcripts, whereas material from four heterozygous females was found to contain an excess of either A or T, two women each, respectively (Fig. 1A).

Because of the limited amount of available samples, 5 heterozygous females were further identified among a total of 42 healthy blood donors. ASTQ with these five samples yielded comparable results (equal expression in two samples, an excess of A in three samples) (data not shown).

On the basis of these findings we decided to restrict analyses to the comparison between A and T homo- and hemizygous individuals.

Epidemiological and Biochemical Characteristics

Demographic analysis revealed a slight trend of the variant allele carriers to be older than those who carry the wild-type allele (47.8 ± 15.0 vs. 42.5 ± 11.2, respectively, $P = 0.069$). This difference, however, was clearer in males ($P = 0.083$) (Table I).

As expected for a European population, most patients (80.1%) were infected with HCV type-1 (including subtypes 1b, 1a, and 1a + 1b), while 19.9% were infected with HCV non-1 (including mainly subtypes 3a, and a minority of 2a and 2b). No significant difference among the distribution of HCV types (or subtypes, data not shown) infections according to patients' TLR7 rs179008 genotype was found (Table I).

AST, ALT, and γ-GT serum activities were recorded as indicators of liver injury in chronic hepatitis C. Although T homo- and hemizygotes seemed to have higher AST and ALT but lower γ-GT serum levels than the A counterparts, the proportion of A or T homo- and hemizygote patients among those with markedly elevated transaminase activities, that is, greater than twofold of the upper normal limit, was not found to be significantly different (Table I).

Genotyping of Individuals With Self-Limited HCV Infection

The T allele is suggested to confer enhanced susceptibility to chronic HCV infection as MAF has been found to be significantly lower in healthy individuals [Schott et al., 2008]. To address the question whether this enhanced susceptibility is due to a higher incidence of infection or to an impaired capacity to self-eliminate

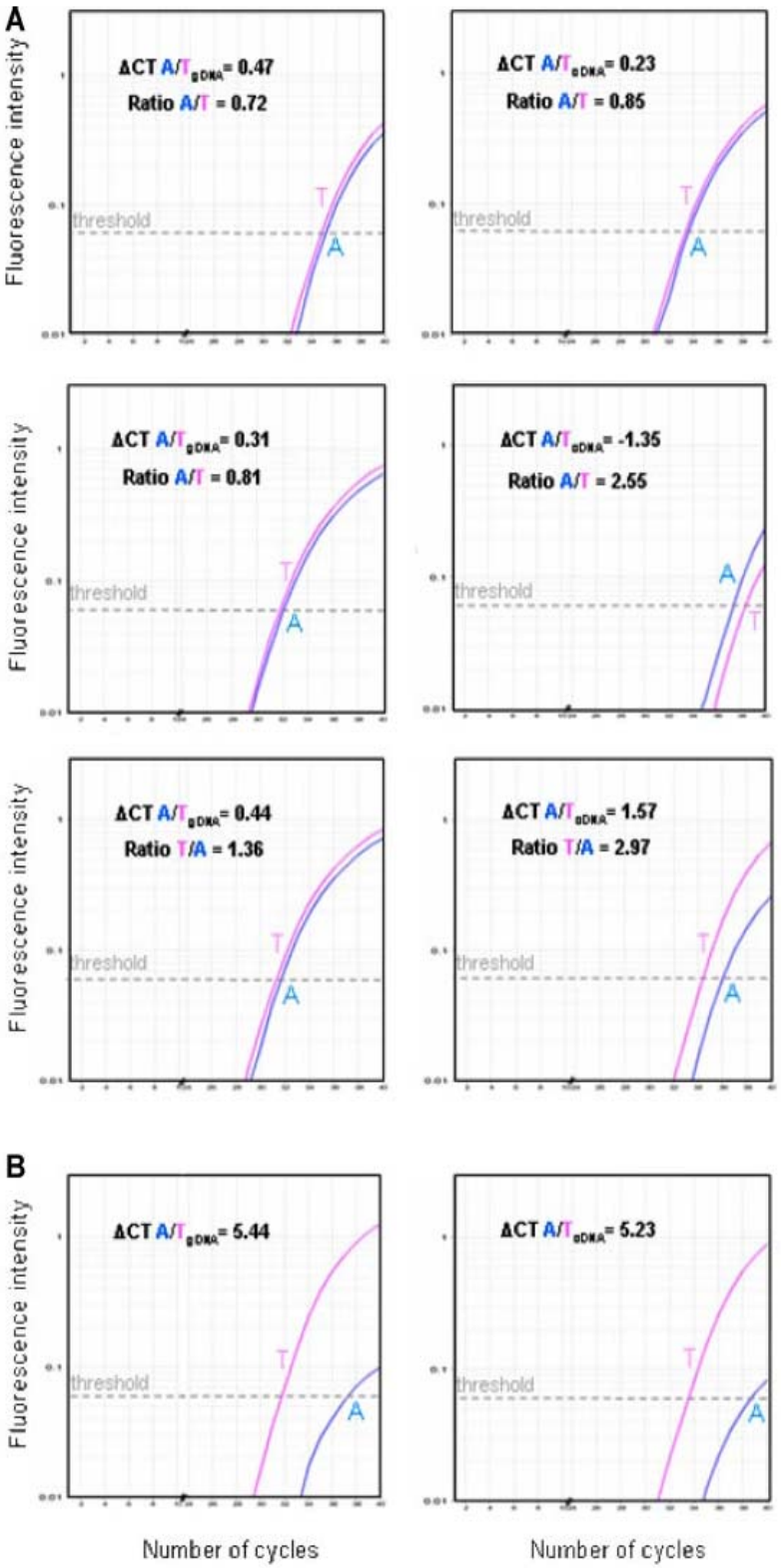

Fig. 1. TLR7 ASTQ in heterozygous female hepatitis C patients. **A**: ASTQ carried out on corresponding gDNA and cDNA samples from three representative TLR7 rs179008 heterozygous female patients revealed either nearly equal amounts of both alleles (**top**), a 2.7-fold excess of the A allele variant (**middle**), or a 2.6-fold excess of the T allele variant (**bottom**). Analyses were made in duplicate, therefore the mean ΔC_T is given but one representative amplification plot is shown. **B**: ASTQ carried out on one representative pair of corresponding gDNA and cDNA samples from a TLR7 rs179008 homozygote T patient yielded only non-specific signal for the allele A as defined by a 10-fold less fluorescence intensity in the plateau phase at the end of the reaction.

the virus, TLR7 rs179008 genotype distribution of the chronic hepatitis C patients was compared to a group of 44 patients with self-limited HCV infection [Wietzke-Braun et al., 2007]. The proportion of T homo- and hemizygous individuals or even of heterozygous female patients was not found to be significantly higher in chronic HCV patients (15.4% and 14.7%, respectively) in comparison to patients with self-limited HCV infection (11.4% and 13.6%, respectively) (Table I).

Hepatitis C Histological Manifestations

To investigate whether a functionally impaired TLR7 protein might be related to histological features of chronic hepatitis C, liver biopsy specimens were taken and evaluated histologically with regard to hepatitis activity, fibrosis progression, steatosis stage, portal lymphoid aggregates, and bile duct damage (Table II). TLR7 rs179008 genotype distribution showed no association with the first three characteristics. Nevertheless, a higher frequency of the minor allele T among patients with portal lymphoid aggregates was observed ($P = 0.013$), this difference, however, was only valid in males ($P = 0.032$) (Table II). Whereas only 30.5% of A carriers were found to have bile duct damage, 52.4% of T carriers did have the lesion ($P = 0.051$) (Table II).

Noteworthy, it was reported recently that patients homozygous for the variant allele T of the endotoxin receptor CD14 SNP rs2569190/C-159T have more frequent portal lymphoid aggregates than C carriers [Askar et al., 2009]. Interestingly, analyzing CD14 C-159T genotype separately in the two genders revealed that the significant association was only confined to males ($P = 0.004$) (unpublished observation).

Response to an IFN-α_{2a} Monotherapy

A total of 55 patients were treated with IFN-α_{2a} as described in the Patients and Methods Section. The initial virological response to therapy is defined as the elimination of HCV-RNA below the limit of detectability during the first 4 months for a period of at least three consecutive months. Some patients kept undetectable viral RNA levels till completing therapy, that is, end-of-treatment response, or even for a period of at least continuous 6 months after the last dose of IFN-α_{2a}, that is, sustained virological response. While 22 (63%) of the A carriers responded, at least initially, to the therapy, only 3 (30%) of the T counterparts (and 30% of the heterozygous females) were responders ($P = 0.069$) (Table III). Similar results were found considering a larger cohort of 145 patients treated with an IFN-α-based therapy (data not shown).

Hepatic Gene Expression

To examine whether a functionally impaired TLR7 protein might be related to hepatic gene expression in chronic HCV infection, innate immunity gene transcripts were quantified in freshly derived liver tissue samples. Data were related both to GAPDH and to albumin as reference genes. When A homo- and hemizygous samples were compared to T homo- and hemizygous samples, no significant difference was found with

TABLE II. Histological Manifestations of Chronic Hepatitis C Patients With Regard to TLR7 rs179008 Genotype

Histological manifestations	TLR7 rs179008 genotype					
	Females n (%)			Males n (%)		
	AA	AT	TT	A	T	P^a
Hepatitis activity						
Mild	20 (57.1)	12 (34.3)	3 (8.6)	35 (85.4)	6 (14.6)	0.156
Moderate	13 (59.1)	6 (27.3)	3 (13.6)	22 (75.9)	7 (24.1)	
Severe	1 (33.3)	2 (66.7)	0 (0)	4 (66.7)	2 (33.3)	
Fibrosis						
Absent	5 (55.7)	3 (33.3)	1 (11.1)	9 (100.0)	0 (0)	0.222
Mild	18 (56.3)	11 (34.4)	3 (9.4)	29 (80.6)	7 (19.4)	
Modorate	6 (54.6)	4 (36.4)	1 (9.1)	11 (37.3)	4 (26.7)	
Marked	4 (66.7)	1 (16.7)	1 (16.7)	5 (62.5)	3 (37.5)	
Cirrhosis	1 (50.0)	1 (50.0)	0 (0)	7 (87.5)	1 (12.5)	
Steatosis						
Absent	15 (60.0)	7 (28.0)	3 (12.0)	27 (77.1)	8 (22.9)	0.509
Mild	12 (50.0)	10 (41.7)	2 (8.3)	20 (83.3)	4 (16.7)	
Moderate	3 (42.9)	3 (42.9)	1 (14.3)	9 (75.0)	3 (25.0)	
Marked	4 (100.0)	0 (0)	0 (0)	5 (100.0)	0 (0)	
Portal lymphoid aggregates						
Absent	25 (64.1)	11 (28.2)	3 (7.7)	39 (88.6)	5 (11.4)	**0.013**[b]
Present	9 (42.9)	9 (42.9)	3 (14.3)	22 (68.8)	10 (31.3)	
Bile duct damage						
Absent	26 (63.4)	12 (29.3)	3 (7.3)	40 (85.1)	7 (14.9)	0.051
Present	8 (42.1)	8 (42.1)	3 (15.8)	21 (72.4)	8 (27.6)	

[a]χ^2-test was applied on A and T homo- and hemizygous patients, to compare mild versus moderate and severe hepatitis activity, absent, mild versus moderate and marked fibrosis and cirrhosis, and absent, mild versus moderate and marked steatosis.
[b]The difference is valid only in males ($P = 0.032$).
Number in bold refers to an analysis irrespective of gender.

TABLE III. Initial Virological Response to an IFN-α_{2a} Monotherapy in Chronic Hepatitis C Patients With Regard to TLR7 rs179008 Genotype

	TLR7 rs179008 genotype					
	Females			Males		
	AA	AT	TT	A	T	*P*
No. non-responsive patients (%)	6 (35.3)	7 (41.2)	4 (23.5)	7 (70.0)	3 (30.0)	0.069[a]
No. responsive patients (%)	4 (50.0)	3 (37.5)	1 (12.5)	18 (90.0)	2 (10.0)	

[a]χ^2-test was applied to compare A with T homo- and hemizygous patients.

regard to the amount of hepatic viral RNA (Fig. 2A), or the genes that have been shown to be enhanced in chronic HCV infection when compared to healthy liver tissue as IP-10, p44, MxA, or IFN-γ [Mihm et al., 2004] (Fig. 2B). In contrast, T homo- and hemizygotes were found to express significant lower amounts of IL-29/IFN-λ_1 ($P = 0.015$), IL-10 receptor beta (IL-10Rβ) ($P = 0.001$), and IL-28 receptor alpha (IL-28Rα) ($P = 0.003$), which constitute the two components of IFN-λ heterodimeric receptor, as well as lower amounts of IFN-α and $IFNAR_2$ (Fig. 2B).

DISCUSSION

The TLR7 rs179008/Gln11Leu is located in the signal sequence of TLR7, adjacent to the typical basic residues in the N-terminal part of this sequence. Signal peptide degeneracy modulates posttranslational modification, localization, quantity, and thus the functionality of the affected protein [Hegde and Bernstein, 2006]. In the studied cohort of 136 chronic hepatitis C patients, no significant association was found between TLR7 rs179008 and any of the epidemiological or biochemical characteristics, inflammation activity (grading), or fibrosis progression (staging). These results are in concordance with previous findings [Schott et al., 2007]. Considering the presence of portal lymphoid aggregates, a significant higher frequency of the T hemizygosity was found among male patients. Portal lymphoid aggregates are defined as densely packed collection of small lymphocytes within the portal tract with or without the formation of a germinal center [Luo et al., 1999]. Their presence, which is suggested to play an immunological albeit indeterminate role in chronic HCV liver injury similar to the mechanism of autoimmune hepatitis [Hino et al., 1992; Mosnier et al., 1993], has been found to be significantly correlated with hepatic inflammatory activity and bile duct damage [Freni et al., 1995; Wong et al., 1996; Luo et al., 1999; Askar et al., 2009]. Interestingly, the common allele A trended to be low-frequented among patients with bile duct damage ($P = 0.051$) (Table II).

Portal lymphoid aggregates have been recently found to be more frequent among patients homozygote for the T allele of CD14 rs2569190/C-159T [Askar et al., 2009], this association, however, was valid only in males as it is for TLR7 rs179008 in the present study. Noteworthy, sex itself is neither found to be associated with portal lymphoid aggregates [Mihm et al., 1997; Luo et al., 1999; Askar et al., 2009], nor with CD14 rs2569190 genotype distribution [Askar et al., 2009]. Freni et al. [1995] described the cellular composition of this manifestation as a core of B cells—which do express TLR7—mixed with many T helper/inducer lymphocytes, and an outer ring prominently formed by T suppressor/cytotoxic lymphocytes, and a rarely identifiable germinal center. Taken together, being the first observation of its kind, although its real biological mechanism is still to be found out, replication in an independent larger cohort, and correction of multiple testing are required, TLR7 rs179008 might have a role in the formation of portal lymphoid aggregates.

TLR7 rs179008 T allele has been found recently to be over-represented and predictive of unfavorable outcome of IFN-α therapy in female patients with chronic HCV infection [Schott et al., 2008]. In the present study, we investigated the distribution of this SNP among patients who spontaneously resolved HCV infection. Comparing the two cohorts with regard to TLR7 rs179008 genotype did not reveal any significant difference (Table I). Unfortunately, this analysis lacks statistical power due to the small cohort of self-limited individuals, yet it did not give any preliminary indication for an altered capacity of resolving the infection spontaneously. Moreover, the observed slight (but still non-significant) trend of the TT/T patients to be non-responders to a mono- or a combined IFN-α-based therapy and to have lower hepatic expression of both IFN-α and $IFNAR_2$ might confirm—in general—the previous findings of Schott et al. [2007] with cautious limitations due to the novel ASTQ analyses in this study that let us omit female heterozygotes from our analyses.

The impaired receptor appeared not to affect HCV hepatic viral load, accordingly, no further effect was observed on p44, MxA, IFN-γ, or IP-10, genes known to be upregulated in chronic HCV infection in the absence of hepatic type I IFN induction [Mihm et al., 2004]. The minor allele T, however, was found to be significantly associated with lower hepatic mRNA expression of IL-29/IFN-λ_1 and both IL-10Rβ and IL-28Rα (Fig. 2B). This suggests that, rather than being useful in forecasting the current IFN-α-based therapy outcomes, genotyping for TLR7 rs179008 might be predictive for response to IL-29/IFN-λ_1-based therapy approaches [Sheppard et al., 2003] currently being in phase 2 of clinical development.

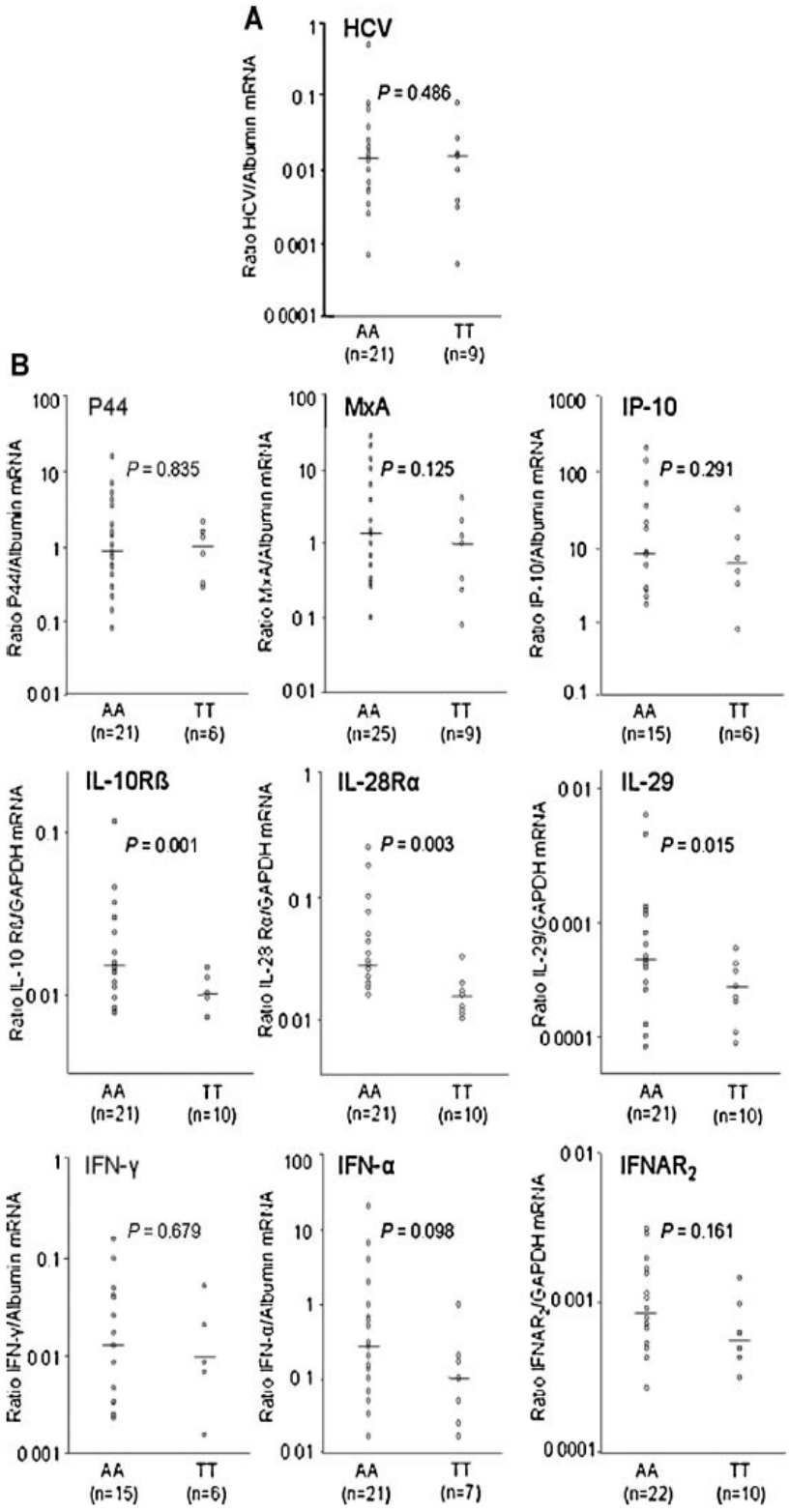

Fig. 2. Hepatic gene expression in chronic hepatitis C patients with regard to TLR7 rs179008 genotype. Total cellular RNA from liver biopsy specimens taken from homo- and hemizygous patients with chronic hepatitis C was quantified with respect to (**A**) HCV RNA, (**B**) p44, MxA, IP-10, IFN-γ, and IFN-α in relation to albumin mRNA transcripts by using competitive quantitative RT-PCR, and IL-10Rβ, IL-28Rα, IL-29, and $IFNAR_2$ in relation to GAPDH mRNA by using quantitative real-time RT-PCR assays. Data are given as ratios of the target to reference gene $\times 10^{-3}$. Medians are indicated by horizontal bars. Levels of significance are given. Similar results were obtained when data were related to β-actin (data not shown).

Three independent genome-wide association studies (GWASs) have reported recently on several SNPs in the intergenic region between the genes coding for IL-28A/IFN-λ_2 and IL-28B/IFN-λ_3 on chromosome 19 to be associated with response outcomes to an IFN-α-based therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous clearance of HCV [Thomas et al., 2009]. The minor non-responder allele of rs8099917 in IL-28B/IFN-λ_3 gene has been found, moreover, to be associated with lower IL-28 mRNA expression in PBMCs [Suppiah et al., 2009; Tanaka et al., 2009]. These GWASs have identified as well many SNPs in several genes to be of minor predictability for IFN-α-based therapy outcomes, TLR7, however, not to be among them.

Taken together, despite of significant decreased hepatic gene expression in TLR7 rs179008 T compared to A allele patients, that might be due to improper virus sensing and that might affect responsiveness to IL-29/IFN-λ_1 rather than IFN-α, differences in phenotype of disease including hepatic viral load, natural outcome of infection, and disease activity and progression appear to be minor with the exception of the presence of portal lymphoid aggregates in T hemizygous males. Further investigations will elucidate the impact of this polymorphism on responsiveness to endogenous and probably exogenous IFN-λ.

ACKNOWLEDGMENTS

We thank all physicians of the Department of Gastroenterology and Endocrinology, UMG, who were involved in patients' care and control for their kind cooperation, PD Dr. Stefan Schweyer, Department of Pathology, UMG, for his help in histological evaluation of patients' liver biopsies, and Waltraut Kopp for expert technical assistance. We also thank Prof. em. Dr. Gerhard Hunsmann, German Primate Center and the Institute of Virology, Goettingen, Germany, for his interest, supervision, and many helpful discussions. E.A. is thankful for her scholarship from Damascus University, Syria.

REFERENCES

Abe T, Kaname Y, Hamamoto I, Tsuda Y, Wen X, Taguwa S, Moriishi K, Takeuchi O, Kawai T, Kanto T, Hayashi N, Akira S, Matsuura Y. 2007. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines. J Virol 81:8953–8966.

Akira S, Takeda K. 2004. Toll-like receptor signalling. Nat Rev Immunol 4:499–511.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124:783–801.

Askar E, Ramadori G, Mihm S. 2009. Endotoxin receptor CD14 gene variants and histological features in chronic hepatitis C virus infection. World J Gastroenterol 15:3884–3890.

Boyum A. 1984. Separation of lymphocytes, granulocytes, and monocytes from human blood using iodinated density gradient media. Methods Enzymol 108:88–102.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303:1529–1531.

Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S, Klucher KM. 2006. Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 44:896–906.

Du X, Poltorak A, Wei Y, Beutler B. 2000. Three novel mammalian toll-like receptors: Gene structure, expression, and evolution. Eur Cytokine Netw 11:362–371.

Fish EN. 2008. The X-files in immunity: Sex-based differences predispose immune responses. Nat Rev Immunol 8:737–744.

Freni MA, Artuso D, Gerken G, Spanti C, Marafioti T, Alessi N, Spadaro A, Ajello A, Ferrau O. 1995. Focal lymphocytic aggregates in chronic hepatitis C: Occurrence, immunohistochemical characterization, and relation to markers of autoimmunity. Hepatology 22:389–394.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Hegde RS, Bernstein HD. 2006. The surprising complexity of signal sequences. Trends Biochem Sci 31:563–571.

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526–1529.

Hino K, Okuda M, Konishi T, Yamashita A, Kayano K, Kubota M, Yasunaga M, Fukumoto Y, Okita K. 1992. Analysis of lymphoid follicles in liver of patients with chronic hepatitis C. Liver 12:387–391.

Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, Endres S, Hartmann G. 2002. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 168:4531–4537.

Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, Endres S, Hartmann G. 2005. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med 11:263–270.

Horsmans Y, Berg T, Desager JP, Mueller T, Schott E, Fletcher SP, Steffy KR, Bauman LA, Kerr BM, Averett DR. 2005. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. Hepatology 42:724–731.

Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, Oki C, Itose I, Hiramatsu N, Takehara T, Kasahara A, Hayashi N. 2004. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. J Infect Dis 190:1919–1926.

Lee J, Wu CC, Lee KJ, Chuang TH, Katakura K, Liu YT, Chan M, Tawatao R, Chung M, Shen C, Cottam HB, Lai MM, Raz E, Carson DA. 2006. Activation of anti-hepatitis C virus responses via Toll-like receptor 7. Proc Natl Acad Sci USA 103:1828–1833.

Luo JC, Hwang SJ, Lai CR, Lu CL, Li CP, Tsay SH, Wu JC, Chang FY, Lee SD. 1999. Clinical significance of portal lymphoid aggregates/follicles in Chinese patients with chronic hepatitis C. Am J Gastroenterol 94:1006–1011.

Migeon BR. 2006. The role of X inactivation and cellular mosaicism in women's health and sex-specific diseases. JAMA 295:1428–1433.

Mihm S, Hartmann H, Fayyazi A, Ramadori G. 1996a. Preferential virological response to interferon-alpha 2a in patients with chronic hepatitis C infected by virus genotype 3a and exhibiting a low gamma-GT/ALT ratio. Dig Dis Sci 41:1256–1264.

Mihm S, Hutschenreiter A, Fayyazi A, Pingel S, Ramadori G. 1996b. High inflammatory activity is associated with an increased amount of IFN-gamma transcripts in peripheral blood cells of patients with chronic hepatitis C virus infection. Med Microbiol Immunol 185:95–102.

Mihm S, Fayyazi A, Hartmann H, Ramadori G. 1997. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology 25:735–739.

Mihm S, Frese M, Meier V, Wietzke-Braun P, Scharf JG, Bartenschlager R, Ramadori G. 2004. Interferon type I gene expression in chronic hepatitis C. Lab Invest 84:1148–1159.

Mosnier JF, Degott C, Marcellin P, Henin D, Erlinger S, Benhamou JP. 1993. The intraportal lymphoid nodule and its environment in chronic active hepatitis C: An immunohistochemical study. Hepatology 17:366–371.

Oh DY, Baumann K, Hamouda O, Eckert JK, Neumann K, Kucherer C, Bartmeyer B, Poggensee G, Oh N, Pruss A, Jessen H, Schumann RR. 2009. A frequent functional toll-like receptor 7 polymorphism is associated with accelerated HIV-1 disease progression. AIDS 23:297–307.

Patzwahl R, Meier V, Ramadori G, Mihm S. 2001. Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: Detection by suppression-subtractive hybridization. J Virol 75:1332–1338.

Schott E, Witt H, Neumann K, Taube S, Oh DY, Schreier E, Vierich S, Puhl G, Bergk A, Halangk J, Weich V, Wiedenmann B, Berg T. 2007. A Toll-like receptor 7 single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection. J Hepatol 47:203–211.

Schott E, Witt H, Neumann K, Bergk A, Halangk J, Weich V, Muller T, Puhl G, Wiedenmann B, Berg T. 2008. Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. J Viral Hepat 15:71–78.

Schwabe RF, Seki E, Brenner DA. 2006. Toll-like receptor signaling in the liver. Gastroenterology 130:1886–1900.

Seki E, Brenner DA. 2008. Toll-like receptors and adaptor molecules in liver disease: Update. Hepatology 48:322–335.

Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, Klucher KM. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4:63–68.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M. 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461:798–801.

Wietzke-Braun P, Manhardt LB, Rosenberger A, Uy A, Ramadori G, Mihm S. 2007. Spontaneous elimination of hepatitis C virus infection: A retrospective study on demographic, clinical, and serological correlates. World J Gastroenterol 13:4224–4229.

Wong VS, Wight DG, Palmer CR, Alexander GJ. 1996. Fibrosis and other histological features in chronic hepatitis C virus infection: A statistical model. J Clin Pathol 49:465–469.

Yonkers NL, Rodriguez B, Milkovich KA, Asaad R, Lederman MM, Heeger PS, Anthony DD. 2007. TLR ligand-dependent activation of naive CD4 T cells by plasmacytoid dendritic cells is impaired in hepatitis C virus infection. J Immunol 178:4436–4444.

6. Acknowledgments

It is a pleasant aspect that I have now the opportunity to express my gratitude to the Syrian ministry of high education for the generous financial support during my study in Germany and to all people who supported me to perform this dissertation.

First, my specific thanks are given to my supervisor Prof. em. Dr. Gerhard Hunsmann, whose interest, recommendations and encouragement were always a motivation for me to go ahead.

I am thankful to Prof. Dr. med. Frank T. Hufert and Dr. rer. nat Manfred Weidmann who invited me initially perform the scientific work in Germany; and to Prof. Dr. med. Giuliano Ramadori for enabling me to perform the experiments in the Gastroenterology and Endocrinology laboratories.

My deepest gratitude is to my direct supervisor Prof. Dr. rer. nat. Sabine Mihm. I have been amazingly fortunate to have an advisor who created me a serious atmosphere of laboratory work and devoted for me a lot of time for helpful discussions. Frau Mihm taught me how to question thoughts and express ideas. Her experience, patience and support helped me to finish this work. She has been always there to listen and give advice apart from carefully reading and commenting on countless revisions of this work. I hope that, one day, I would be as responsible an advisor to my students as Frau Mihm has been to me.

Many other people have helped me during this work. I would like to express my heart-felt thanks to the members of our group in the hepatitis C laboratory in the Department of Gastroenterology and Endocrinology of Georg-August University in Goettingen, especially Mrs. Valtraut Kopp and Dr. med. Rusudan Bregadze for their support to overcome many problems in the lab-related work.

7. Curriculum Vitae

I obtained the Syrian secondary education diploma in Fairoza, Homs, Syria in 1993. Then I studied human medicine in Damascus University, Syria for 6 years, and in July 1999, I did the final medical graduate examination. Thereafter (1999-2002), I followed a postgraduate training in microbiology in the Department of Laboratory Medicine, Faculty of Human Medicine, Damascus University, Syria.
In June 2003, I was assigned as a postgraduate assistant (lecturer) at the Department of Laboratory Medicine, Faculty of Human Medicine, Damascus University where I worked until the end of 2006.
In 2007 I got a scholarship from the Syrian government to make a doctoral project in Germany.

I have worked on the current doctorate thesis since January 2008 under the supervision of Prof. Dr. rer. nat. S. Mihm and Prof. Dr. med. G. Hunsmann at the Department of Gastroenterology and Endocrinology, University Medical Center Goettingen, Georg August University, Germany.

Publications

1. Askar E, Bregadze R, Mertens J, Schweyer S, Rosenberger A, Ramadori G, Mihm S (2009): TLR3 gene polymorphisms and liver disease manifestations in chronic hepatitis C. J Med Virol 81, 1204-11.

2. Mertens J, Bregadze R, Mansur A, Askar E, Bickeböller H, Ramadori G, Mihm S (2009): Functional impact of endotoxin receptor CD14 polymorphisms on transcriptional activity. J Mol Med 87, 815-24.

3. Askar E, Ramadori G, Mihm S (2009): Endotoxin receptor CD14 gene variants and histological features in chronic HCV infection. World J Gastroenterol 15, 3884-90.

4. Askar E, Ramadori G, Mihm S. (2010): Toll-like receptor 7 rs179008/Gln11Leu gene variants in chronic hepatitis C virus infection. J Med Virol 82, 1859-68.