HEPATOCELLULAR CARCINOMA

VIRAL ETIOLOGY AND CELLULAR MECHANISMS

A THESIS SUBMITTED TO
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
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SEPTEMBER, 2002

TO MY SON AND MOM

MY FUTURE AND PAST,

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

Hepatocellular Carcinoma
Viral Etiology and Cellular Mechanisms
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Hepatocellular carcinoma (HCC) is one of the most frequent carcinomas throughout the world, being responsible for more than 1 million deaths annually and has a strong association with several etiological factors including aflatoxinB1, alcohol and Hepatitis virus B and C. Several studies suggested that HCV subtype 1b causes more severe liver diseases including HCC in a high manner and resistance to antiviral therapy. So, it is important to know genotype and some characteristics of HCV which are unique for the countries to develop better strategies regarding public health. By using direct sequencing information from 5`UTR and NS5B regions we identified subtype 1b as a predominant hepatitis C virus genome in Turkey. Next, the full genome sequence of a Turkish 1b isolate (HCV-TR1) was obtained by cloning of polypeptide-encoding region into 7 overlapping fragments. Although major structural and functional motifs of HCV proteins were maintained in HCV-TR1, it displayed amino acid substitutions in 6 out of 9 major cytotoxic T-cell epitopes. Several HCV proteins have been reported to contribute hepatocellular malignancy by interaction with critical cellular proteins involved in hepatocyte proliferation and survavil. Such studies often use HCC-derived cell lines as experimental models. As a prerequisite to future studies about the Turkish HCV 1b isolate in term of its contribution to HCC developments we investigated on phenotypic characterization of HCC cell lines. We provide experimental evidence that α-fetoprotein-producing HCC lines display *in*

vitro liver stem cell-like properties with self-renewing capability and multi-lineage differentiation potential, even after single-cell cloning. However, their ability to generate fully differentiated normal progeny was disrupted, even if they modulate their differentiation program in response to external factors. These features qualify AFP-producing HCCs as "mis-specified" liver stem cell cancers whose cellular programs are deviated from repopulating liver to forming malignant tumors. Interestingly, stem-like cells described here have been used extensively to study the role HCV proteins. Our observations offer new opportunities for addressing the potential role of HCV in the misspecification of liver stem cells in relation with viral hepatocellular carcinogenesis.

ÖZET

Karaciğer kanseri
Viral Etiyoloji ve Hücresel Mekanizmalar
Esra Erdal-Yıldız

Doktora Tezi, Moleküler Biyoloji ve Genetik Bölümü
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Hepatosellular karsinoma (HCC), yılda 500.000'den fazla kişinin ölümünden sorumlu olan ve aflatoksin B1, alkol, Hepatit C ve B virusları gibi birçok etiyolojik faktörle ilişkili, tüm dünyada en sık görülen karsinomalardan biridir. HCC patogenezinde HCV'nin rolü tüm olarak aydınlatılamamışsa da, birçok çalışma HCV altgrup 1b'nin büyük oranda HCC olmak üzere, ağır karaciğer hastalıklarına ve antiviral terapiye direnc sebep olduğunu öngörmektedir. Bundan dolayı, HCV'nın ülkelere özgün bazı yapılarının ve genotipinin bilinmesi toplum sağlığı ile ilgili stratejilerin daha iyi geliştirilmesi için önemlidir. 5'UTR ve NS5B bölgelerinden direk sekanslama bilgisi kullanılarak Türkiye'de genotip 1b'nin baskın Hepatit C virus genomu olduğunu tespit ettik. Daha sonra proteın 7 birbirini takip eden parçalar şeklinde klonlanması ile Türk 1b isolatının (HCV-TR1) tüm genom sekansı elde edildi. HCV-TR1'de HCV proteinlerinin temel yapısal ve işlevsel motifleri bulunsa da 9 temel sitotoksik T-hücre epitoplarından 6'sında amino asit değişiklikleri görülmüştür. Birçok HCV proteininin hepatosit çoğalması ve yaşamasında rol alan önemli hücresel proteinlerle ilişkiye girerek hepatosellular malignansı oluşturduğu rapor edilmiştir. Bu çalışmalarda sıklıkla deneysel model olarak HCC kaynaklı hücre hatları kullanılmıştır. HCC gelişimindeki rolü ile ilgili Türk HCV izolatının gelecekteki çalışmalarına ön hazırlık olarak, HCC hücre hatlarının fenotipik karakterizasyonu incelendi. Alfafeto protein üreten HCC hatlarının *in vitro* koşullarda, tek hücre

klonlamasından sonra bile, farklı hücre tipleri verebilme ve kendini yenileyebilme gibi karaciğer kök hücre benzeri özellikler gösterdiği deneysel olarak kanıtlandı. Fakat, dış faktörlerle module edilebilmesine rağmen, tamamen farklılaşmış progeni oluşturma özelliği kaybolmuştur. Bu özellikler AFP üreten HCC hücre hatlarını, hücresel programları habis tümor oluşturmak üzere tekrar popule olabilen "yanlış özelleştirilmiş (mis-specified)" karaciğer kök hücre kanserleri olarak belirlemiştir. İlginç olanı, burada tanımlanan kök hücre benzeri hücreler çoğunlukla HCV proteinlerinin rolünü çalışmak için kullanılmıştır. Bizim gözlemlerimiz, viral hepatosellular karsinogenez ile ilgili karaciğer kök hücrelerinin, yanlış özelleşmesinde HCV'nin muhtemel rolünü göstermek için yeni ufuklar açmıştır.

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ABBREVIATIONS

ARF	Alternative Reading Frame
BSA	Bovine Serum Albumin
C	Capsid
CDK	Cyclin Dependent Kinase
C-terminus	Carboxy terminus
DNA	Deoxyribonucleic acid
E	Envelope
ED	Embriyonal day
HRP	Horse Reddish Peroxidase
MDM2	Mouse Double Minute 2
MMLV	Murine Maloney Leukemia Virus
NS	Nonstructural
N-terminus	Amino terminus
O/N	Over Night
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20
PCR	Polymearase chain reaction
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
S/N	Supernatant
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween-20
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	Tris (hydroxymethyl)-methylamine
UV	Ultraviolet

CHAPTER 1. INTRODUCTION

1-1 HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) accounts for 80-90% of liver cancers and is one of the most frequent carcinomas worldwide, with an estimated 564,000 new cases per year and almost as many deaths in 2000 (Parkin, 2001). In developing countries the incidence rates are two to threefold higher than in developed countries. The disease is more prevalent in parts of Africa and Asia than in continental America and Europe with a strong etiological association with viral hepatitis, hemochromatosis, known liver (hepatic) carcinogens, and toxins (aflatoxin).

Hepatocarcinogenesis is a slow process during which genomic changes progressively alter the hepatocellular phenotype to produce cellular intermediates that evolve into hepatocellular carcinoma. During the long preneoplastic stage, in which the liver is often the site of chronic hepatitis, cirrhosis, or both, hepatocyte cycling is accelerated by upregulation of mitogenic pathways. It is believed that this chronic regeneration process leads to the production of aberrant and dysplastic hepatocytes that have telomere erosion and telomerase re-expression, sometimes aberrant methylation or occasionally structural changes in genes and chromosomes. Development of dysplastic nodules and hepatocellular carcinoma are associated with the accumulation of irreversible structural alterations in genes and chromosomes, but the genomic basis of the malignant phenotype is heterogeneous. The malignant hepatocyte phenotype may be produced by the disruption of a number of genes that function in different regulatory pathways, producing several molecular variants of hepatocellular carcinoma (Thorgeirsson and Grisham, 2002).

1-1.1 Genetic Mechanisms of Hepatocarcinogenesis

As with other kinds of cancer, the etiology and carcinogenesis of HCC are multifactorial and multistage. The multistep process of HCC may be divided into chronic liver injury that produces inflammation, cell death, cirrhosis and regeneration, dysplasia, and finally HCC (Figure 1.1).

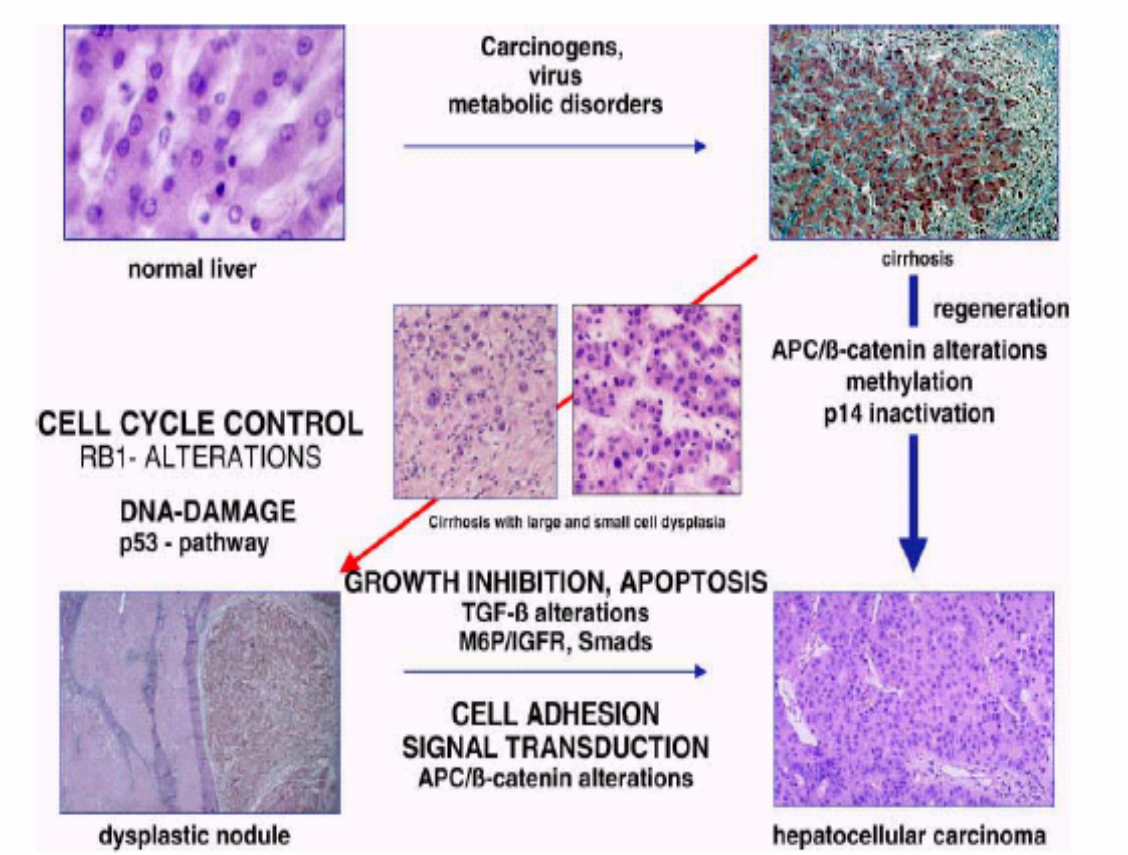

Figure 1.1. Multistage process of carcinogenesis (Tannapfel and Wittekind, 2002)

It has been proposed that six essential alterations in normal cell physiology state progression of liver malignancy, including independence towards growth, anti growth and apoptotic signals, unlimited division, and angiogenetic and metastatic capacities (Ozturk and Cetin-Atalay, in press). Mutations in critical genes may range from subtle sequence changes at a few nucleotides to gross chromosomal abnormalities including deletions, amplifications, and translocations of large DNA fragments.

Allelic imbalance and microsatellite instability

Most of the genes mutated in HCC are tumor suppressor genes, and frequent allelic loses (loss of heterozygosity, LOH) have been described. By comparative genomic hybridization, chromosomes 1q, 8q, and 17q show gene dose increase while chromosomes 1p, 4q, 8p, 9p, 13q, 16p, 16q, and 17p show gene dose loss. Frequent LOH, or more comprehensive, allelic imbalance (AI), is consistently observed on chromosomes 1p, 4q, 6p, 8p, 13q, 16q, and 17p by whole-genome allelotyping (Tannapfel and Wittekind, 2002). The chromosome regions with gene dose increase may contain critical oncogenes while those with gene dose loss may contain tumor-suppressor genes. For chromosomes 17p, 13q, 9p, 6q, and 16p, LOH could be related to p53, Retinoblastoma 1(RB1), p16, Insulin-like growth factor-2 receptor (IGF2R) and E-cadherin inactivation (Feitelson et al., 2002). In dysplastic nodules LOH has been observed with a prevalence of 50-80%. (Thorgeirsson and Grisham, 2002).

In HCC, chromosome 2 and 3 on which DNA mismatch repair genes are located are not frequently affected by allelic loses. But mutations in a mismatch repair gene known as Human Mut S homolog-2 (hMSH2) have been reported at about 30% of HCCs examined (Yano et al., 1999).

Cell cycle regulation

Cells respond to proliferative or antiproliferative signals through the cyclin D1-RB-CDK4/6 and the $p14^{ARF}$/mdm2/p53 pathways. When quiescent cells in G0 are stimulated to enter cell cycle, genes encoding cyclin-D type cyclins are induced in response to mitogenic signals (Figure 1.2). These cyclins associate with either CDK4 or CDK6 subunits and the complex becomes activated by phosphorylation. Active cyclin/CDK complexes drive the cell cycle forward via phosphorylation of substrates such as Rb in early G1 phase (Weinberg, 1995). Rb is thereby inactivated, and its growth repressive functions abolished, resulting in release of a class of associated transcription factors known as E2Fs. Then “free” E2F transactivate cyclin E gene and promote DNA synthesis necessary for cell cycle progression. According to this, loss of Rb or its aberrant phosphorylation leads to a loss of growth control at the G_1 phase. To maintain Rb protein in its active, anti-proliferative state, p16

(INK4a) inhibits the activity of CDK4 by specific binding thus preventing its association with cyclin D and/or blocking the catalytic activity of the kinase (Hirai et al., 1995). The $p14^{ARF}$ tumor suppressor, encoded by an alternative reading frame of the INK4a-ARF locus (9q21), senses "mitogenic current" flowing through the Rb pathway and is induced by abnormal growth promoting signals. By antagonizing Mdm2, a negative regulator of the p53 tumor suppressor, ARF triggers a p53-dependent transcriptional response that diverts incipient cancer cells to undergo growth arrest or apoptosis. Although ARF is not directly activated by signals that damage DNA, its loss not only dampens the p53 response to abnormal mitogenic signals but also renders tumor cells resistant to treatment by cytotoxic drugs and irradiation.

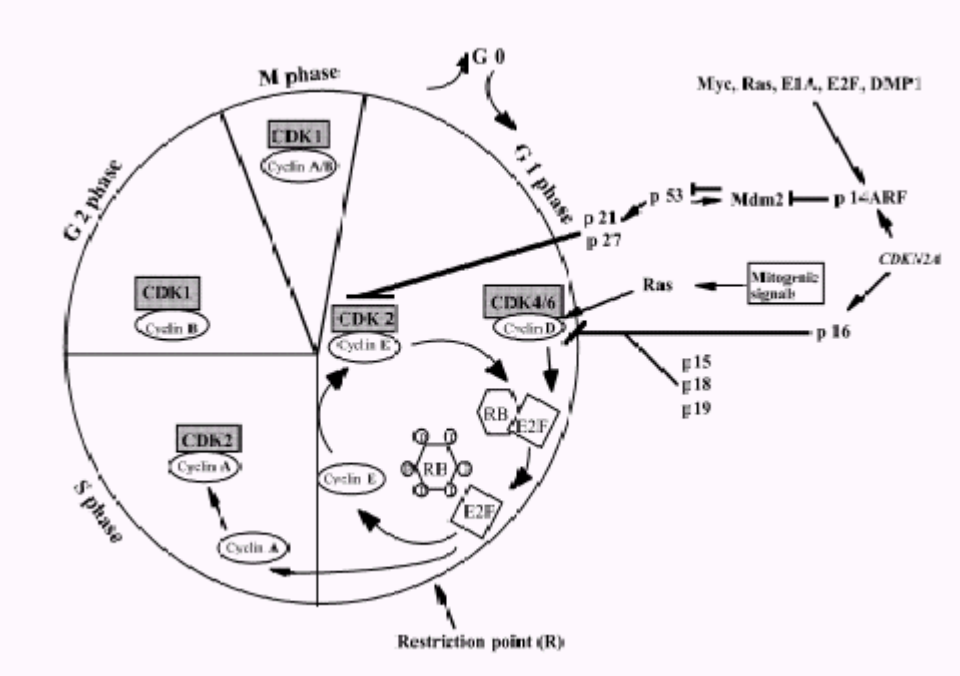

Figure 1.2 Schematic representation of the cell cycle and G1/S controlling elements (Hashemi, 2002)

→ activation ⊢ inhibition

Taken together, disturbances in the p16-cyclin D-CDK4-Rb and p14-Mdm2-p53 pathways could be a main axis of genetic events in HCC because all players in these pathways seem to be altered in HCC. The Rb gene, one of the main player, is localized to chromosome 13q, which is a common deletion site for HCC and Rb

mutations are also observed in 15% of HCCs (Ozturk M., 1999). Moreover, Rb protein is a target for ubiquitin-dependent degradation and this degradation mechanism was shown to be dysregulated in HCCs by overexpression of a pRb specific ubiquitin ligase, gankyrin (Higashitsuji et al., 2000). Also, overexpression of cyclin D_1 has been observed in about 10-13% of HCC cases (Ozturk, 1999). It has recently been shown in a transgenic mouse model that overexpression of cyclin D_1 is sufficient to initiate hepatocellular carcinogenesis (Deane et al., 2001). The transduction of antisense cyclin D_1 inhibits tumor growth in a xenograft hepatoma model. Correcting alterations that have occurred in the G_1 phase regulatory machinery may therefore provide a novel weapon to treat and prevent HCC (Deane et al., 2001). Also, it was reported that about 50% of HCC displays *de novo* methylation of INK4a-ARF locus that encodes $p16^{INK4}$ and $p14^{ARF}$ and LOH at the same locus was 20 % (Ozturk, 1999; Liew, 1999)

p53 and homologues

The protein product of p53 gene is activated by different stimuli such as oncogenic activation, DNA damage, decrease in nucleotide pools and oxidative stress and induces cell cycle arrest or apoptosis, depending on the cell context (Blagosklonny, 2002). p53 mutations are found in about 30% of HCC cases worldwide. Until now all reported mutations (mostly missense, leading to stabilization of protein) have been somatic, indicating that germline p53 mutations do not appear to be predisposition for HCC. Tumor-specific p53 mutations have been identified in several studies, linking the mutation pattern to suspected etiological factors. A selective guanine-to-thymine transversion mutation in codon 249 AG*G* to AG*T* (transversion italicized) leading to an arginine-to-serine substitution of the p53 gene has been identified as a "hotspot" mutation for HCC. Epidemiological and experimental evidence suggests that in HCC this mutation is strongly associated with exposure to aflatoxin B_1 in combination with a high level of chronic hepatitis B virus infection in the population (Bressac et al., 1991; Hsu et al., 1991). Moreover, there is a strong correlation between p53 mutations, large tumor size, and poor differentiation state.

No specific mutations or interactions have yet been described for p73 and p63 which are homologs of p53. However, overexpression of p73 (wild type) has been described in a subset a HCC, indicating a poor prognosis in these patients (Tannapfel et al., 1999). More recently Sayan et al., identified that it is the transcriptionally active (TA) form of p73 which is upregulated in HCC, probably because of Rb pathway dysregulation (Sayan et al., 2001).

Wnt pathway: APC, β-catenin, axin 1, and E-cadherin

Somatic mutations of β-catenin have been observed in 19-26% of HCC cases, mostly missense mutations and interstitial deletions of exon 3 (Tannapfel and Wittekind, 2002). These mutations cause nuclear accumulation of aberrant β-catenin proteins that stimulates the activity of LEF-TCF family of transcription factors which in turn transactivate a series of cell cycle progression genes such as cyclin D and myc as shown in colorectal cancers (Calvisi et al., 2001). Axin, an important regulator of β-catenin, is mutated in about 10% of HCC cases, leading to an activation of the Wnt pathway. Axin1 and β-catenin mutations are mutually exclusive in HCC, suggesting that they affect the same (presumably Wnt) pathway (Morin et al., 1997; Satoh et al., 2000). It has recently been shown that ectopic expression of the wild-type axin gene (AXIN1) induces apoptosis in HCC cells, indicating that axin 1 may be an effective growth suppressor of hepatocytes (Satoh et al., 2000)

Somatic APC mutations are rare events in HCC, but it was recently reported that biallelic inactivation of the APC gene contributed to the development of HCC in a patient with familial adenomatous polyposis and a known germline mutation of the APC gene at codon 208 (Su et al., 2001). E-cadherin, a receptor in adherence junctions, which is essential both for maintenance of tissue structure and regulation of free cytoplasmic β-catenin level, is rarely mutated in HCC. However, loss of function due to LOH or *de novo* methylation occurs in about 30% of HCC cases (Tannapfel and Wittekind, 2002).

Alterations of the TGF-β/IGF-axis

Transforming growth factor (TGF) β initiates signaling through heteromeric complexes of transmembrane type I and type II serine/threonine kinase receptors. Activated TGF-β receptors phosphorylate receptor-regulated Smads which induces both inhibition and apoptosis in hepatocytes. Genetic alterations of the TGF-β pathway are mediated by mutations of the Smad2 and Smad4 gene, which occur in about 10% of HCC cases (Yakicier et al., 1999). Mutations of the TGF-β receptor (TGF-β1RII) gene itself are detected in patients with HCC and may also abrogate TGF-β signaling (Enomoto et al., 2001).

A potent activator of TGF-β is the mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) which suppresses cell growth through binding to the insulin-like growth factor (IGF) 2 and latent complex of TGF-β. The deregulation of the IGF axis, including the autocrine production of IGFs, IGF binding proteins (IGFBPs), IGFBP proteases, and the expression of the IGF receptors, has also been identified in the development of HCC. Also, both LOH and mutations of the M6P/IGF2R have been reported in about 30% HCC patients (Oka et al., 2002; De Souza et al., 1995; Piao et al., 1997).

PTEN/MMAC1/TEP1 (PTEN) tumor suppressor gene has recently been shown to block growth-stimulatory and survival signals mediated by PI-3 kinase and to converge the activation of protein kinase B/Akt. Alterations, mainly mutations but also LOH, of PTEN have been reported in about 27% of HCC cases (Kawamura et al., 1999). Recently it was demonstrated that PTEN significantly lowers IGF secretion and also expression of secretory and cellular vascular endothelial growth factor proteins in HCC cell lines and could therefore inhibit tumorigenicity (Kawamura et al., 1999).

1-1.2 Significance of Hepatitis B and C viruses in HCC

From the information released by the World Health Organization, it is estimated that the numbers infected with HBV and HCV worldwide are 180 and 300

millions, respectively. Together, the two viruses contribute to the etiology of about 80% of global HCC (World Health Organization., 2000). They contribute to hepatocarcinogenesis indirectly, by causing chronic necroinflammatory hepatic disease. They may also display direct hepatocarcinogenic activity.

Hepatitis B Virus (HBV) is a partially double-stranded DNA virus belonging to the Hepadnaviridae and approximately 25% of chronic carriers of the virus develop the tumor (Beasley and Hwang, 1984). Oncogenic mechanism of HBV infection may be simply defined as releasing the growth control of hepatocytes by coding for a factor that activates otherwise dormant genes or activates proto-oncogenes or silences anti-oncogenes; by inserting its DNA sequences that can activate and influence the transcription of cellular genes; by causing chronic inflammation with cell death and hepatocyte regeneration with fibrosis; as well as by activation of the immune system liberating cytokines at the wrong time in the wrong place. Transcriptional activation of a wide range of viral, as well as cellular genes such as *c-fos, c-myc,* IGF2, insulin-like growth factor I receptor (IGF_{R1}) and β-interferon, by HBV encoded X antigen (HBxAg) was shown in many studies (Caselmann, 1996; Colgrove et al., 1989; D'Arville et al., 1991; Kim et al., 1996; Twu and Schloemer, 1987). In chronic HBV infection, it has been shown that HBxAg binds and functionally inactivates the tumor suppressor p53 (Huo et al., 2001; Ueda et al., 1995) and the negative growth regulator $p55^{sen}$ (Feitelson, 1999; Ueda et al., 1995), both of which are involved in senescence-related pathways. The activation of the Rb tumor suppressor by hyperphosphorylation resulting in the activation of E2F1 has been reported in HBxAg positive HCC cells (Sirma et al., 1999). It has also been shown that HBxAg can down regulate the expression of translational factor, sui1, and cyclin dependent kinase inhibitor, $p21^{WAF1/CIP1/SDI1}$(Feitelson et al., 1999; Sirma et al., 1999). As with HBxAg, carboxyterminal truncated middle hepatitis B surface protein ($MHBS^{t}$) can activate various viral and cellular gene promoters (Caselmann et al., 1990; Kekule et al., 1990). Recent data suggests that HBxAg contributes to HCC development also by mechanisms other than transactivation. HBxAg binds to the X-associated protein 1 and possibly disturbs its function in nucleotide excision repair mechanism (Becker et al., 1998) and also it has been show that HBxAg stimulated cell growth is associated with constitutive activation of the ras/raf/MAPK and NKκ-B signal transduction

pathways (Benn and Schneider, 1994; Lucito and Schneider, 1992; Shirota et al., 2001).

Hepatitis C Virus is a more important causal association of the tumor than is HBV, and in Japan, Italy, and Spain the virus accounts for as much as 80% of HCC (Kew, 1998). It has been postulated that HCC largely develops indirectly as a result of the inflammatory responses that lead to hepatocyte destruction, regeneration and fibrosis. Since there is no evidence that HCV RNA is integrated into host genome as stated in HBV, the virus may play a more direct role in neoplastic transformation of hepatocytes. HCV proteins are shown to interact with various cellular proteins: 14-3-3 protein, apolipoprotein AII, Tumor necrosis factor (TNF) receptor, lymphotoxin-ß receptor, DEAD domain of RNA helicase, nuclear ribonucleoprotein K for core protein, double stranded RNA protein kinase (PKR) for E2 and NS5A, p53 for NS3 and possibly core, and SNARE-like protein for NS5A. (Ghosh et al., 1999; Ray and Ray, 2001; Shimotohno, 2000). Interactions with these cellular proteins which seem to be important for the function of HCV proteins in the regulation of cell proliferation will be summarized in 1.2.3.

Recently developed transgenic mice model for the viral hepatocarcinogenesis showed that HBx protein and HCV core protein may be capable of inducing HCC in the absence of a complete set of genetic aberrations which are necessary for a multistage development of all cancer types as proposed by Vogelstein. (Koike et al., 2002; Moriya et al., 1998)

1-2 HEPATITIS C VIRUS

Hepatitis C was first recognized as a separate disease entity in 1975 when the majority of cases of transfusion-associated hepatitis were found not to be caused by the only two hepatitis viruses recognized at the time, hepatitis A virus and hepatitis B virus. The disease was called "non-A non-B hepatitis," and it was demonstrated to be transmissible to chimpanzees. It was not until 1989, however, that the cloning and

sequencing of the viral genome of the non-A non-B hepatitis virus was first reported and the virus was renamed "hepatitis C virus" (Choo et al.,1991).

Hepatitis C virus is a positive strand RNA virus of Filaviviridae, genus hepacivirus, approximately 9.6 kb in length, and is most closely related to the pestiviruses.

1-2.1 Epidemiology

Hepatitis C is a major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer. An estimated 170 millions of persons are chronically infected with HCV worldwide and 3-4 million persons are newly infected each year (World Health Organization, 2000).

HCV infection appears to be endemic in most parts of the world, with an estimated overall prevalence of 3%. However, there is considerable geographic and temporal variation in the incidence and prevalence of HCV infection. In United States, 1.8% of population is infected with HCV (Alter, 1997) while the HCV prevalence rate is in between 0.2-3% in Europe (Memon and Memon, 2002). In Turkey the prevalence of HCV is % 0.3-1.8 (Sharara et al., 1996).

There are a few countries with very high HCV prevalence rate, such as Egypt, where 10-30% of population is infected with HCV (Arthur et al., 1997; el-Sayed et al., 1996). The nationwide campaing to treat schistosomiasis infections by inoculation of needles in 1970s is hypothesized to be responsible for this high prevalence, as lower HCV prevalence among individuals born after the end of campaign was observed as a preliminary evidence (el-Zayadi et al., 1997).

1-2.2 Genomic Organization of HCV

The genome of HCV is a single-strand linear RNA of positive sense. The functional and structural units of the HCV genome are schematically depicted in

Figure1.3. A 5' untranslated (UTR) region consists of approximately 340 nucleotides, which has stem-loop structure and contains an apparent internal ribosomal entry site (IRES). Immediately downstream is a single large open reading frame (ORF) of approximately 9,600 nucleotides, encoding a large polyprotein precursor of approximately 3,200 amino acids that is cotranslationally or posttranslationally cleaved into separate proteins by a combination of host and viral proteases. The genomic order of HCV has been shown to be C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. Capsid protein (C), two envelope proteins (E1 and E2) are the virion structural proteins. The function of p7 is currently unknown. These proteins have been shown to arise from the viral polyprotein via proteolytic processing by the host signal peptidases.

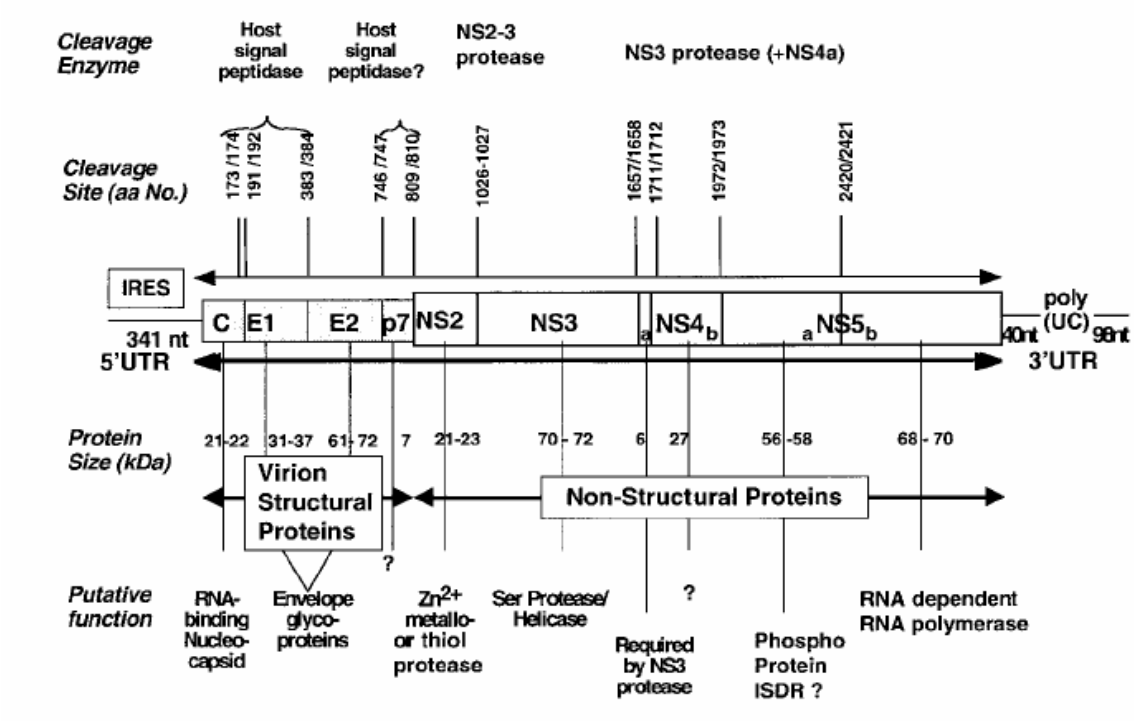

Figure 1.3 Genomic map of the Hepatitis C virus (Rosenberg, 2001).

Generation of the mature nonstructural protein, NS2 to NS5B, relies on the activity of viral proteinases. Cleavage at the NS2/NS3 junction is accomplished by metal-dependent autocatalytic proteinases encoded within NS2 and the N-terminus of NS3. The remaining cleavages downstream from this site are affected by a serine proteinase also contained within the N-terminal region of NS3. NS3 also contains an

RNA helicase domain at its C-terminus. NS3 forms a heterodimeric complex with NS4A. The latter is a membrane protein that has been shown to act as a cofactor of the proteinase. While no function has yet been attributed to NS4B, it has been suggested that NS5A is involved in mediating the resistance of the HCV to interferon. And the NS5B protein has been shown to be the viral RNA-dependent RNA polymerase. Finally, there is a 3' UTR region that consists of approximately 40 nucleotides, a polypyrimidine track and a highly conserved terminal sequence of approximately 90 nucleotides.

1-2.3 Genetic Heterogeneity and Classification systems

After the complete HCV genome was determined by Choo et al. (1991), several HCV isolates from different parts of the world were obtained and sequenced. Comparison of the published sequences of HCV has led to the identification of several distinct types that may differ from each other by as much as 33% over the whole viral genome (Okamoto et al., 1992). Sequence variability is distributed equally throughout the viral genome, apart from the highly conserved 5` UTR and core regions and the hypervariable envelope (E) region that is the most heterogeneous portion of the genome.

As different investigators developed and used their own classification for HCV strains, a confusing literature developed by Okamoto, Simmonds, Enomato, Choo. However, at the 2nd International Conference of HCV and Related Viruses, a consensus nomenclature system was proposed to be used in future studies of HCV genotypes and subtypes (Simmonds et al., 1994). According to this system, HCV is classified on the basis of the similarity of nucleotide sequence into major genetic groups designated genotypes. HCV genotypes are numbered (arabic numericals) in the order of their discovery. The more closely related HCV strains within types are designated subtypes, which are assigned lowercase letters (in alphabetic order) in the order of their discovery. The complex of genetic variants found within an individual isolate is termed the quasispecies (Table 1.1).

The heterogeneity of HCV within an individual can be attributed to error prone RNA-dependent RNA polymerase (RdRp) of HCV, NS5B. The absence of proof reading activity of NS5B creates *de novo* mutations. Recently, HCV RNA turnover rate is calculated in humans as 4-7 hours and this high turnover rate leads to production of 300 billion HCV RNA molecules per day (Neumann et al., 1998), which contributes significantly to the heterogeneity of HCV. It was hypothesized that the existence of HCV as quasispecies may be the basis of the mechanism for viral persistence. With high numbers of RNA produced per day and NS5B without proofreading activity would lead to production of escape mutants, therefore, HCV establishes chronic infection.

According to this classification, there are 6 main genotype and approximately 70 subtypes and some of the recently identified isolates from Vietnam, Thailand and Indonesia were identified as subtypes of HCV genotype 6.

TABLE 1.1. Terminology commonly used in studies related to HCV genomic heterogeneity

Terminology	Definiton	%Nucleotide similarity[a]
Genotype	Genetic heterogeneity among different HCV isolates	66-69
Subtype	Closely related isolates within each of the major genotypes	77-80
Quasispecies	Complex of genetic variants within individual isolates	91-99

[a] % Nucleotide similarity refers to the nucleotide sequence identities of the full-length sequence of the HCV genome

1-2.4 Importance of HCV genotypes on disease progression and treatment

The role of HCV genotypes in the progression of liver disease is one of the most controversial areas of HCV research. Several studies showed that subtype 1b infections proceed much faster to severe forms of chronic hepatitis, cirrhosis (Watson et al., 1996) and hepatocellular carcinoma (Silini et al., 1996; Zein et al., 1996). However, there are also reports that failed to show the association between subtype 1b infections and faster progression into cirrhosis (Mita et al., 1994) or HCC

(Lee et al., 1996; Yotsuyanagi et al., 1995). Moreover, in the patients with chronic HCV, infection with genotype 1b is reportedly associated with a more severe liver disease and a more aggressive course than is infection with other HCV genotypes (Nousbaum et al., 1995; Pozzato et al., 1995). Similar to these studies (Silini et al., 1995; Zein et al., 1995), HCV genotype 1b was significantly more prevalent among patients with liver cirrhosis and those with decompensated liver disease requiring liver transplantation than among those with chronic active hepatitis C (Zein et al., 1995; Belli et al., 1996) and was associated with earlier recurrence and more severe hepatitis than other genotypes in liver transplant recipients (Pageaux et al., 1997). Although these are indirect evidence, they suggest an association between HCV genotype 1b and the development of these complications. Furthermore, HCV genotype 1b was shown to be present in most of the patients in approximately 60 to 70 % with HCV associated hepatocellular carcinoma (Zein et al., 1996; Reid, 1994). However, some reports refute the associations related with HCV subtype 1b, which was mentioned above (Benvegnu et al., 1997; Brechot, 1997; Naoumov et al., 1997; Yamada et al., 1994). Since the patients infected HCV 1b generally were older than those infected with other genotypes and genotype 1b may have been present before the other genotypes, whether HCV genotype 1b is a marker for severe HCV-associated liver disease remains unclear, because it may have been a reflection of a longer time of infection rather than a more aggressive form of hepatitis C.

Enomoto et al by comparing full length genome sequences of HCV isolates obtained from different Japanese patients, identified a 39 amino acid long region (2209-2248) in carboxyl terminus of N55A, which is associated with sensitivity to interferon and referred as interferon sensitivity determining region (ISDR) (Enomoto et al., 1996). Other groups working with Japanese patients infected with genotype 1b, 2a, or 2b HCV strains confirmed that the IFN-α resistant strains had same sequences with HCV- 1b prototype strains in the ISDR region (Chayama et al., 1997; Kurosaki et al., 1997; Halfon et al., 2000). However, the correlation is substantially weaker or lacking in patients infected with genotype 1a HCV strains or European patients infected with HCV strains of 1b, 2b, or 3a (Casato et al., 1997; Pawlotsky et al., 1998). The reason for these discrepancies are not clear, but differences in the interferon doses of these patients and the lower mutation rate of the ISDR in European HCV-1b patients relative to their Japanese counterparts are likely to be

contributing factors. In summary, ISDR sequences may affect the response of the individual HCV isolates to interferon treatment.

1-2.5 Geographic Distribution of HCV Genotypes

At least six major genotypes of HCV, each comprising multiple subtypes, have been identified worldwide. The geographical distribution of different genotypes and subtypes differs greatly from one region to the other. The reasons of this differential distribution are ill known, but the profile of geographical distribution could reflect the different modes of viral transmission as well as the host immune response variations. For example, HCV 1a subtype, which is seen frequently in North America, could have been transmitted to other regions of the world, especially to Europe, by contaminated blood-derived products (Brechot et al., 1998). In contrast, subtype 1b appears to be dominant in Japan and Southern Europe. In Europe, HCV 1 is the major genotype and there is a south-north gradient for 1a and 1b subtypes, the prevalence of subtype 1b being increasingly higher in southern Europe. The data on HCV subtypes in the Middle Eastern countries is limited. In Egypt, Hepatitis C is an endemic disease that is associated with genotype 4, almost exclusively (Ray et al., 2000). Similarly, genotype 4 is also predominant in the Gaza region, but not in Israel where subtype 1b is predominant (Shemer-Avni et al., 1998). Moreover, genotypes 5 and 6 seem to be confined to South Africa and Hong Kong, respectively. HCV genotype 7, 8, and 9 have been identified only in the Vietnamese patients, and genotypes 10 and 11 were identified in patients from Indonesia.

The HCV genotype distribution of patients living in Turkey is not well known. To our knowledge, there are only two published reports concerning Turkish patients, which indicated a high frequency (75-87 %) of subtype 1b (Abacioglu et al., 1995; Simsek et al., 1996). Regarding the distribution of HCV genotypes in neighbors of Turkey, subtype 4a and 1b are predominant in Syria and there is no data for Iraq and Iran (Abdulkarim et al., 1998). On the other hand, in countries located on the northern frontiers of Turkey as well as in Greece, subtype 1b appears to be the dominant form (Viazov et al., 1997; Andonov et al., 1996).

The geographical distribution and diversity of HCV genotypes may provide clues about the historical origin of HCV. The presence of numerous subtypes of each HCV genotype in some regions of the world, such as Africa and Southern Asia, may suggest that HCV has been endemic for a long time. Conversely, the limited diversity of subtypes observed in United States and Europe could be related to the recent introduction of these viruses from areas of endemic infection.

1-2.6 Methods for HCV Genotyping

Molecular genotyping

Because differences in geographical distribution, disease outcome, and response to therapy among HCV genotypes have been suggested, reliable methods for determining the HCV genotype may become an important clinical test. In theory, the most accurate method for HCV genotyping is the sequencing of whole genome and phylogenetic tree construction. However, in practice this method is not appropriate and feasible in genotyping large numbers of samples as full-length genome sequencing is too laborious and expensive. Hence, subgenomic regions representative of whole genome have been investigated. To this date, phylogenetic analysis E1, NS5B, and 5'UTR sequences correlated with whole genome based phylogenetic analysis suggesting the equivalence of sequence relationships between HCV genotypes in different regions of genome. This finding formed the basis of the proposal by Simmonds et al. that a new HCV genotype should be assigned only if phylogenetic analysis of at least two genomic regions showed a distinct phylogenetic branch (Simmonds et al., 1994). 5'UTR region is the most conserved region of HCV genome. Therefore, it may have identical sequence in different subtypes but contains variations aiding determination of 6 genotypes and some subtypes.

Amplification of NS5B, core, E1 regions, followed by sequence comparison and phylogenetic tree construction for confirmation, is currently considered "gold-standard" for the assignment of HCV genotypes. However, there are limitations of this method. Amplification of some genomic regions may not be efficient. In this case, use of different sets of primers would be needed, which may lead to the selection of certain genotypes or quasispecies. In a large-scale genotyping study,

mixed infections would not be identified. In these studies, the cost of the study and labor-intensive nature of this technique also limits to its usage.

Other methods that depend mainly on the amplification of HCV RNA followed by either reamplification with type specific primers or hybridization with type-specific probes or by digestion of PCR products with restriction endonucleases that recognize genotype-specific cleavage site. HCV genotyping by using type-specific primers was first introduced by Okamoto et al. and used primer specific for the core region (Okamoto et al., 1992a). This method lacked sensitivity and specificity and was only able to detect subtypes 1a, 1b, 1c, 2a, 2b, and 3a. New variations of this method from both core and NS5B region have been developed to increase genotyping of more types or subtypes (Ohno et al., 1997). A commercial kit (InnoLipa) for HCV genotyping has been introduced in Europe and is based on hybridization of 5`UTR amplification products with genotype specific probes. Although initial version of InnoLipa had lower sensitivity, the newer version is capable of discriminating among HCV subtypes 1a, 1b, 2a to 2c, 3a to 3c, 4a to 4h, 5a, and 6a.

Restriction Fragment Length Polymorphism analysis of HCV genome for genotyping was initially reported by Nokao et al. (1991). Subtype or type-specific restriction enzyme recognition sites present in amplified PCR products are utilized to cut PCR fragments with different restriction endonucleases. Electrophoresis profiles of these are used to identify the genotype of the sequence. In the RFLP analysis, 5'UTR or NS5B region has been used widely with variations.

Although all these methods are able to identify correctly the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating among subtypes. Moreover, all of these PCR-based methods have the shortcomings and advantages of PCR. They are expensive and time-consuming and require specialized facilities to ensure accurate results and prevent contamination. The advantages of PCR-based methods include reliability if performed accurately and the ability to obtain information relevant to the molecular pathogenesis of HCV.

Serological genotyping

Serological genotyping has several advantages that make it suitable for large epidemiological studies, especially. These advantages include the low risk for contamination and the simplicity of the assay. However, it seems to lack specificity and sensitivity, which limits its usefulness.

Two commercially available serological genotyping assays have been introduced over the past 3 to 4 years. The RIBA SIA was introduced by Chiron Corp. and contained five different serotype-specific peptide sequences taken from the NS4 region and two taken from the core region of the HCV genomes for genotype 1, 2, and 3. The second serological genotyping assay is the Murex HCV serotyping enzyme immune assay, which is based on the detection of genotype-specific antibodies, directed to epitopes encoded by the NS4 region of the genomes for genotypes 1 through 6.

1-2.7 Role of Genomic Heterogeneity in HCV Persistence and Vaccine Development

With the rarity of severe acute or fulminant HCV infections, the significance of this infection in humans is its tendency to become persistent and to induce chronic liver disease. The mechanisms whereby HCV circumvents the immune response, persists and causes chronic inflammatory liver disease are currently undefined.

One hypothetical explanation of HCV persistence, sequence variation due to the quasispecies nature and the high mutation rate of HCV, has often been discussed. Amino acid changes in immunodominant epitopes may permit HCV to escape from the antiviral immune response. The most convincing evidence of this phenomenon is the lack of the immune protection and the infectibility of chimpanzees rechallenged with the same HCV inoculum (Prince et al., 1992). In addition to the lack of protection by the humoral immune response, there is also evidence that the cellular immune response may be subverted during HCV infection, since subsequent experiments Weiner et al., 1995 have described the emergence of an HCV mutant that was able to escape the HCV-specific CTL response in an infected chimpanzee.

Possible targets for HCV-specific CTL recognition within the conserved core protein and additional epitopes in the more highly variable region E2 protein were also identified (Koziel et al., 1993). In a chronically infected chimpanzee, CTLs obtained from the liver were initially able to recognize an epitope in the NS3 protein. Over a period of years, a new strain of the virus emerged with a mutation in the CTL epitope that was no longer recognized by the CTLs isolated earlier. Although direct evidence for the presence of CTL escape mutants in human HCV infection is lacking, it has been shown that single-amino-acid changes in CTL epitopes result in failure of recognition by HCV-specific CTLs (Koziel et al., 1998). These single-amino-acid changes are found in natural isolates of HCV, hence the need to address the problem of type specificity of immune responses.

1-2.8 Molecular mechanisms of HCV related hepatocarcinogenesis

Role of Hepatitis C virus proteins in the modulation of proliferation

Core protein of HCV has been shown to play various roles in the regulation of cell proliferation including activation of the Ras/Raf kinase cascade, regulation of p53 function, modulation of apoptosis, oncogenic functions in transgenic mice and certain cell lines, and transactivation or transexpression of certain cellular genes as well as HBV and HIV genes. Recently, the envelope protein of E2 was shown to suppress RNA-activated protein kinase (PKR) function, which is important to disrupt viral gene expression, by acting as a decoy of elF2-α. Also, NS3 protein of HCV interacts with p53 and has the ability to transform NIH3T3 cells. Finally, NS5A protein interacts with SNARE-like protein, which may be important for membrane fusion (Shimotohno K. 2000).

Anti-apoptotic function of HCV core protein

Core protein of HCV plays a role in the suppression of caspase activation induced by anti-Fas and TNF-α in some of the cell lines, and that the suppression was likely to be achieved upstream of caspase-8 in the caspase cascade. It also binds to the death domain of tumor necrosis factor receptor 1 (TNFR1) and the cytoplasmic

tail of lymphotoxin-β receptor, implying that it may be involved in anti-apoptotic signaling pathways (Zhu et al., 1998). Finally, hepatitis C virus core protein activates the cellular transcriptional factor, NF-κB and it may be another mechanism to suppress apoptotic mechanisms (Tai et al., 2000).

Possible roles of HCV proteins in liver dysfunction

When HCV-specific T cells migrate into hepatocytes and recognize the viral antigen via T-cell receptor, they become activated and express Fas ligand that can transduce the apoptotic death signal to Fas-bearing hepatocytes. The cells in which NF-κB is activated by core protein may escape from Fas-mediated apoptosis and contribute to virus replication and release of virus particles. Persistent infection with HCV may be explained by such a mechanism, in addition to insufficient activation of CTL to clear HCV-infected cells. Core protein also has the potential to activate the MAK kinase cascade, which may have a mitogenic effect. The liver undergoes persistent regeneration following hepatic injury and growth factors stimulate this liver regeneration in hepatitis. It is possible that HCV core protein, in regenerating hepatocytes, enhances growth stimuli and repeated hepatocyte proliferation may cause disorder of the gene in the hepatocytes, thus causing hepatocellular carcinoma.

In conclusion several HCV proteins have been shown to interfere with the function of cellular proteins involved in malignant transformation of hepatocytes. However, currently there is no experimental model for HCV infection of hepatocytes, in vitro or in vivo. Therefore, it is unknown whether such virus-cell protein interactions also occur during HCV infection. This is why, it is not possible at present time to classify HCV as an oncogenic virus. It is also important to pay attention to the fact that molecular and cellular events involved in the initiations of human HCC (either viral or nonviral) are not known at all. Although we know several genes that are mutated in HCC, we do not know whether and how they contribute to the initiation of hepatic neoplasia.

1-3 LIVER CANCER STEM CELL

One of the most debated issues in HCC, is its origin. Dedifferentiation of mature hepatocytes was proposed by some authors as a main cause of HCC, although others believe that HCC results from incomplete differentiation of hepatic stem cells. We will first describe “stem cell and cancer stem cell” concepts.

1-3.1 Stem Cell, Cancer Stem Cells

Potten and Loeffler define stem cells as undifferentiated cells capable of (a) proliferation, (b) self-maintenance, (c) the production of a large number of differentiated, functional progeny, (d) regenerating the tissue after injury, and (e) flexibility in the use of these options, but make a distinction between actual and potential stem cells- the latter being cells possessing, but not expressing, all these capabilities (Potten and Loeffler, 1990).

Recently, it was suggested that tumours might contain “cancer stem cells”- rare cells with indefinite proliferative potential that drive the formation and growth of tumours. For example; it was shown that lymphoblastic, acute and chronic myeloid leukemias originate from haematopoietic stem cells (HSCs) (Bonnet and Dick, 1997; Mauro and Druker, 2001; George, 2001). Moreover, there are many similarities in the mechanisms that regulate self-renewal of HSCs and cancer cells. For instance, the prevention of apoptosis by enforced expression of the oncogene bcl-2 results in increased numbers of HSCs in vivo, suggesting that cell death has a role in regulating the homeostasis of HSCs (Domen and Weissman, 2000). Some other pathways associated with oncogenesis such as the Notch, Sonic hedgehog (Shh) and Wnt signaling pathways, may also regulate stem cell self renewal (Taipale and Beachy, 2001). Furthermore, it is well documented that many types of tumours contain cancer cells with heterogenous phenotypes reflecting aspects of the differentiation that normally occurs in the tissues from which the tumours arise. The variable expression of normal differentiation markers by cancer cells in tumour suggests that some of the heterogeneity in tumours arises as a result of the anomalous differentiation of tumour cells. Examples of this include the variable expression of

myeloid markers in chronic myeloid leukaemia, the variable expression of neuronal markers within peripherial neurectodermal tumours, and the variable expression of milk proteins or the oestrogen receptor within breast cancer. In other words, both normal stem cells and tumorigenic cells give rise to phenotypically heterogeneous cells that exhibit various degrees of differentiation. Tumorigenic cells can be thought of as cancer stem cells that undergo an aberrant and poorly regulated process of organogenesis analogous to what normal stem cell do (Sell and Pierce, 1994).

1-3.2 Origin of Hepatocellular carcinoma

As stated earlier, one of the most debated issues concerning HCC is its cellular origin. Various rodent models of chemically induced liver tumors are currently used to clarify this issue.

According to the conventional theory, HCC results from "**dedifferentiation**" of mature hepatocytes into a less differentiated state without the involment of other cell types (Aterman, 1992). According to this model, cancer results from a multistep process. Chemical carcinogens act by modifying DNA of mature hepatocytes to form DNA adducts during the first step of initiation. In the second step of carcinogenesis, often referred to as promotion, other noncarcinogenic compounds increase hepatocarcinogenic effects, most of them being able to induce cellular proliferation. Arguments of this hypothesis are the findings that exposure of rats to carcinogens results in sequential liver alterations as follows; (a) focal proliferation of altered hepatocytes, (b) appearance of preneoplastic nodules, and (c) rise of cancer from persistent nodules (Farber and Sarma, 1987).

A "**stem cell origin**" has been proposed as an alternative mechanism (Sell and Dunsford, 1989, Sell, 2001). According to this theory, HCC originates from "oval" cells displaying stem cell-like properties that are detected in the liver prior to the chemical induction of HCC in rats. First, Solt and Faber showed that administration of 2-acetylaminofluorene (2-AAF) together with partial hepatectomy results in suppression of hepatocyte proliferation and stimulation of oval cell proliferation (Solt et al., 1977) Later, oval cells have been observed in various

models of rodent experimental carcinogenesis, including exposure to DIPIN (Factor and Radaeva, 1993) and choline-deficient, ethionine-supplemented (CDE) diet (Shinozuka et al., 1978). Oval cells are small, oval shaped epithelial cells identified in the liver during normal embryonic development (Tian et al., 1997) and in some of the adult liver pathologies, such as hemochromatosis and alcohol liver disease (Lowes et al., 1999). Moreover they arise in the periportal region of the liver probably derived from cells of Hering canal, bile ducts, intraportal or periportal ductules, or from periductular cells (Paku et al., 2001; Sarraf et al., 1994; Sell and Salman, 1984; Vessey and Hall, 2001)

Recently, it has also been suggested that oval cells may derive from bone marrow cells (Peterson et al., 1999). Evidence suggests that the liver oval cell is at least bipotential, capable of differentiating into mature hepatocytes (Evarts et al., 1989, Coleman et al., 1993) or cholangiocytes (Germain et al., 1988a; Germain et al., 1988b; Lenzi et al., 1992) under different experimental regimens. Progenitor cells in the liver are identified by their ability to express markers characteristic of immature hepatocytes. Such markers include albumin, transferin, alpha fetoprotein (AFP), and cytokeratin19 (CK19) all of which are expressed by the first primitive liver stem cells as well as oval cells (Cascio and Zaret, 1991).

The stem cell theory is not readily applicable to human HCC that occurs mostly as a virally induced disease. Consequently, the “dedifferentiation” is still considered as the major mechanism of HCC development in man (Kojiro, 2002). Although “oval” cell-like structures have been described in association with different liver diseases (Hsia et al., 1994; Roskams et al., 1998; Lowes et al., 1999; Theise et al., 1999; Libbrecht et al., 2001b), these descriptive observations do not provide convincing evidence for a direct contribution of liver stem cells to HCC. Moreover, in the absence of isolated human liver stem/progenitor cells, their occurrence remains hypothetical, although bone morrow-derived putative stem cells appear to generate hepatocytes, and bile duct cells (Alison et al., 2000; Theise et al., 2000).

1-3.3 Stem Cells during liver embryonic development

Hepatogenesis can be considered in distinct developmental stages: competence, commitment, differentiation and morphogenesis. Although these stages reflect key aspects of hepatic development it is worth bearing in mind that they are closely related, interdependent, and often overlapping processes.

From Endoderm to Liver; Determination, Commitment, and Differentiation

This process begins on embryonal day (ED) 8.5 in the mouse with proliferation of undifferentiated endodermal cells of the ventral foregut and their migration into the septum transversum, where they come into contact with mesenchymal cells (Figure 1.4).

At this point, they are already specified to enter the liver lineage (determination) and form the hepatic diverticulum (Zaret, 2001). Mouse foregut-derived cells begin to express AFP at ED9.0 and then albumin at ~ED9.5, followed by placental alkaline phosphatase and intermediate filament proteins, cytokeratins 14, 8 and 18 (Cascio and Zaret, 1991). Fibroblast growth factors (FGFs), particularly FGFs 1 and 2, can cause cardiac mesenchyme in inducing albumin gene expression (Jung et al., 1999). The morphology of the cells then changes to that of the hepatoblast (an early progenitor or stem/progenitor cell), expressing δ-glutamyl transpeptidase, α-antitrypsin, glutathione-S-transferase and fetal isoforms of aldolase, lactic dehyrogenase and pyruvate kinase in addition to AFP and albumin (Fausto, 1990; Brill et al., 1995; Thorgeirsson, 1996). The cells proliferate rapidly between ED12 and ED16 and subsequently diverge along two distinct lineages, the hepatocyte and cholangiocyte, beginning just prior to ED16. This time period has been referred to as a differentiation window in hepatic development (Marceau et al., 1992). In cell culture prior to ED16, fetal liver epithelial cells (early progenitor cells) appear to have the ability to change their phenotypic gene expression pattern from hepatocytic to ductular or reverse, depending on the environmental (cell culture) conditions (Marceau et al., 1992; Blouin et al., 1995). However, after ED16, the cells are committed to progress along one or the other of these lineages and no longer

retain their bipotential properties, although they continue to proliferate (i.e. "committed" or late progenitor cells). However, the irreversible nature of these changes no longer holds, as even phenotypically fully mature cells isolated from the adult liver appear to switch their phenotype in culture between hepatocytic and ductal, depending on experimental conditions (Michalopoulos et al., 2001).

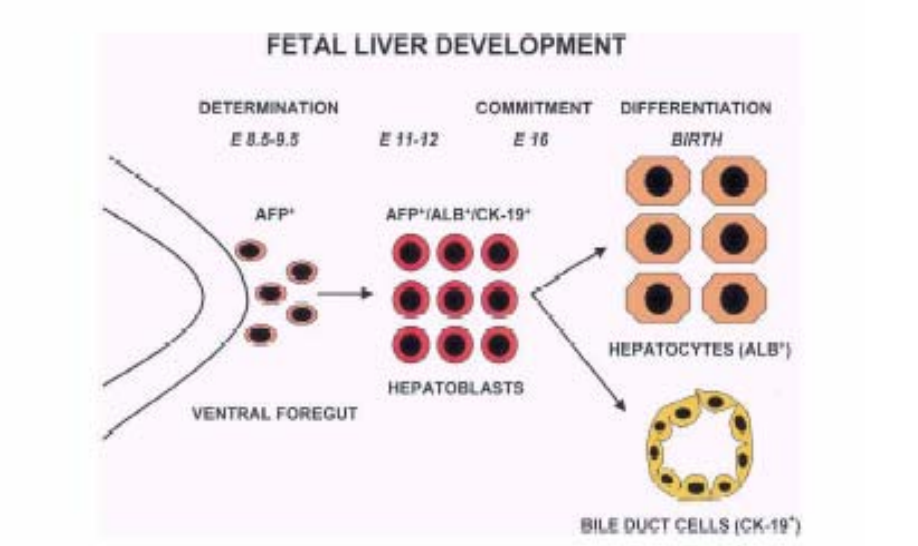

Figure 1.4 Schematic diagram of fetal liver development in the mouse (Shafritz and Dabeva, 2002)

After commitment, hepatocyte progenitor cells express antigens HBD-1, H-2, transferrin receptor, c-CAM and HES6 in addition to the proteins mentioned above and biliary epithelial cell progenitors also express CK7 and 19, OC-2, OV-6 and BD-1 (Shiojiri et al., 1991; Fausto, 1990; Thorgeirsson, 1996; Marceau et al., 1992; Blouin et al., 1995). As organogenesis proceeds, intrahepatic bile ducts are formed in the vicinity of large portal vein branches, beginning on ED17. This is evidenced by formation of ductular structures containing CK19 positive epithelial cells that have the appearance of "strings of pearls". The basic lobular structure is then formed, although the hepatic parenchymal plates or cords do not become fully mature until several weeks after birth.

Transcriptional Regulation of Liver Development

Although the different steps involved in liver development are well described from the hepatic specification of ventral endoderm to the generation of hepatoblasts and the differentiation of mature hepatocytes, the control mechanisms underlying these steps are still poorly understood. Recent experiments with primary tissue explants of foregut endoderm have suggested the influence of positive and negative extracellular signals, respectively provided by cardiac and dorsal mesoderm, during early hepatic specification (Gualdi et al., 1996). Gene disruption in mice has demonstrated those factors as diverse as c-jun (Hilberg et al., 1993), RelA (Beg et al., 1995), and both hepatocyte growth factor/scatter factor (HGF/SF; (Schmidt et al., 1995) and its receptor Met (Bladt et al., 1995). It is generally accepted that particular combinations of members of four families of liver-enriched transcription factors (LEFT), including hepatocyte nuclear factor (HNF)3, HNF4, HNF1, and CCAAT/enhancer binding protein (C/EBP) control critical steps in hepatic differentiation, and that a hierarchy of expression of these transcription factors exists (Cereghini, 1996)(Figure 1.4)

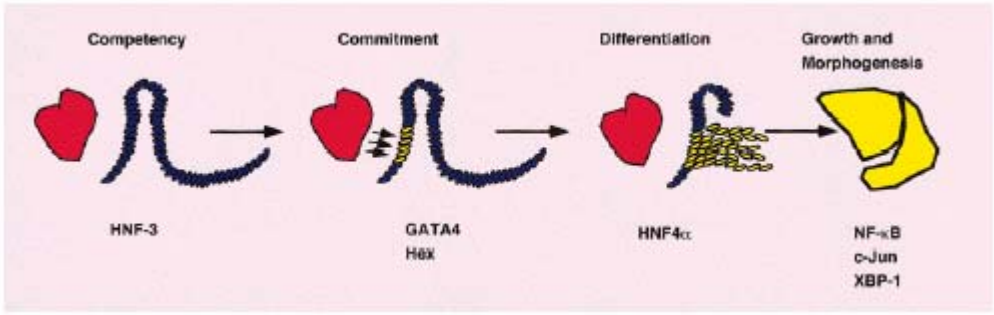

Figure 1.5 Key stages in liver development (Duncan, 2000)

The HNF3 family of transcription factors has important roles in determining hepatic competency of endoderm. The initial expression of *hnf-3* occurs before the onset of hepatic development with *hnf-3β* preceding to that of *hnf-3α* in mouse. HNF3 proteins share a highly conserved novel DNA binding domain and, along with the *Drosophila* forkhead protein, are the founding members of the winged helix family of transcription factors. HNF3 remodels the configuration of chromatin around transcriptional regulatory regions containing *hnf-3* binding sites. It can interact with its DNA binding element within the context of chromatin. Upon

binding, HNF3 acts to disrupt linker histone binding and repositions nucleosomes around the regulatory region. This enhances the accessibility of other transcription factors such as GATA4 which acts cooperatively with HNF3 to regulate gene expression (Cirillo and Zaret, 1999) and Hex (hematopoietically expressed homeobox) that is also essential for the earliest stages of liver development (Martinez Barbera et al., 2000) then commitment of endoderm to a hepatic fate has started by the expression of AFP and Albumin.

Once the pre-hepatic cells become committed to the liver-cell fate, the process of differentiation starts. HNF4α, which is required for complete differentiation of hepatocyte, binds DNA strictly as a homodimer. Expression of HNF4α closely correlates with the expression of the transcription factor HNF1α (Bulla, 1997). HNF4α and 1α combination has a key role in the expression of diverse sets of genes that control multiple aspects of metabolism, detoxification, and serum factor secretion in normal liver physiology (Spath and Weiss, 1997).

1-3.4 Identification of Stem-Like Cells in Animal and Mouse Models

In response to the loss in the hepatic mass after partial hepatosectomy, hepatocytes readily proliferate to reconstitute the liver. However, when proliferation of hepatocytes was inhibited, a novel cell population proliferates in the liver; these cells are collectively termed “oval cells”. It is suggested that the oval cell compartment contains putative hepatic stem cells and/or their partially committed progeny (Sell and Pierce, 1994; Fausto, 1994).

When the hepatic cells were labeled in vivo using retroviral vectors to follow their fate during early stages of chemical induced hepatocarcinogenesis in rats, it has been demonstrated that preneoplastic foci can originate from mature hepatocytes as similar as what dedifferentiation theory tells about the origin of HCC. However, workers still can not exclude the possibility that oval cells may contribute to the generation of foci either directly or by differentiation of oval cells to hepatocytes that will subsequently dedifferentiate and give rise to foci (Gournay et al., 2002)

Nonparenchymal liver epithelial (oval) cell lines derived from the livers of adult rats fed by a choline-deficient diet containing ethionine have been used to provide hepatocytic differentiation under different in vitro culture conditions and they demonstrated that cultured oval cells have capacity toward hepatocytic differentiation and suggested that these cells also have the capacity to differentiate along the bile duct lineage (Lazaro et al., 1998). Later, Kubota and Reid (2000) used colony-forming assay and flow cytometry to identify rat hepatoblast, common precursors for hepatocytic and biliary lineages. Fetal epithelial liver cells with a dual hepatocytic biliary phenotype also have been isolated from monkey fetus and immortalized by retrovirus-mediated transfer of SV40 large T antigen to provide unlimited expansion capacity (Allain et al., 2002). In both of the studies, the cells express the liver epithelial cell markers CK8/18, the hepatocyte specific markers albumin and AFP, and the biliary specific markers CK7 and 19 and bipotentiality of gene expression was confirmed by clonal analysis initiated from a single cell. Recently, it was shown that hepatic stem cells isolated from mouse fetus by using flow cytometry and single cell-based assays are pluripotent besides having self-renewal capacity (Suzuki et al., 2002) which means they can differentiate to the cells of other organs of endodermal origin under appropriate microenvironment.

Haruna et al., identified bipotential progenitor cells in the developing human liver and studied the expression of CK14, CK19, HepPar1 and Vimentin on 19 human fetal liver tissues taken from different periods of gestation, 4 to 40 weeks (Haruna et al., 1996). Since in rat experiments, activation of progenitor cells is seen in conditions associated with hepatocyte injury, or inhibited replication, regenerating human liver models are used to identify existence of hepatic stem cells in man. Human putative progenitor cells have been shown in chronic cholestasis, in regenerating human liver after submassive necrosis, hepatocellular adenomas and in several human liver diseases by immunostaining with cell lineage specific markers (Theise et al., 1999; Roskams et al., 1998; Libbrecht et al., 2001a).

CHAPTER 2. AIMS AND STRATEGY OF THE STUDY

This thesis work had two main objectives. Initially, we focused on the genomic characterization of HCV subtype 1b which affects >90 % of hepatitis C patients in Turkey. This was important to study, because subtype 1b appears to be a potent form for hepatocellular carcinogenesis. As a continuation of this project, we aimed to define the cellular phenotype of HCC cells, as a critical step in the overall HCC programme at Bilkent University. Prior to addressing specific questions related to the role of HCV in HCC, it was important to define the nature of HCC cell lines to be used as an experimental model for future studies. To our great surprise, we obtained unexpected results in this second step, so that we had to extend our second aim to a careful investigation of HCC stem cell lines.

Specific Aims I:

Hepatitis C virus is the most important etiologic factor for the occurrence of hepatocellular carcinoma. And there are controversial studies regarding the different role of HCV genotypes on the diagnosis, disease progression and treatment. Therefore, determination of genotypes in selected population would be helpful to design the prevention policy for hepatitis infection accordingly. At the time we started our study, there was only one reported genotyping study in Turkey (Abacioglu et al., 1995) and it showed that the predominant genotype is subtype 1b which is the form of hepatitis C virus having severe end liver disease and resistant to antiviral therapy. First aim of this part of the study was to determine genotypes of HCV in Turkey by using direct sequencing of 5`UTR and NS5B region of genome and second is to sequence full genome of one of the HCV isolates as a predominant form by cloning of polypeptide-encoding region into seven overlapping fragments. Then further characterization of the clone was done by comparing major structural and functional motifs that are important for the

processing of polyprotein, replication and host response of the virus with the other sequences available in database.

Specific Aims II:

It was proposed that mutations affecting genes involved in *wnt*, *hedgehog* and TGF-β signaling pathways contribute to the "mis-specification" of stem cells (Taipale and Beachy, 2001, Reya et al., 2001). In this regard, it is noteworthy that HCC cells display mutations in genes involved in *wnt* and TGF-β signaling pathways. In the second part of study, we aimed to analyze HCC-derived lines for stem cell-like properties. For this, expression analysis was performed for early and late hepatic lineage markers such as HNF1α, 3α, 3β,.. by using semi-quantitative PCR and for the liver stem cell markers such as AFP, CK19 by western blotting in HCC cell lines. Then, self-renewing capability and multi-lineage differentiation potential of candidate HCC cell lines for being stem cell was tested by using *in vitro* colony assay at clonal density in which colonies initiated from a single cell would be stained by ALB/CK19 and ALB/AFP antibodies. Finally, we decided to further analyse the clonolality and *in vitro* modulation of differentiation program of stem cell-like HCC cells.

CHAPTER 3. MATERIAL AND METHODS

3-1. Genotyping of Hepatitis C Virus and Analysis of Full Genome Turkish HCV 1b Isolate

3-1.1 Patients

A total of 79 HCV-positive patients from the Gastroenterology Department of Çukurova University in Turkey were investigated for HCV genotyping. The great majority of these patients lived in South Anatolia and Southeast Anatolia regions at the time of diagnosis. All patients were positive for anti-HCV antibodies, which were determined using a second-generation ELISA test. Among this group of patients, a serum sample from a 59 year old woman suffering from chronic hepatitis C which was collected prior to any treatment for her disease was used for HCV cloning studies.

3-1.2 Viral RNA Extraction from serum and cDNA synthesis

We used a modified and optimized RNA extraction protocol derived from a previously published procedure (Ausubel, 1987). RNA was extracted from 300 µl of serum with the freshly made lysis buffer containing 4M guanidine-HSCN, 0.5% N-laurylsarcosine, and 1% mercaptoethanol in the presence of an RNA carrier (1µg/ml) and 2M sodium acetate pH: 4.0. After extraction with distilled water saturated phenol pH: 8.0 and chloroform/isoamyl alcohol (24:1) the RNA was recovered by isopropanol precipitation and resuspended in 10 µl DEPC–ddH2O. First strand cDNA synthesis was performed using a commercial kit (MBI). Briefly, 10 µl

resuspended RNA was treated in a 20 µl reaction volume with 0.2 µg of random primers, 40 U of M-MuLV Reverse Transcriptase, 20 U of Ribonuclease inhibitor, and 1mM (each) deoxyribonucleotides at 37 °C for 1 hr after brief denaturing at 90 °C.

3-1.3 PCR amplification of 5`UTR and NS5B regions of HCV for genotyping studies

Initially we used sequence information at 5`UTR for genotyping studies. Sequence information derived from NS5B region was used for confirmation studies. A 285 bp fragment from the 5`UTR of the HCV genome was generated with PCR amplification of one fourth of cDNA. The first round of "nested" PCR was performed using outer primers F1 (5'-ATCACTCCCCTGTGAGGAAC-3') and R1 (5'-TGCTCATGGTGCACGGTCTAC-3'), after denaturation at 94°C for 5 min, with 25 cycles, each containing 45s at 94°C, 45 s at 55 °C and 45 s at 72°C, followed by a final extension for 10 min at 72°C. For the second amplification, 8 µl of the first reaction mixture was further amplified with inner primers F2 (5'-GAGGAACTACTGTCTTCACGC-3') and R2 (5'- TCTACGAGACCTCCCG GGGCA-3') under the previous conditions, except the annealing temperature, which became 60°C to generate a fragment of 285 bp. For PCR amplification of NS5B region, two-step PCR with the same primer set was established to generate a 400 bp DNA fragment-covering region 7904 to 8304 (Position of 5` base relative to HCV genomic sequence in Choo et al., 1991) by using NS5B Forward (5'-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3') and NS5B Reverse (5'-GGCGGAATACCTGGTCATAGCCTCCGTGAA-3') primers. PCR amplification was done after denaturation at 94°C for 4 min, with 30 cycles, each containing 40s at 94°C, 40 s at 58 °C and 40 s at 72°C, followed by a final extension for 10 min at 72°C. For the second amplification, 4 µl of the first reaction mixture was further amplified with the same set of primers in a total volume of 50 µl, using Taq *Polymerase* (MBI). Specific PCR amplification of correct sized DNA fragments was confirmed by agarose gel electrophoresis and appropriate amount of PCR products (usually 100 ng) were subjected to automated nucleic acid sequencing using the same

sets of primers and cycle sequencing kits from Perkin Elmer and Amersham following the manufacturer's instructions. Sequencing reaction products were analyzed on ABI-377 DNA sequencer (Perkin Elmer).

3-1.4 Agarose Gel Electrophoresis

DNA fragments were separated by gel electrophoresis using agarose at concentration of 0.8- 2.0 w/v in 1x TBE buffer. Samples were mixed with loading dye (8% deionized formamide, 1xTBE and 0.1 % bromophenol blue) and the samples were loaded onto the gel and electrophoresis was performed at 100V. When the run was complete, the gel was stained in 1xTBE containing 0.1 % EtBr for 5 minutes and rinsed with ddH_2O. DNA fragments were visualized by using UV transilluminator. The gel photos were captured with BioRad Multi-Analyst Software.

TBE Buffer:

Working Solution	*10X Stock Solution (1lt)*
45 mM Tris-borate	108 g Tris Base
1mM EDTA	55 g Boric Acid
	40 ml 0.5 M EDTA, pH:8.0

3-1.5 HCV genotype identification and phylogenetic sequence analysis

The 5'UTR and NS5B regions of all available genotypes at NCBI Taxonomy Homepage were aligned using MULTIALIN multiple alignment program to reveal the subtype-specific consensus sequences (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalinan.html). Using the PHYLIP program to determine the genotypic distribution of all samples also used the same groups of sequences to construct the phylogenetic tree. In order to compute the distance matrix, we used DNADIST module from PHYLIP software package with Kimura 2-parameter model (http://sdmc.krdl.org.sg:8080/~lxzhang/phylip).

3-1.6 Molecular cloning and characterization of a Turkish HCV 1b isolate (HCV-TR1)

A nearly full length HCV isolate from a 59 years old female Turkish patient was amplified in 7 overlapping PCR fragments Figure 3.1. Primer sets for each fragment, which were designed from the most conserved regions in the desired area, are given Table 3.1. Fragments smaller than 1 kb were amplified with *Pfu* DNA *Polymerase* in the first round in order to decrease the PCR-mediated mutation risk and in the second round *Expand High fidelity PCR System* (Boehringer Mannheim) was used to obtain PCR products with adenine overhangs. Fragments larger than 1 kb were amplified with *Expand High Fidelity System*. PCR products were cloned into pGEM®-T Easy vector (Promega) which is a linearized vector with T overhangs in the multiple cloning site. Selected plasmids containing desired inserts were purified by using QIAfilter plasmid midi kit (Qiagen) and the automated DNA sequencing of the inserts were performed with sequencing primers by ABI Prism 310 Sequencer**.** (Asli Öztan, M.S. Thesis, MBG, Bilkent) For sequencing, M13 and T7 universal primers that are present at the two ends of the cloning site, on the vector sequence and HCV sequence-derived sense and anti-sense primers were used initially. Then internal primers, which were designed and synthesized according to the outer sequencing data obtained from previous reactions, were used to complete full genome sequence data.

The obtained nucleic acid sequence was translated into protein sequence by Translator program (http://www.expasy.ch/tools/dna.html). Inferred amino acid sequence of Turkish HCV-1b isolate were aligned with 48 protein sequences of other HCV isolates in NCBI database (http//:www.ncbi.nlm.nih.gov) by Clustal W program (http://workbench.sdsc.edu) to identify the differences in amino acid sequences, that may be specific to the Turkish isolate. At each position, we compared the amino acid sequences and we defined an amino acid difference if and only if none of the amino acid sequence agrees with that of Turkish HCV-1b isolate.

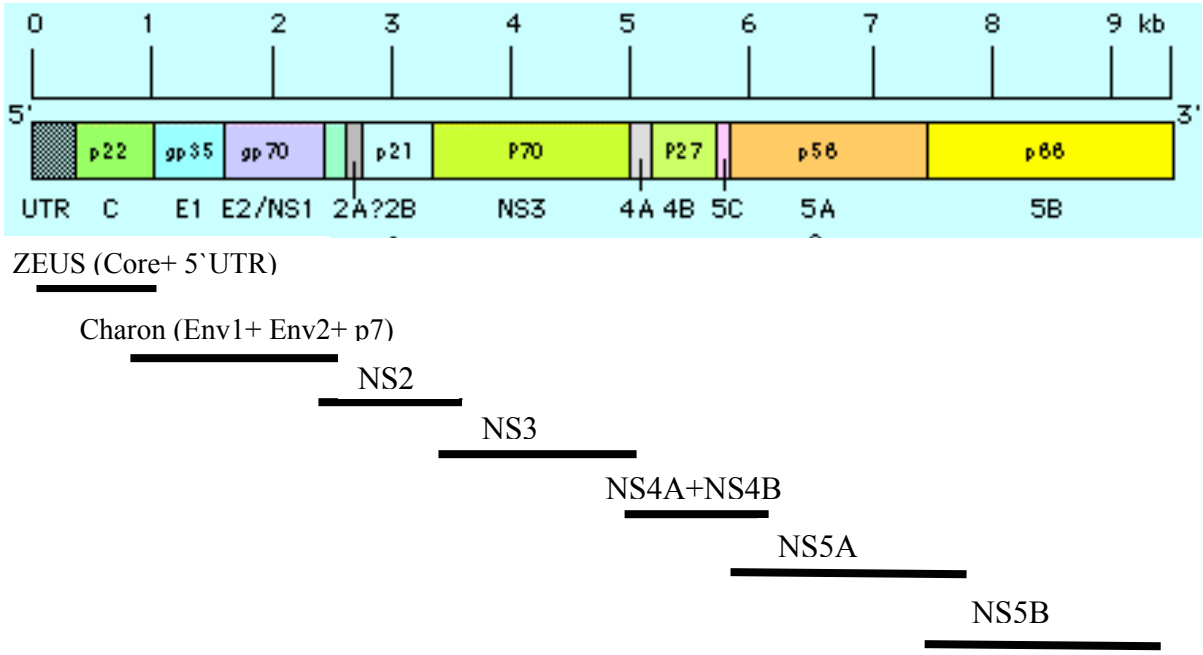

Figure 3.1 : Amplification strategy of HCV genome

Table 3.1 : Sequences of primers used for PCR amplification of overlapping cDNA regions of the genome of HCV isolate HCV-TR1

Primer	Sequence (5`→ 3`)	Position*	Amplified Fragment
F1	ATCACTCCCCTGTGAGGAAC	-306	5`UTR &
CoreR	(G/A)GAGCA(G/A)TCGTTCGTGACAT	964	Core
E1F	CCCGGTTGCTCTTTCTCTATC	850	E1 & E2
E2R	ATGC(A/G)GCCATCTCCCGGTC	2791	& p7
NS2F	T(C/T)CT(G/A)(C/T)TG(G/T)C(G/A)TTACCACC	2738	NS2
NS2R	GT(C/T)TG(C/T)TG(G/A/T)G(A/C)GTAGGCCGT	3449	
NS3F	CCGAAGGGGGA(A/G)GGAGAT	3354	NS3
NS3R	GCACCCA(G/A)GTGCT(A/C/G)GT(G/A)ACGAC	5326	
NS4F	ATGCATGTCGGC(C/T)GACCT	5283	NS4A &
NS4R	TG(G/A)AGCCA(G/A)GTCTTGAAGTC	6329	NS4B
NS5AF	TATGTGCCTGAGAGCGACG	6142	NS5A
NS5AR	(A/G)CG(C/T)AGCAAAGAGTTGCTCA	7695	
NS5BF	AGCGACGGGTC(C/T)TGGTCTAC	7543	NS5B &
NS5BR	CCTGGAGTG(G/T)TT(A/G)GCTCCC	9397	3`UTR

*Nucleotide numbers according to (Choo et al., 1991).

3-2 Identification of Stem-like Cells in Hepatocellular Carcinoma Lines

3-2.1 Maintenance and Subculturing of Cells

All cells (listed in table 3.2) were grown in RPMI-1460 media (Sigma) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 100 μg/ml penicillin/streptomycin at 37^{o}C and 5% CO_2. The cells were routinely subcultured at 2-4 day intervals depending on the growth rate.

Defrosting Cells

One vial of the frozen cell line from the nitrogen tank was taken and immediately put into ice. The vial was placed into 37^{o}C water bath until the external part of the cell solution is thawed (takes approximately 1-2 minutes). The cells were resuspended gently using a pipette and transferred immediately into a 15 ml. sterile tube containing 10 ml cold medium. The cells were centrifuged at 1500 rpm at 4^{o}C for 5 minutes. Supernatant was discarded and the pellet was resuspended in 10 ml 37^{o}C culture medium to be plated into 100 mm dish. Cells were left O/N in culture. The following morning culture medium was refreshed.

Subculturing Of Cells

Culture renewal was done when the cells reached 80-90% confluency. For splitting, the medium was aspirated and the cells were washed with sterile PBS pH: 7.4 for three times. PBS was removed and trypsin was added to the plates. Plates were incubated in the incubator for 3-5 minutes until the cells are detached. Cells were plated in the desired dilution into new plates.

Freezing Cells

The cells to be frozen were grown in a 150 mm cell culture dish to 70-80% confluency at a concentration of $4x10^6$ cells/ml for 2 or 3 days. After washed two times with PBS, the cells are trypsinized and detached from the plate. The suspension of cells are centrifuged at 1500 rpm for 5 minutes and resuspended in 1.5 ml freeze medium containing 20% FCS, 10% DMSO, 80% DMEM unless otherwise indicated at ATCC definition. Then the cells are immediately placed in

-20°C, and kept 5 hours at this temperature. Consequently, the cells are moved to -70°C and kept O/N at this temperature and next day transferred to liquid nitrogen tank.

Table 3.2 : The cell lines used in this thesis

Cell Line	Age(yr)[1]	Tumor[2]	HBV[3]	AFP [4]	Differentiation status
Huh7	57	HCC	-	+	Differentiated
HepG2	15	HCC/HB	-	+	Differentiated
Hep3B	8	HCC	+	+	Differentiated
Hep3B-TR	*	HCC	+	*	Differentiated
Hep40	?	HCC	+	+	Differentiated
PLC/PRF/5	24	HCC	+	+	Differentiated
FOCUS	63	HCC	+	-	Undifferentiated
Mahlavu	?	HCC	-	-	Undifferentiated
SK-Hep-1	52	AC	-	-	Undifferentiated
SNU-449	52	HCC	+	-	Undifferentiated
SNU-475	43	HCC	+	-	Undifferentiated
SNU-387	41	HCC	+	-	Undifferentiated
SNU-423	40	HCC	+	ND	Undifferentiated
SNU-398	42	HCC	+	ND	Undifferentiated
SNU-182	24	HCC	+	ND	Undifferentiated

[1]Age of the patients at the time of tumor surgery; [2]HCC: hepatocellular carcinoma; HB: hepatoblastoma; AC: adenocarcinoma; [3]HBV: presence of integrated hepatitis B virus DNA; [4]production of α-fetoprotein
*; The TGF-β resistance Hep3B clone
ND; not determined

3-2.2 In vitro Colony assays

All cell lines were grown in standard culture medium with 10% FCS, as described previously. For low density clonal culture analyses, cells were plated on six well plates at a density of ~30 cells/cm^2, as described by Suzuki et al (2002), except that cells were grown on uncoated glass coverslips in standard cell culture medium for 7-10 days with a medium change at day 4. The individual clones were then analyzed by dual immunofluorescence staining for ALB/CK19 and ALB/AFP, respectively.

3-2.3 Immunofluorescence staining

Cells were seeded on the autoclaved-sterilized coverslips which were placed into the well of 6-multiwell plates at a proper concentration and grown for a certain time interval in 2 ml growth medium. Cells were washed with PBS and fixed with methanol at –20 °C for 5 min, and blocked against nonspecific binding with PBS containing 10% FCS. Then cells were incubated with polyclonal rabbit anti-human ALB antibody (1: 1000) (ABCAM/ ab1217), in combination with either mouse anti-CK19 (1: 500) (Santa Cruz/ sc-6278) or mouse anti-AFP (gift from Dr.Bellet) (1:1000) antibody diluted in PBS-T (PBS+ 0.1% Tween-20). After washing, cells were incubated with a mixture of FITC-conjugated anti-rabbit (1:100) (ABCAM/ ab6717)) and rhodamine-conjugated anti-mouse (1:100) (ABCAM/ ab6786) antibodies diluted in PBS-T, for 30 minutes. Nuclear DNA was stained with Hoechst 33258 (3 μg/ml; Sigma). Hoechst 33258 was aspirated and destaining was done in double-distilled water for 15 min. Immediately after coverslips were taken out from the well and excess water removed by tissue paper, coverslips were mounted onto slides containing 10 μl 80% glycerol. All steps after the addition of FITC-conjugated secondary antibody, were performed in the dark. Each immunostaining experiment was done using appropriate antibody (no first antibody) and cell line controls, in parallel. Stained cells were examined under fluorescence microscope (ZEISS) and pictures captured in a digital Kodak Camera (DC290, Eastman Kodak Co.), using Adobe Photo Deluxe (Adobe Systems Inc.) software. Pictures were edited using

Adobe Photoshop 5.0 (Adobe Systems Inc.) software. Digital images were magnified when needed during picture editing.

Phosphate-Buffered Saline (PBS)

Working solution (pH:7.4)	x10 Stock Solution (1lt)
137 mM NaCl	80 g NaCl
2.7 mM KCl	2 g KCl
4.3 mM $Na_2HPO_4.7H_2O$	11.5 g $Na_2HPO_4.7H_2O$
11.4 mM KH_2PO_4	2 g KH_2PO_4

3-2.4 Preparation of Huh7-derived stable clones

Huh7-derived clones were obtained by transfection with pCI-Neo plasmid (Promega) or pCI-Neo-S33Y (kindly provided by B.Vogelstein, Bethesda, USA), and selection of antibiotic-resistant colonies, as described (Morel et al., 2000). G-418-resistant colonies were collected 6 weeks later into 6-well plates using cloning cylinders. Huh7-SC1 and Huh7-SC3 were obtained from pCI-Neo and pCI-Neo-S33Y transfections, respectively. Although transfected with a mutant β-catenin plasmid, Huh7-SC3 cells did not overexpress β-catenin, and displayed no increased TCF4 reporter activity, compared to parental Huh7 cells (T. Cagatay & M. Ozturk, unpublished).

3-2.5 External modulation of cell differentiation fate

For western blot studies, Huh7 cells were grown for 3-6 days in three different culture media including standard culture medium, and FCS-free standard medium supplemented with 100 nM $NaSeO_3$ (Sigma), or OPI (0.15 mg/ml oxaloacetate, 0.05 mg/ml pyruvate, 0.0082 mg/ml bovine insulin; Sigma-Catalog No: O-5003). Then total cell lysates were prepared and blotting was done with anti CK19 antibody as described below. Anti CK18 antibody was used in parallel as an equal loading control.

For immunofluorescence studies, cells were grown on Matrigel-coated cover-slips. Glass cover-slips were coated with ten-fold diluted Matrigel (Becton-Dickinson), following instructions recommended by the supplier, and placed in 6-well plates. Huh7 cells were seeded on cover-slips and left overnight in standard culture medium. The following day the medium was changed and cells were grown for 16 days in either OPI or selenium-complemented FCS-free medium in parallel standard culture medium. Culture media were changed every 4 days. Then the cells were analyzed by dual immunofluorescence staining for ALB/CK19 and ALB/AFP, respectively.

3-2.6 Immunoblotting

Crude Total Protein Extraction from Cultured Cells

Cells were grown to 70-80% confluency and washed two times with ice-cold PBS. Cells were scraped in ice-cold PBS and centrifuged at 1500 rpm for 5 minutes at 4^{o}C. Pellet was either frozen in liquid nitrogen or lysed immediately in NP-40 lysis buffer (250 mM NaCl, 1.0% NP-40, 50 mM Tris (pH 8.0), Protease Inhibitors coctail(Sigma)) which is prepared freshly just before use. For lysis, the pellet was resuspended in 4-5 volume of NP-40 lysis buffer and incubated in ice for 30 minutes by stirring the cell within 10 minutes periods. The lysate was centrifuged at 14.000 rpm for 15 minutes at 4^{o}C. Supernatant was taken to a fresh tube and following protein quantitation, aliquoted and stored at –70^{o}C.

Bradford Assay for Protein Quantification

Stock Bovine Serum Albumin (BSA) was prepared as in concentration 1.0 mg/ml and kept at -20°C.

Different dilutions of BSA was prepared as described below:

Tubes nb.s	1	2	3	4	5	6	7	8
BSA stock (µl)	0	2.5	5	7.5	10	12.5	15	20
ddH_2O (µl)	100	97.5	95	92.5	90	87.5	85	80
Bradford working(µl)	900	900	900	900	900	900	900	900

Protein samples were prepared as described below:

Tubes numbers	1	2	3	4	5
Sample(µl)	0	2	2	2	2
DdH_2O(µl)	98	98	98	98	98
Bradford working(µl)	900	900	900	900	900
Lysis buffer	2	-	-	-	-

(1= blank in both tables)

Blanks, BSA standards, test samples were prepared in disposable plastic cuvettes and were allowed to incubate at room temperature in dark for 5 minutes. The absorbance of each sample was measured at 595 nm using a UV-visible spectrophotometer. The absorbance of each BSA standard was ploted linearly as a function of its theoretical concentration and the best fit of the data was determine to a straight line in the form of the equation "y = mx + b" where y = absorbance at 595 nm and x = protein concentration. Finally, this equation was used to calculate the concentration of the protein sample based on the measured absorbance.

Bradford Stock Solution

100 ml 95 % ethanol
200 ml 88% phosphoric acid
350 mg Serva Blue G
Stable indefinitely at RT

Bradford Working Solution

42.5 ml ddH_2O
1.5 ml 95% ethanol
3 ml 88% phosphoric acid
3 ml Bradford stock solution
Filter through Whatman No:1 paper; Store at RT in a brown glass bottle. Usable for several weeks but may need refiltered.

SDS-Polyacrylamide Gel Electrophoresis of Proteins

The glass plates were assembled according to the manufacturer's instructions (EC). The volume of the gel mold was determined according to the information provided by the manufacturer (EC). In an Erlenmeyer flask, the

appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel was prepared. Effective range of separation of SDS-PAGE gels due to different acrylamide concentrations are summarized in Table 3.3 and concentrations of components of the 10% resolving gel, which was used in this thesis, are summarized in Table 3.4.

Table 3.3 : Effective range of separation of SDS-PAGE gels

Acrylamide concentration (%)	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Without delay, the mixture was swirled rapidly and the acrylamide solution was poured into the gap between the glass plates. Sufficient space (the length of the teeth of the comb plus 1 cm.) for the stacking gel was left. The acrylamide solution was overlayed by using a pasteur pipette with isobutanol. The gel was placed in a vertical position at room temperature. After polymerization was complete, the overlay was poured off and the top of the gel was washed several times with deionised water to remove any unpolymerized acrylamide. As much as possible fluid was drained from the top of the gel and then any remaining water was removed with the edge of a paper towel. Stacking gel was prepared in a disposable plastic tube at an appropriate volume and at desired acrylamide concentration. Concentrations of components of the stacking gel at different volumes are summarized in Table 3.5. Without delay, the mixture was swirled rapidly and the stacking gel solution was poured directly onto the surface of the polymerized resolving gel. The comb was immediately inserted into the stacking gel, being careful to avoid trapping air bubbles. The gel was placed in a vertical position at room temperature. While the stacking gel was polymerizing, the samples to be loaded were prepared by heating them to 100^{o}C for 5 minutes in 1X SDS gel-loading buffer to denaturate the proteins. After polymerization was complete, the comb was removed carefully. By using a squirt bottle, the

wells were washed with deionized water to remove any unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. The bubbles that were trapped at the bottom of the gel between the glass plates were removed by a bent hypodermic needle attached to a syringe. 30-200 μg of protein was loaded in a predetermined order into the wells. The electrophoresis apparatus was attached to an electric power supply and the gel was run at 80 volts until the dye front has moved to the resolving gel, after the voltage was increased to 15 V/cm, until the bromophenol blue reaches the bottom of the resolving gel. Then the power supply was turned off. The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. By using a spatula, the plates were pried apart. Orientation of the gel was marked by cutting a corner from the bottom.

Tris-glycine electrophoresis buffer:

25 mM Tris

250 mM glycine (electrophoresis grade)

0.1% SDS

30% mix (Acrylamide and bis-acrylamide solution)

A stock solution composed of 29% (w/v) acrylamide and 1% (w/v) bis-acrylamide, prepared in water. Solution was stored in dark bottles at 4^{o}C.

10% SDS

A 10% (w/v) stock solution was prepared in ddH2O

APS

A small amount of 10% stock solution was prepared freshly in ddH2O

5X gel loading buffer

3.8 ml ddH2O

1.0 ml 0.5 M Tris-HCl

0.8 ml glycerol

1.6 ml 10% SDS

0.4 ml 0.05% BPB fluid

400 μl β-Mercapto ethanol (added freshly)

Table 3.4 Solution of preparing 10% resolving gel for Tris-glycine SDS-PAGE

Solution components	Component Volumes (ml)							
10%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
ddH_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Table 3.5: Solution of preparing 5% stacking gels for Tris-glycine SDS-PAGE

Solution components	Component Volumes (ml)					
5% gel	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml
ddH_2O	0.68	1.4	2.1	2,7	3.4	4.1
30% mix	0.17	0.33	0.50	0.67	0.83	1.0
1.0 M Tris (pH 6.8)	0.13	0,25	0.38	0.50	0.63	0.75
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06
10% APS	0.01	0.02	0.03	0.04	0.05	0.06
TEMED	0.001	0.002	0.003	0.004	0.005	0.006

Semi- Dry Transfer of Proteins from SDS-Polyacrylamide Gels to Solid Supports

As the SDS-polyacrylamide gel was approaching the end of its run, four pieces of Whatman 3MM paper and one piece of transfer membrane (PVDF) was cut to the exact size of the SDS-SDS-polyacrylamide gel by wearing gloves. One corner of the membrane was marked with a soft-lead pencil. The membrane was left in methanol for 1 minutes and then washed with deionized water and soaked

into transfer buffer for 15-20 minutes. Meanwhile the Whatman 3MM papers were soaked into a shallow tray containing a small amount of transfer buffer and kept shaking for 1 minute.

The transfer apparatus was set as follows:

- 2 layers of Whatman 3MM paper that have been soaked in transfer buffer were put onto the plate which will be positively charged (anode). All air bubbles were squeezed.

- The membrane was placed onto the Whatman 3MM papers. (The transfer membrane should be exactly aligned and the air bubbles trapped between it and the Whatman 3MM paper should be squeezed out.)

- The glass plates holding the SDS-polyacrylamide gel were removed from the electrophoresis tank, and the gel was transferred to a tray of deionized water.

- SDS-polyacrylamide gel was placed onto the transfer membrane. Any trapped air bubbles were squeezed out with a gloved hand.

- 2 layers of Whatman 3MM paper were placed to the top of the sandwich (this side will be negatively charged during the transfer (cathode side)).

The upper plate of the apparatus will be the cathode during the transfer. The electrical leads of the apparatus were connected to the power supply and the transfer was carried out at a current of 3.5 mA/cm^2 of the gel for a period of 30-45 minutes. The electric current was turned of at the end of the run time and the transfer apparatus was disassembled from top downward, peeling off each layer in turn. The gel was transferred to a tray containing Coomassie Brilliant Blue and stained in order to check if the transfer is complete or not. The top left-hand corner of the membrane was cut as insurance against obliteration of the pencil mark.

Transfer Buffer:

2.9 g Glycine

5.8 g Tris base

0.37 g SDS

200 ml methanol

Adjust volume to 1 lt with ddH_20

Immunological detection of immobilized proteins (Western Blotting)

The membrane was washed gently with deionized water and neutralized with the blocking buffer containing 3% milk powder in 0.1% Tween 20-PBS solution for 5 minutes. In order to inhibit non-specific binding sites, the membrane was immersed in the blocking solution for one hour. Primary antibody (Table 3.6) was diluted according to instructions in blocking solution and incubated with the membrane at room temperature for one hour or at 4^{o}C O/N on a slowly rotating platform. Afterwards the membrane was washed three times, once for 15 minutes and twice for 5 minutes, with PBS-T. Following the washes, the membrane was incubated with an secondary antibody HRP-conjugated anti-mouse Ig (DAKO) which is diluted as 1:1500 in blocking solution for 1 hour at room temperature and then washed three times with PBS-T. Then the membrane becomes ready for incubation with substrate and development

Table 3.6 The primary antibodies used in this thesis for the western blotting

Company	Origin	Against/ clone name	Clonality	Final concentration
*	Mouse	AFP /AF01	Monoclonal	0.5 µg/ml
*	Mouse	Cytokeratin18/JAR13	Monoclonal	0.5 µg/ml
Santa cruz/	Mouse	Cytokeratin19/ A53-B/A2	Monoclonal	0.2 µg/ml
Transduction Labs.	Mouse	GSK-3b/clone7	Monoclonal	0.1 µg/ml

** both kindly provided by D.Bellet, France*

For detection ECL (**E**nhanced **C**hemi**L**uminescence; Amersham Pharmacia Biotech. Cat. No. RPN 2106) was carried out according to the manufacturer`s instructions.

3-2.7 Expression analysis of a gene by semi-quantitative PCR

3-2.7.1 Extraction of total RNA from tissue culture cells

The isolation of RNA requires pure reagents and care in preparation due to the sensitivity of RNA to chemical breakdown and cleavage by nucleases. Therefore, all the solutions (except Tris) were made either with DEPC-treated water or treated with 0.1% DEPC overnight at 37 °C. The centrifuge tubes were soaked overnight in solution of 0.1% DEPC prior to autoclaving and all glass ware were baked at 180°C overnight.

Exponentially growing monolayer cultures were washed twice with ice-cold PBS, scraped with scraper, pelleted and snap frozen in liquid nitrogen, and stored at -80°C until needed for RNA preparation. Frozen cells quickly thawed and lysed with Tripure. Alternatively cells were lysed directly in 150 mm tissue culture dishes with Tripure reagent. The Tripure (Roche) isolation reagent was used for isolation of total RNA from cultured cells. The Tripure is a monophasic solution of phenol and guanidine thiocyanate, which allows the isolation of total RNA, DNA and protein from the same sample in single-step liquid separation. Tripure reagent RNA isolation protocol was followed as manufacturer's instruction. Total RNA pellets were dissolved in 50 µl of DEPC-treated water. This procedure yields approximately 5-10 µg RNA /10^6 cells. Integrity and quality of total RNAs was checked with gel electrophoresis (as described in section 3.2.7.2 and spectrophotometric quantification was performed.

3-2.7.2 Formaldehyde Containing RNA Gel and RNA Electrophoresis

1% Formaldehyde containing agarose gel is prepared as follows: 11 ml 5X Forrmaldehyde gel running buffer (10 ml of 2M Sodium Acetate, 10.3 gr MOPS and 390 ml DEPC treated distilled water is mixed and pH is adjusted to 7.0 with Sodium Hydroxide (~15 ml 5M NaOH). 5 ml, 0.5 M, pH. 8.0 EDTA is added and the

solution is added up to 500 ml), 35 ml DEPC water and 0.5 gr agarose is added and the sample is heated to boil in a microwave owen. When the solution is cooled to 60-70 degrees, 10 ml Formaldehyde is added and the gel is immediately poured into the casted apparatus.

RNA samples for loading are prepared as follows: 15 µl of RNA loading buffer (50% formamide, 20% Formaldehyde, 15% 5X running buffer, 15% glycerol-dye, premixed and stored at -20 degrees) and 5 µl of RNA sample is mixed, heated at 70 degrees for 5 minutes, chilled on ice. The prepared samples are loaded to the gel and run at 80V for 45 minutes and stained in Et-Br containing water for 5 min and destained overnight in distilled water.

3-2.7.3 First strand cDNA sythesis

First strand cDNA synthesis from total RNA was performed using RevertAid First Strand cDNA synthesis kit from MBI. The RevertAid kit relies on genetically engineered version of Moloney Murine Leukemia Virus reverse transcriptase (RevertAid M-MuLV RT) with low RNase H activity. This allows the synthesis of full-length cDNA from long templates. The first stand reactions were primed with oligo(dT) primer to specifically amplified mRNA population with 3'-poly(A) tails. As the reaction conditions and components of this kit and those of conventional PCR are compatible, first strand synthesized with this system can be used as a template for the PCR.

3.5 µg total RNA was used to synthesize the first stand cDNA following the manufacturer's instruction. After 1:1 dilution of total reaction products in DEPC-treated water, 2 µl of diluted first strand cDNA was used for PCR.

3-2.7.4 Primer design for expression analysis by semi-quantitative PCR

Expression profile analysis of a gene can be performed by semi-quantitative PCR of first strand cDNAs. The primer pairs will be used in such study should be

design carefully. Forward and reverse primer should be positioned on different exons of the gene of interest, that the primer pair should either be able to produce a longer amplicon from genomic DNA or should not be able to amplify from the covered genomic DNA region in a given PCR conditions (critical parameter is extension time) therefore the amplicon, which will be amplified from cDNA, should not be longer than 1500 bp. Primers were used for expression analysis have been designed strictly considering these criteria as in Figure 3.2 and listed in Table 3.7

3-2.7.5 Fidelity and DNA Contamination control in first strand cDNAs

The fidelity and genomic DNA contamination of in first strand cDNAs were checked before making expression analysis. 2µl of diluted first strand cDNA was used for PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a ubiquitously expressed transcript. GAPDH primer pair for this analysis was designed to produce a 151 bp fragment from cDNA and 250 bp fragment from genomic DNA (Table 3.7).

3-2.7.6 PCR Amplification using cDNA

Target DNA sequences between a pair of oligonucleotide primers were amplified by using thermostable DNA Polymerase of *T. aquaticus* (Taq) (MBI). A typical 50 µl PCR reaction contained 2 µl cDNA as DNA template, 25 pmol of each primer, 2 mM of each dNTP (200mM), 1.5 mM $MgCl_2$ (25mM), 1x Taq Pol buffer, and 2 units Taq DNA Polymerase (5u/µl). PCR amplification was done after denaturation at 94 °C for 4 min, with optimized number of cycles, each containing 30 sec at 94 °C, 30 sec at proper Tm of primer and 30 sec at 72 °C, followed by a final extension for 10 min at 72°C by using an automated thermal cycler (Perkin Elmer Gene Amp PCR System 9600). The product was assessed by agarose gel electrophoresis and EtBr staining as described above.

3-2.7.7 Determination of optimal cycle number for semi-quantitative PCR of a gene

Using an equal amount of templates for PCR amplifications of a gene of interest gives comparable results at a specific number of PCR cycles. The number of optimal PCR cycle was determined by an initial study for each gene by performing 35-cycle PCR during which PCR amplicon samples were collected by 2 cycle intervals. Agarose gel analysis of samples from 20^{th}, 23^{rd}, 26^{th}, 29^{th}, 32^{nd}, and 35^{th} cycles of PCR with an equal load defined the minimum number of cycle to visualize the product on agarose gel and the saturation cycle. Agarose gels were analyzed by Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software (BioRad). The determined cycle number was used for amplification of gene of interest.

3-2.7.8 GAPDH Normalization

Equal volume (2µl) of all first strand cDNA samples was used for cold-PCR amplification of GAPDH transcript using the pre-determined optimal cycle number for GAPDH. Then equal volume of each sample was loaded onto agarose gel and intensity of each band was analyzed by Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software. After intensities were determined, intensity of sample with the highest densitometric reading and 2 µl loading volume were used as reference points for normalization of input loading volume of other samples for expression analysis of both GAPDH and gene of interest by cold PCR amplification. Amplification products were analyzed in computer.

3-2.7.9 PCR amplification of selected transcripts using the optimized cDNA

The PCR reactions were carried out as described above. The transcript specific primers are summarized in Table 3.7. Position of primers and size of the amplified fragments can be seen as a schematic representation in Figure 3.2.

Table 3.7 Primers Used In the semi-quantitative RT-PCR analysis of hepatocyte nuclear factors

NAME	SEQUENCE	Tm(°C)
HNF-3β- Forward	5`-CCGTCCGACTGGAGCAGCTACTAT-3`	62
HNF-3β- Reverse	5`-CGTGTAGCTGCGCCTGTAGGTCTT-3	
HNF-3α- Forward	5`-GGAACTGTGAAGATGGAAGG-3`	59
HNF-3α- Reverse	5`-GTGATGAGCGAGATGTACGAGTAG-3`	
HNF-1α- Forward	5`-CGGAGGTGCGTGTCTACAACTGGT-3`	62
HNF-1α- Reverse	5`-CAGGAAGTGAGGCCATGATGAGGT-3`	
HNF-4α- Forward	5`-AGACAAGAGGAACCAGTGCCGCTA-3	62
HNF-4α- Reverse	5`-CCTTCATGGACTCACACACATCTG-3`	
GAPDH- Forward	5'-GGCTGAGAACGGGAAGCTTGTCAT-3'	64
GAPDH- Reverse	5'-CAGCCTTCTCCATGGTGGTGAAGA-3'	

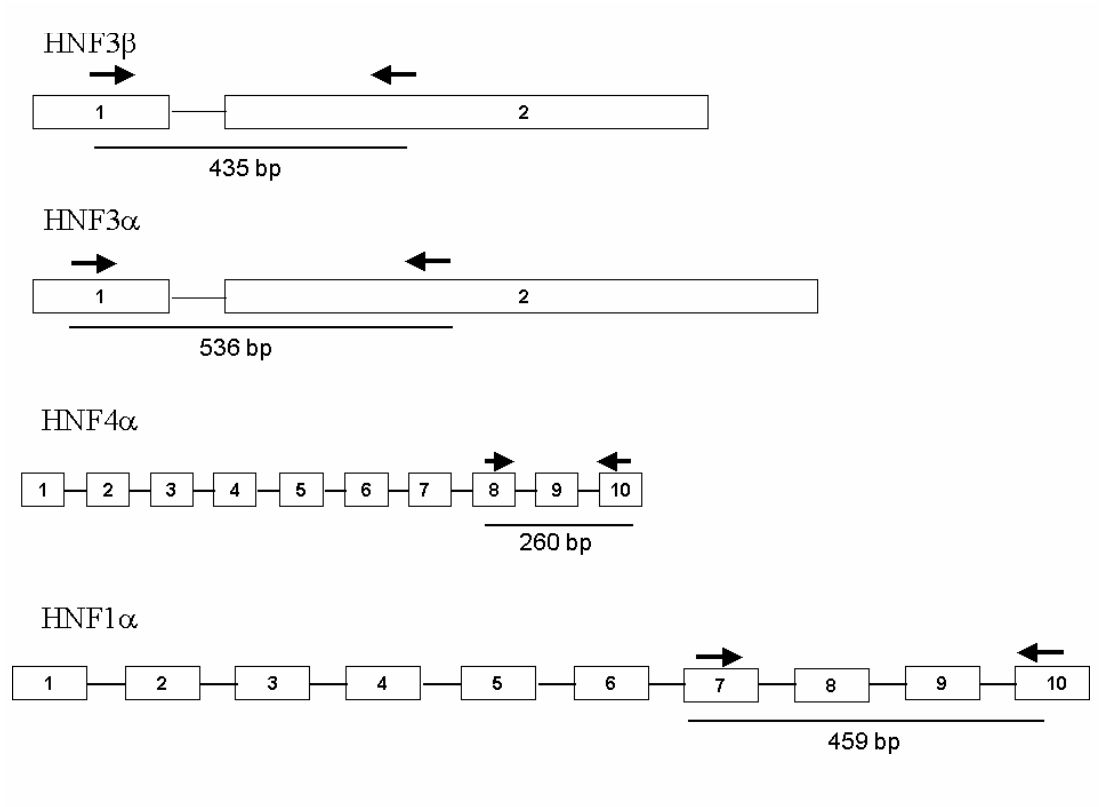

Figure 3.2 Schematic representation of the primers position on the exonic structure of hepatocyte nuclear factors 3β, 3α, 1α and 4α, respectively.

CHAPTER 4. RESULTS AND DISCUSSION

As explained in the "Aims and strategy of the study" section, this Ph D project is composed of two separate, but complementary subjects. The first was related to genomic identification of a Turkish HCV 1b isolate as a predominant viral form in Turkey. As a part of a long term project on HCC at Bilkent University, we aimed to characterize HCC cell lines as potential experimental models of viral hepatocarcinogenesis. This characterization work led us to discover that α-feto protein producing HCC cells act as liver stem cells in vitro.

For these reasons, "Results and Discussion" will be presented under two separate sections.

4-1 Molecular Characterization of a Full Genome Turkish Hepatitis C virus 1b isolate (HCV-TR1): a predominant viral form in Turkey

4-1.1 Hepatitis C virus genotyping in Turkish patients

A total of 79 HCV RNA-positive sera from patients living in southern Turkey were used for genotyping. The amplified 5`UTR region was analyzed by automated DNA sequencing. The sequence data was compared with HCV subtype-specific consensus sequence data as described in 'Material and Methods'. A phylogenetic tree for HCV subtypes in our sample population was constructed in comparison with consensus HCV genotypes. For the analysis, a 100-base region located between nucleotides -172 and -72 of HCV was selected, since this region was variable enough between HCV subtypes. Out of 79, 70 samples displayed unambiguous nucleotide

sequence at this region. The sequence similarities between our sample population ranged from 84 and 100 %. In order to build the pyhlogenetic tree, we included 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b and 4c subtype consensus sequences into our population data, and examined the distances between sequence clusters. Prior to this analysis, we calculated sequence similarities between subtype consensus sequences, which ranged from 75 % (between 2a and 3b) to 99 % (between 1a and 1b). The phylogenetic tree indicated that out of 70 nucleic acid sequences, 3 were clustered with 1a subtype and displayed 0 to 0.0102 evolutionary distances. Sixty-five sequences were grouped together with 1b subtype showing a distance rate of 0 to 0.0219. One sequence was grouped with 2a subtype with a zero evolutionary distance, while another sequence grouped with subtype 4c consensus with zero evolutionary distance. There was no sequence clustered with 2b, 3a, 3b and 4a subtypes (Figure 4.1). This phylogenetic tree analysis showed that 5`UTR data can be used for subtype identification. Accordingly, the remaining 9 samples were genotyped by manual alignment with subtype-specific consensus sequences. To confirm HCV genotyping results obtained by 5`UTR sequence data, 19 randomly selected samples were genotyped using NS5B region sequence data and all samples displayed the expected genotype (data not shown). When combined together, these analyses indicated that, 72 of 79 patients (91%) displayed 1b genotype of HCV, and 5/79 (6 %) had genotype 1a. Thus, all but 2 of 79 HCV genotypes analyzed had genotype 1. The other two samples displayed subtypes 2a and 4c, respectively.

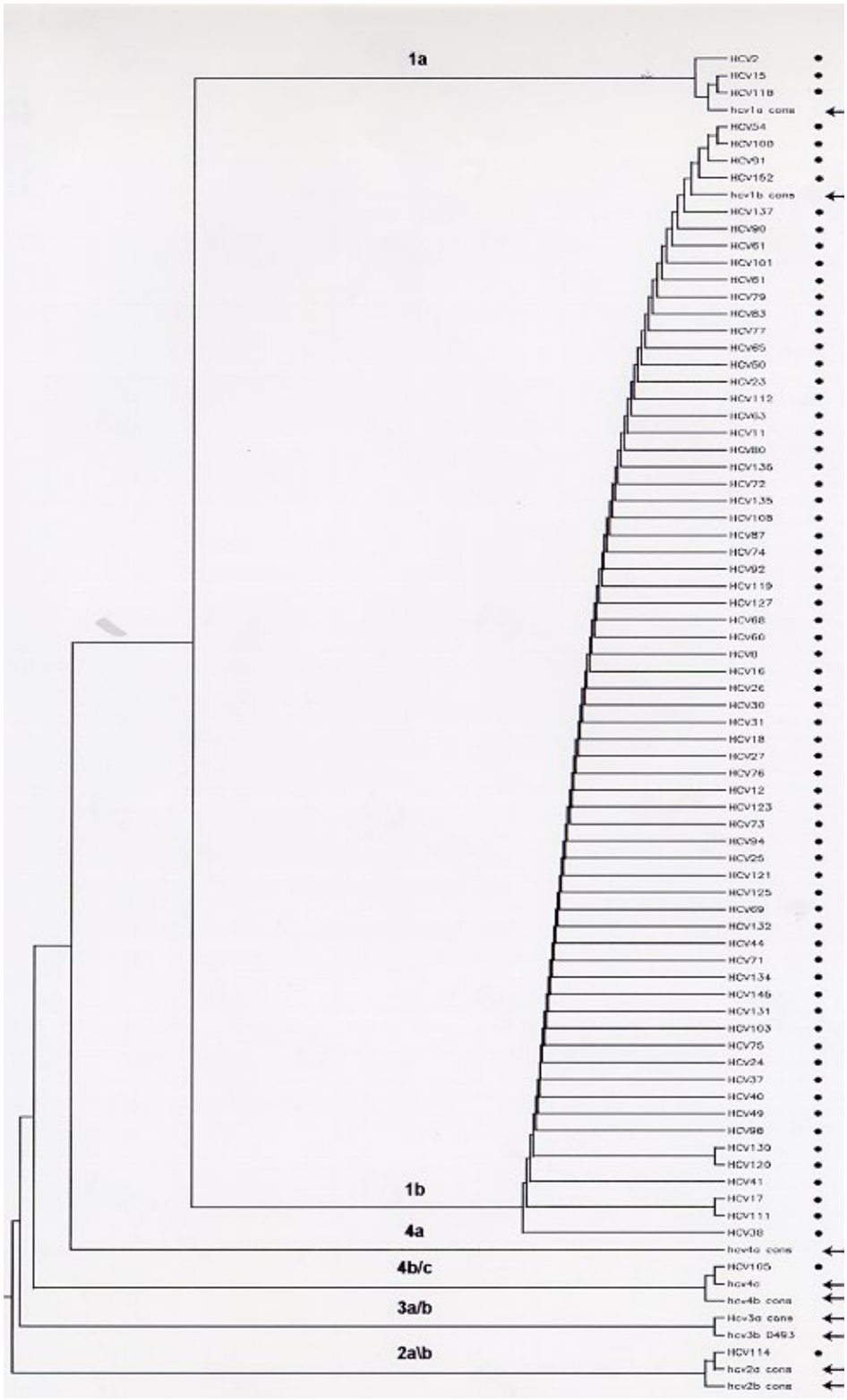

Figure 4.1 Pylogenetic tree of the 5`UTR sequences from 70 isolates (filled circles) of HCV from Turkey. The consensus sequence data for subtypes 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b and 4c (arrows) were included as reference sequences. The genetic distances were calculated with the DNADIST module from PHYLIP software package and were based on a Kimura 2-parameter matrix with a transition to transversion ratio of 2.

4-1.2 Molecular Characteristics of Turkish isolate of HCV 1b (HCV-TR1)

Following our identification of subtype 1b as the main HCV genotype affecting Turkish patients, we decided to obtain full genome sequence information from a Turkish HCV 1b isolate. Using a single serum sample obtained from a patient prior to any treatment, we cloned the major portion of HCV genome into 7 overlapping fragments. When combined together, these clones covered the entire sequence for HCV polyprotein, as well as most of the nucleotides of the 5`UTR and 3`UTR regions. The overlapping regions all contained identical sequences. The Turkish 1b isolate which we named HCV-TR1, comprises 9361 nucleotides, including 306 nucleotides of 5`UTR, a single long open reading frame of 9033 nucleotides, and 22 nucleotides of 3`UTR (data submitted to GenBank nucleotide sequence database and assigned the accession number AF483269) (Figure 4.2). This genomic sequence showed highest homology (91 % identity) to a reported HCV 1b isotype (strain HCV-1b, clone HCV-K1-R2) from Japan, when tested by BLAST using GenBank database. The HCV-TR1 displayed a single open reading frame encoding a 3010-amino acid polyprotein which showed 93 % identity to clone HCV-K1-R2.

LOCUS AF483269 9361 bp RNA linear VRL 21-MAR-2002
DEFINITION Hepatitis C virus type 1b isolate HCV-TR1 from Turkey, complete genome.
ACCESSION AF483269
VERSION AF483269.1 GI:19568932
KEYWORDS .
SOURCE Hepatitis C virus type 1b.
ORGANISM Hepatitis C virus type 1b
Viruses; ssRNA positive-strand viruses, no DNA stage; Flaviviridae; Hepacivirus.
REFERENCE 1 (bases 1 to 9361)
AUTHORS Yildiz,E., Oztan,A., Akkiz,H. and Ozturk,M.
TITLE Molecular chracterization of a full genome Turkish Hepatitis C virus 1b isolate (HCV-TR1): a dominant viral form in Turkey
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 9361)
AUTHORS Ozturk,M.
TITLE Direct Submission
JOURNAL Submitted (13-FEB-2002) Molecular Biology and Genetics, Bilkent University, Bilkent, Ankara 06533, Turkey
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1921 ctcaacaaca cgcggccacc gcaaggcaac tggttcggct gtacatggat gaatagcact
1981 gggttcacca agacgtgcgg gggccccccc tgtaacatcg ggggggccgg caacaacacc
2041 ttgctatgcc ccacggattg cttccggaag caccccgacg ccacatacac caaatgcggt
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2161 ccctgcactg tcaactttac cgtcttcaag gtgaggatgt acgtgggggg cgtggaacac
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```

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3181 tgggcccacg cgggcctgcg ggacctcgcg gtggcagttg agcccgtcat cttctctgac
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3721 gtcattccgg tgcgccggcg gggcgacagc agggggagcc tgctctctcc caggcccatc
3781 tcctatttaa agggttcttc gggtggacca ctgctctgcc cctcggggca cgctgtgggc
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5641 tccactctgc ctgggaaccc cgcaatagca tcactgatgg cattcacagc ctctatcact
5701 agcccgctca ccactcaaca caccctcctg tttaatatct tgggggggatg ggtagccgcc
5761 caactcgcgc cccccagcgc cgcttctgcc ttcgtgggcg ccggcattgc aggtgcggct
5821 gttggcagta taggccttgg gaaagtgctt gtggacatcc tggcgggcta tggagcgggg
5881 gtggcaggcg cgctcgtggc ctttaaggtc atgagtggcg agatgccctc tacagaggat
5941 ctggtcaact tactccctgc tatcctctct cctggtgccc tggtcgtcgg agtcgtgtgc

6001 gcagcaatac tgcggcggca tgtgggccca ggagaggggg cggtacaatg gatgaaccgg
6061 ctgatagcgt tcgcttcacg gggtaaccac gtttccccca cgcactacgt gcctgagagc
6121 gacgctgcag cgcgtgtcac tcagatcctc tctagcctta ccatcactca gctgctgaag
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7561 tgctcgatgt cctacacatg gacaggcgcc ctgatcacgc catgcgccgc ggaggagagc
7621 aagctgccca tcaatgcgct gagcaactct ttgctgcgtc accataacat ggtgtacgcc
7681 acaacatccc gcagcgcaag tcagcggcag aagaaggtca cctttgacag actgcaggtc
7741 ctggatgacc attaccggga cgtgcttaag gagatgaagg cggaggcgtc cacagttaag
7801 gctaaacttc tatccgtgga agaagcctgc aagctgacgc ccccacactc ggccaagtct
7861 aaatttggct atggggcaaa ggacgtccgg aacctatcca gcaaggccgt taaccacatc
7921 cgctccgtgt gggaggacct gctggaagac actgagacac caatcgagac cgtcatcatg
7981 gcaaaaaatg aggttttctg cgtcaaacca raaatgggag gccgcaagcc agctcgctta
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8101 tccacccttc ctcaggccgt gatgggctcc tcatatggat tccagtactc tcctgcacag
8161 cgggtcgagt tcctggtgaa tgcctggaaa tcaaagaaaa accctatggg tttctcgtat
8221 gacacccgct gttttgactc gacggttact gagaatgaca tccgtgttga ggagtcaatt
8281 tatcaatgtt gtgacttgac ccccgaagcc agacaggcta taaggtcgct cacggagcgg
8341 ctttacatcg ggggccccat gaccaattca aaagggcaga actgcggtta tcgccggtgc
8401 cgcgcgagcg gcgtgctgac gactagctgc ggtaataccc tcacatgtta cttgaaagcc
8461 tctgcggcct gtcgagctgc aaagctccag agctgcacga tgctcgtgaa cggagacgac
8521 ctcgtcgtta tctgtgagag cgcgggaacc caagaggacg cggcgagcct acgagtcttc
8581 acggaggcta tgactaggta ctctgccccc cccggggacc tgccccaacc agaatacgac
8641 ttggagttga taacatcatg ctcctccaat gtgtcagtcg cgcacgatgc atctggcaaa
8701 agagtatact acctcacccg cgaccccacc acccccccttg cacgggctgc gtgggagaca
8761 gctagacaca ctccagttaa ctcctggcta ggcaacatca tcatgtacgc gcccaccata
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9121 acatgtggta gatacctctt caactgggca gtaaggacca agctcaaact cactccaatt
9181 ccggctgcat ctcggttgga cttgtccggc tggtttgttg ctggttacag cgggggagac
9241 atatatcaca gcctgtctcg tgcccgaccc cgctggttta tgttgtgcct cttcctactt

9301 tccgtagggg taggcatcta tctgctcccc aaccgatgaa cggggagcca accactccag
9361 g

Figure 4.2 Complete sequence of HCV-TR1

Proteolytic processing of HCV polypeptide between C-E1, E1-E2, E2-p7 and p7-NS2 is performed by host cell proteases, whereas viral proteases cleave between the nonstructural proteins NS2-NS3, NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B (Hagedorn and Rice, 2000). HCV-TR1 polypeptide displayed no major amino acid change in boundary regions at proteolytic cleavage sites (data on cleavage sites of nonstructural proteins shown in Table 4.1), suggesting that it can be correctly processed into HCV proteins, including core (1-191; 191 aa), E1 (192-383; 192 aa), E2 (384-746; 363 aa), p7 (747-809; 63 aa), NS2 (810-1026; 217 aa), NS3 (1027-1657; 631 aa), NS4A (1658-1711; 54 aa), NS4B (1712-1972; 261 aa), NS5A (1973-2419; 447 aa), NS5B (2420-3010; 591 aa) proteins.

Table 4.1 Comparison of inferred amino acids at proteolytic cleavage sites between HCV-TR1, HCV-J and HCV-BK

Genotype	**NS2/NS3**	**NS3/NS4A**
HCV-TR1	ADSFKGQGWRLL ↓	APITAY MACMSADLEVVT ↓ STWVLV
HCV-J	ADSFGEQGWRLL ↓	APITAY MACMSADLEVVT ↓ STMVLV
HCV-BK	ADSLEGRGLRLL ↓	APITAY MACMSADLEVVT ↓ STMVLV

Genotype	**NS4A/ NS4B**	**N4SB/NS5A**
HCV-TR1	ALYQAFDEMEEC ↓	ASHLPY HQWINEDCSTPC ↓ SGSWLR
HCV-J	VLYQEFDEMEEC ↓	ASHPLY HQWINEDCSTPC ↓ SGSWLK
HCV-BK	LLYQEFDEMEEC ↓	ASHPLY HQWINEDCSTPC ↓ SGSWLR

Genotype	**NS5A/NS5B**
HCV-TR1	SEEASEDVVCC ↓ SMSYTW
HCV-J	SGEAGEDVVCC ↓ SMSYTW
HCV-BK	SEEASEDVVCC ↓ SMSYTW

↓ : denotes cleavage sites. Amino acid residues of HCV-TR1 those are different from that of two other HCV-1b isolates (Kato et al., 1990, Takamizawa et al., 1991) are underlined.

Because of nucleic acid sequence ambiguities, we were not able to determine the amino acid residue in 3 positions. The alignment of this polyprotein sequence with that of other 48 HCV-1b isolates available at the GenBank database revealed that there was overall 36 amino acid substitutions in the Turkish isolate. 20 of these

substitutions occurred at amino acid residues that showed variations among different 1b isolates. In contrast, the remaining 16 amino acid changes of HCV-TR1 occurred at conserved amino acid residues. Out of 16 unique amino acid substitutions, half were conserved, the other half not being conserved (Table 4.2).

Table 4.2 Summary of amino acid differences between the HCV-TR1 Turkish isolate and other characterized HCV-1b genomes

Region	Amino Acid Position	Other HCV 1b isolates[1]	HCV-TR1	Type of Substitution[2]
E2	591	E	D	C
E2	728	D	Y	NC
E2	750	N	I	NC
NS3	1075	N	S	C
NS3	1539	L	V	C
NS3	1628	L	M	C
NS4A	1704	E	A	NC
NS5A	2170	V	T	NC
NS5A	2176	T	I	NC
NS5B	2493	K	E	NC
NS5B	2556	T	V	NC
NS5B	2567	Q	K	C
NS5B	2570	K	M	NC
NS5B	2838	L	I	C
NS5B	2897	S	P	C
NS5B	2996	L	F	C
Core	187	I, T, V	M	
E2	397	F, G, H, I, L, M, Q, R, S, Y	A	
E2	478	D, G, H, N, Q, R, S, V	E	
E2	493	P, Q, R	K	
E2	580	I, T	L	
E2	626	I, L	V	
NS2	828	F, L	I	
NS2	857	L, M	V	
NS2	861	I, V	T	
NS2	949	I, V	L	
NS3	1290	P, S	G	
NS3	1382	I, L	V	
NS3	1636	I, T, V	N	
NS5A	2030	P, Q	S	
NS5A	2079	S, F	T	
NS5A	2302	K, R	E	
NS5B	2554	D, N, Q	E	
NS5B	2617	G, K	A	
NS5B	2665	A, S, V	T	
NS5B	2736	D, N	S	

[1]Data obtained from 48 HCV 1b isolate amino acid sequences available at the GenBank database.

[2]Conserved (C) and non-conserved (NC) amino acid substitutions at residues conserved in all published sequenced were based on PAM250 amino acid scoring matrix (Dayhoff et al., 1978).

4-1.3 Main features of HCV-TR1 polyprotein primary structure

When compared to consensus HCV 1b polypeptide sequence, ten conserved cystein residues (14, 89, 170, 223, 243, 274, 279, 295, 366, 521) of HCV (Okamoto et al., 1992c), five N-glycosylation motifs (NXS) of E1 protein (Dubuisson et al., 2000), the catalytic triad (His_{57}, Asp_{81}, Ser_{139};), AX_4GKS motif involved in ATP binding, as well as Cys_{97}-Cys_{99}-Cys_{145}-His_{149} metal binding site of NS3/NS4A heterodimeric serine protease (De Francesco et al., 1996, Kim et al., 1996, Kim et al., 1997) were conserved. In addition, motifs I (207-GXGKS/T-211), II (290-DECH-293), III (322-TATPP-326), V (410-ATDALMTGFTGDFD-423), VI (460-QRXGRXGR-467) and putative Motif IV (370-SK-371) of NS3 helicase were maintained in HCV-TR1 (De Francesco et al., 1996, Kim et al., 1996, Kwong et al., 2000). The ISDR (Interferon Sensitivity Determining Region) of NS5A (2209-PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN-2248) was also conserved (Enomoto et al., 1996). The HVRI region which is the most prone to mutation in HCV, was also maintained for 25 of 27 residues in HCV-TR1 (Puntoriero et al., 1998). These comparative data indicates that viral proteins encoded by HCV-TR1 share the main structural and functional features with other HCV isolates.

The HCV virus was shown to harbour several Cyotoxic T-Cell (CTL) and T-Helper Cell-specific dominant epitopes which may play a major role in host immunity toward viral infection (Urbani et al., 2001). Table 4.3 shows immunodominant CTL epitopes of HCV, in comparison with corresponding amino acid residues of HCV-TR1. There are 8 known major CTL epitopes of HCV. Among those, one epitope located on NS4B protein (aa 1671-1680), and 2 epitopes located on NS5A protein (aa 1992-2000 and 2145-2154) were fully conserved in HCV-TR1. In contrast, HCV-TR1 displayed amino acid substitutions in the five remaining epitopes located on core, E2, NS3, NS5A and NS5B proteins, respectively. Of particular interest, 6 out of 11 (55%) amino acid residues of an E2 epitope (aa 402-412) were different in HCV-TR1. Similarly, two NS3 epitopes (aa 1073-1081 and 1406-1415, respectively) displayed amino acid changes in three positions (30-33 % difference). In contrast to high rate of mutations in CTL epitopes, the dominant T-

Helper Cell-specific epitope located on NS3 protein (aa 1251—1259; VLVLNPSVA) was conserved in HCV-TR1 (Rehermann and Chisari, 2000)

Table 4.3 Comparisons of immunodominant Cytotoxic T cell epitopes of HCV with corresponding amino acid residues in Turkish isolate HCV-TR1

Viral Protein	*Aminoacid No*	*Epitope*[1]	*HCV-TR1**
Core	178-187	LLALLSCLTV	LLALLSCLT**M**
E2	402-412	SLLAPGAKQNV	SL**F**A**S**G**PT**Q**RI**
NS3	1073-1081	CINGVCWTV	C**VS**G**A**CWTV
	1406-1415	KLVALGINAV	KL**SG**LG**L**NAV
NS4B	1671-1680	VLAALAAYCL	VLAALAAYCL
NS5A	1992-2000	VLSDFKTWL	VLSDFKTWL
	2145-2154	LLREEVSFRV	LLREEVSFRV
	2221-2231	SPDAELIEANL	SPDA**D**LIEANL
NS5B	2594-2602	ALYDVVTKL	ALYDVV**S**TL

[1] From refs. (Rehermann and Chisari, 2000; Urbani et al., 2000).

*Amino acid residues of HCV-TR1 that are different from the known epitope are underlined

4.1.4 Discussion on Turkish HCV 1b viral genome

Hepatocellular carcinoma (HCC) is one of the most frequent carcinomas worldwide, with an estimated 564,000 new cases per year and almost as many deaths in 2000 (Parkin et al., 2001). It has strong association with several etiologic factors such as aflatoxin, alcohol and viral hepatitis. Indeed, both hepatitis C and B viruses contribute to the etiology of about 80% of global HCC.

Hepatitis C virus chronically infects at least 1% of the world`s population and causes end liver disease. It has been postulated that HCC largely develops indirectly as a result of the inflammatory responses due to persistent infection of HCV that lead to hepatocyte destruction, regeneration and fibrosis. HCV displays a remarkable degree of genomic diversity, with the six major genotypes and numerous subtypes

differing in geographical distribution. Moreover, there is an argument about the role of HCV genotypes in the progression of severe liver disease and in the resistantance to antiviral therapy. So, it is important to know the genotype of HCV which infect a certain population to develop better strategies regarding public health.

Although a variety of methods have been used for genotyping HCV, nucleotide sequencing of a phylogenetically informative region remains the gold standard. The first observation of this study was that a 100 bp sequence region (-172 to -72) of HCV 5`UTR carries enough sequence variations for differential analysis of 9 subtypes of genotypes 1 through 4, as shown in Figure 4.1. The phylogenetic analysis also showed that the HCV isolates identified in Turkish patients did not diverge from other known and commonly found HCV isolates. Indeed, the great majority of these isolates (91 %) were identified as subtype 1b with a maximum evolutionary distance of 0.0219 within this group based on kimura2 parameters. The subtype 1a was rare (6 %), while genotypes 2 and 4 were exceptional (1/79 for each case). Thus, more than 90 % of HCV infections in Turkish patients living in the southern region are caused by a single subtype, namely 1b. Since 5`UTR region of genome has only one base pair difference in between subtype 1a and 1b, confirmation experiments performed by using NS5B region were required. There is 100% concordance between the results of these two methods and they permitted a correct characterization of genotypes. Also, our results confirm earlier reports for Turkish patients that showed 1b as a predominant subtype in other regions of Turkey (Abacioglu et al., 1995, Simsek et al., 1996). Thus, it appears that HCV infections in Turkey are due almost exclusively to a single subtype, namely subtype 1b.

The predominance of 1b subtype in Turkey correlates with the north-south gradient of increased 1b subtype occurrence in Europe. For example, a similarly high frequency of 1b subtype (91%) was reported for Sicilian patients, while only 8 % of HCV infections in Finland are due to the same subtype (Maertens and Stuyver, 1997). It was hypothesised that HCV infections with 1a subtype in Europe are due to the use of blood products originating from the USA. The low prevalence of 1a subtype in Turkey may support this hypothesis and indicates that HCV infections in Turkey are due to the local propagation of a 1b subtype. The exceptional occurrence of genotype 4 in southern Turkey is in favour of such a hypothesis. This particular

genotype is endemic in Egypt and highly prevalent in other Middle East countries with the exception of Israel (Shemer-Avni et al., 1998; Abdulkarim et al., 1998). It appears that the genotype 4 did not propagate from these countries towards Turkey.

After identification of subtype 1b as the predominant genotype, whole genome sequencing of a Turkish HCV-1b isolate from a single human carrier was performed. When compared to other variants of 1b subtype, the Turkish HCV-1b isolate displayed highest homology to a Japanese 1b strain. The reasons of this close relationship between Turkish and Japanese HCV isolates are presently unknown. As reported by Smith et al. (1997), the average time of divergence of variants of subtype 1b was about 70-80 years ago. In addition, the absence of country-specific groupings by phylogenetic analysis of subtype 1b sequences suggested that the spread of this genotype occurred on a worldwide basis at a similar time (Simmonds and Smith, 1997). Thus, the high homology of a Turkish isolate (HCV-TR1) with a Japanese isolate provides further evidence for this prediction. The HCV-TR1 polyprotein displayed amino acid substitutions at 36 positions when compared to other 1b variants. More than 50% of these substitutions occurred at residues that were heterogenous among different isolates. However, HCV-TR1 displayed specific changes in 16 positions and 8 of them were nonconserved amino acid substitutions (Table 4.2). To further characterize HCV-TR1 isolate, we examined the amino acid sequence at the proteolytic cleavage sites and we found high conservation at the amino acid level, suggesting no alteration in the processing of the polyprotein. In the ISDR there was only one amino acid difference between HCV-BK isolate and Turkish HCV-1b isolate and also the amino acid sequence of Turkish isolate was identical to that of HCV J isolate at the ISDR. It was reported that HCV-1b isolates with more than 4 amino acid difference form HCV-J isolate are related to higher response to interferon treatment (Enomoto et al., 1996). As we found no substitution, we expect to have lower response to interferon treatment. Lastly, we investigated the conservation of 26 cysteine residues in envelope proteins of Turkish HCV-1b isolate. Cysteine residues are conserved in four types of HCV, which is thought to be important for intra- and interchain disulfide bond formation. Disulfide bonds may provide the proper folding of envelope proteins, E1 and E2 (Okamoto et al., 1992c). There was no substitution in the cysteine residues and this is a promising evidence for proper folding of the E1 and E2 proteins of the Turkish HCV1b.

Therefore, it appears that the Turkish HCV-TR1 share similar features with other HCV isolates in terms of amino acid residues directly involved in protein function.

In contrast to the conservation of functional characteristics of viral proteins, a high number of immunodominant epitopes of HCV-TR1 displayed structural changes. Of particular interest, three CTL epitopes, one located on E2 and two on NS3 viral proteins displayed a high rate of amino acid substitutions (30-55%). Based on the fact that major functional features of HCV proteins are conserved, but many CTL-epitopes displayed substitutions at several amino acid residues, we believe that mutations affecting immunodominant viral epitopes in HCV-TR1 are not due to experimental errors and represent true changes in the immunogenicity of this strain.
HCV-specific CD8+ cytotoxic T lymphocytes are believed to play an important role in the pathogenesis of liver cell injury and viral clearance in HCV infection (Rehermann and Chisari, 2000). The efficacy of anti-viral cytotoxic immune response relies on the availability of viral epitopes to be recognized by specific CTLs. The fact that 6 out of 9 major CTL epitopes of HCV-TR1 are different from the consensus epitope sequence (Table 4.3), strongly suggests that the host immune response to this viral strain is defective or deficient. Further studies are needed to know whether mutations affecting immunodominant CTL epitopes may serve as a basis for unusually high frequency of HCV infections with 1b subtype in Turkish patients. More importantly, immunodominant CTL epitopes are considered as candidates for design of therapeutic vaccines for HCV (Urbani et al., 2001). Such vaccines may not be efficient against strains such as HCV-TR1 since they display major amino acid changes at candidate vaccine epitopes.

4-2 α-Fetoprotein-Producing Hepatocellular Carcinomas As Liver Stem Cell Cancers

4-2.1 AFP-producing HCC cell lines express early and late hepatic lineage markers

We initially screened liver cancer-derived cell lines (*n*=15) for the expression of several hepatocyte nuclear factors (HNFs), because of their known roles in the formation of embryonic liver stem cells and hepatocyte differentiation. HNF-3 (FOXA) family of proteins are among the earliest factors expressed in the endoderm from which the liver bud is derived (Zaret, 2001), and HNF-3β acts as a hepatic competency factor (Duncan, 2000) whose activity must be critical for stem cell properties of embryonic hepatoblasts, progenitors of both hepatocytes and bile duct cells. On the other hand, HNF-4α and HNF-1α are necessary for hepatocyte-specific expression of genes encoding proteins involved in liver functions (Kaestner, 2000; Duncan, 2000). On the basis of HNF expression, cell lines formed two groups. The first group including Huh7, Hep40, HepG2, PLC/PRF/5, Hep3B and its TGF-β-resistant clone Hep3B-TR expressed all four HNFs tested. The second group formed by the remaining cell lines displayed an incomplete expression pattern (Figure 4.3a). These observations suggested that a subset of liver cancer cell lines (first group) have in common to specifically express the factors needed for hepatic lineage competency, as well as liver differentiation.

Next, we selected five cell lines from each group on the basis of their reported tumor origin as adult HCC (Figure 4.3b) to compare their ability to express liver stem cell markers AFP and cytokeratin (CK)19. AFP, the earliest known marker for hepatic lineage competency, marks specifically hepatoblasts and “oval” cells, whereas CK19 is a marker for late hepatoblasts and “oval” cells, but also bile duct cells (Fausto, 1994, Shafritz and Dabeva, 2002). As tested by western blotting, HCC cell lines formed two homogeneous groups, the first expressing both AFP and CK19, the second negative for both markers (Figure 4.3b).

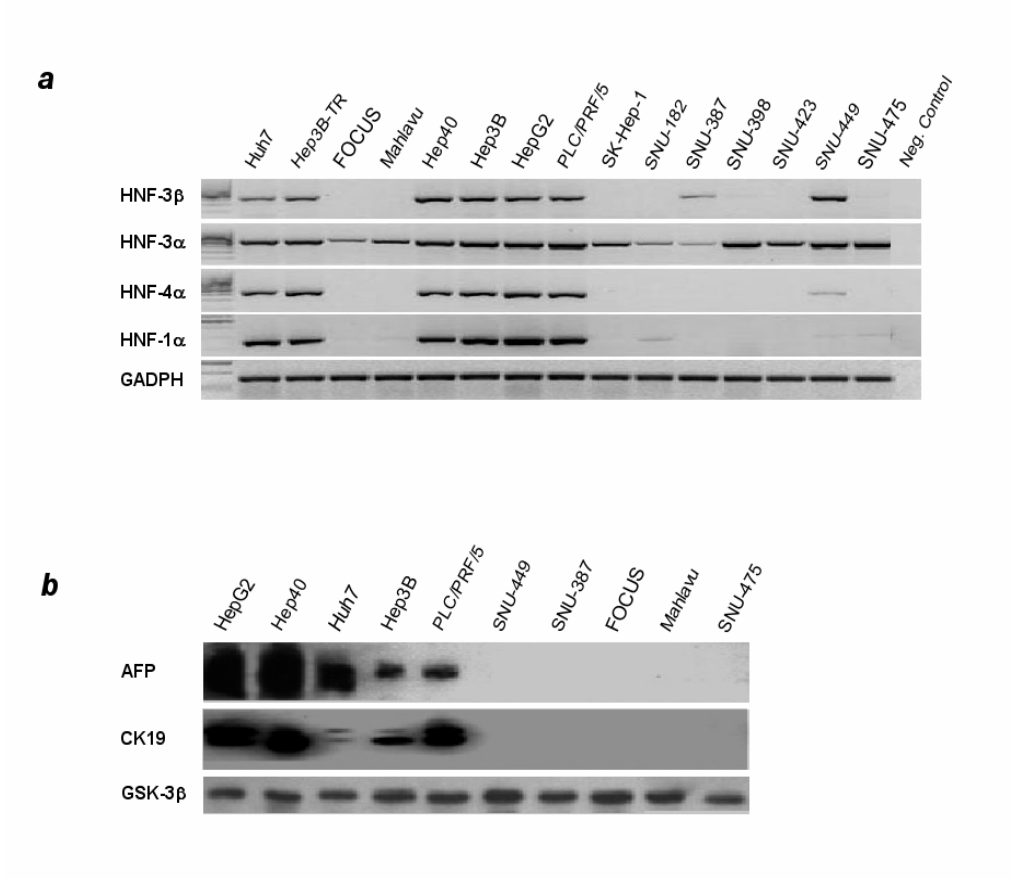

Figure 4.3: Hepatic transcription factor and lineage marker expression in liver cancer cell lines. **(*a*)** Expression analysis of HNF-3β, HNF-3α, HNF-4α and HNF-1α transcripts in 15 liver cancer-derived cell lines by semi-quantitative RT-PCR. GAPDH was used as a cDNA input control for PCR. **(*b*)** Further characterization of 10 HCC-derived cell lines by western blot analysis of hepatic lineage markers AFP and CK19. GSK-3β was used as a loading control. The adult HCC origin of these cell lines has been clearly established, with the exception of HCC-derived HepG2 that is considered to be a hepatoblastoma cell line (Miyazaki and Namba, 1994; Aden et al., 1979; Nakabayashi et al., 1982; Bouzahzah et al., 1995; Park et al., 1995; Bressac et al., 1991). **(*c*)** Expression of hepatic lineage markers during embriyonic liver development

This observation led us to further consider the cell lines of the first group as potential liver stem-like cells. Next, we subjected all 5 cell lines of this group to double-labeling immunofluorescence staining with CK19 and AFP, in conjugation with a third marker, namely albumin (ALB) which marks both liver stem cells and mature hepatocytes (Fausto, 1994, Shafritz and Dabeva, 2002). This method, based on differential staining of individual cells with rhodamine- and FITC-conjugated secondary antibodies, revealed that all tested cell lines contain different cell types (Figure 4.4a/b). This cellular heterogeneity could be considered as a sign of multiclonality, but also as an indication that AFP-producing HCC cell lines have the capability to generate multiple hepatic progeny, a common feature of mammalian liver stem/progenitor cells (Lazaro et al., 1998; Spagnoli et al., 1998; Kubota and Reid, 2000; Allain et al., 2002 ; Dumble et al., 2002; Suzuki et al., 2002).

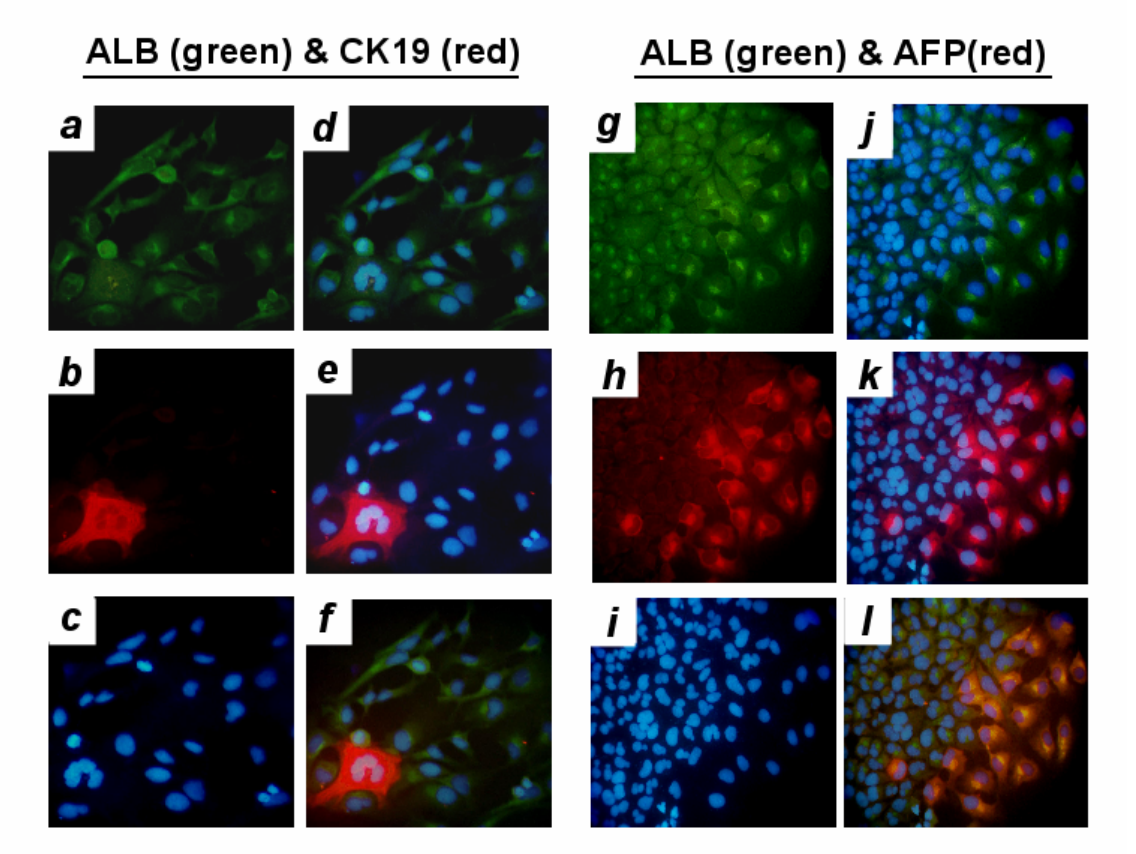

Figure 4.4: Heterogeneously staining cell types in AFP-producing human HCC cell lines: **(a)** Experimental approach to investigate the occurrence of multiple cell types in a cell line. The example illustrates the results obtained with Huh7 cells, following double-labeling immunofluorescence staining for either ALB/CK19 (*a-f*), or ALB/AFP (*g-l*). Original pictures from ALB (*a, g*), CK19 (*b*), AFP (*h*) and nuclear DNA (c, i) staining were merged successively (*d, e, j, k*) to generate the multi-color digital images (*f, l*) that were used in the following figures.

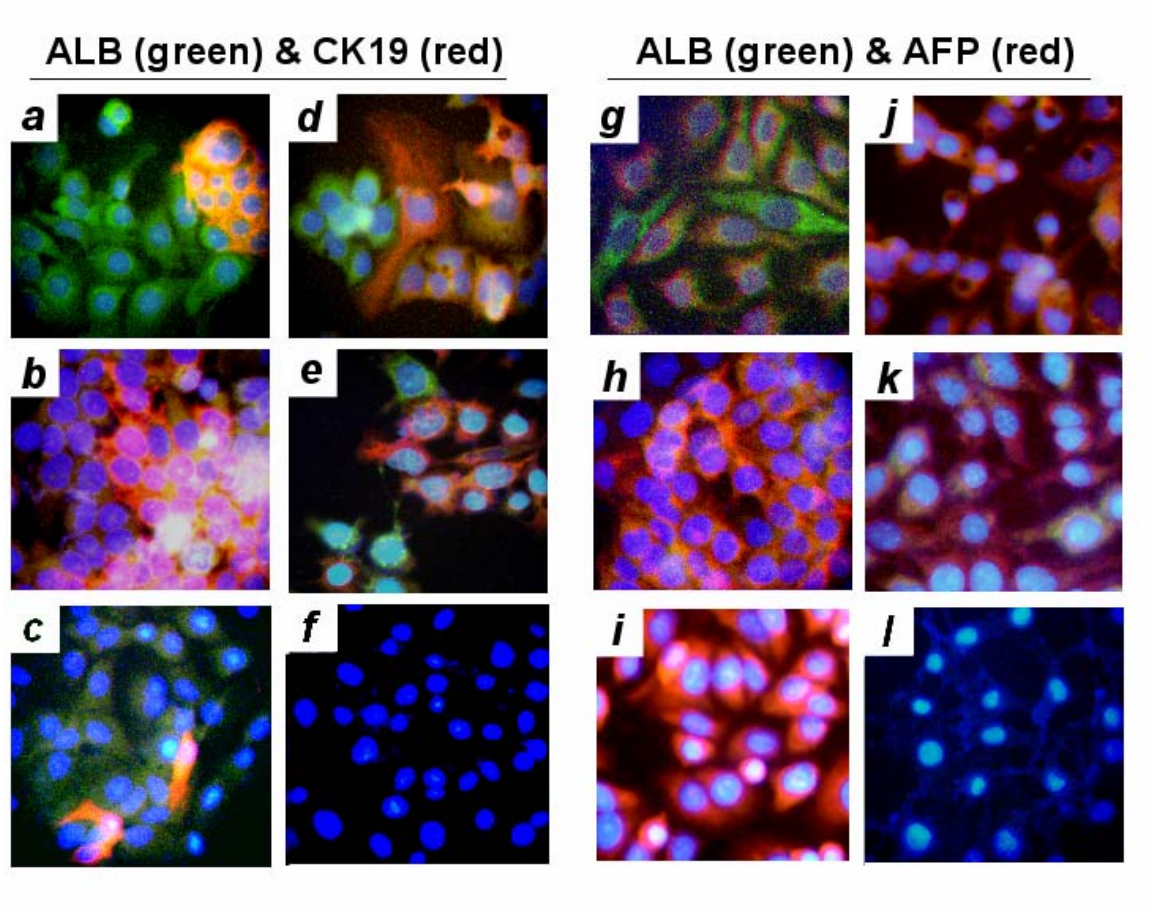

Figure 4.4 cont`: Heterogeneously staining cell types in AFP-producing human HCC cell lines: **(b)** All AFP-producing HCC cell lines are composed of cell types staining heterogeneously either for ALB/CK19 (*a-e*) and/or ALB/AFP (*g-k*). Huh7 (*a, g*), Hep40 (*b, h*), PLC/PRF/5 (*c, i*), HepG2 (*d, j*) and Hep3B (*e, k*) were AFP-positive cell lines. FOCUS served as a negative control (*f, l*).

4-2.2 Self-renewal and multi-lineage differentiation potential of AFP-producing HCC Cell Lines

We next tested self-renewing capability and multi-lineage differentiation potential of three different AFP-producing cell lines using *in vitro* colony assay at clonal density. Colonies generated by different cell lines were immunostained and counted. Figure 4.5 shows several representative examples of marker expression patterns. Most colonies were composed of homogeneously stained cells (Figure 4.5 *a, b, d, f, h, i*), but we also observed heterogeneous colonies with differentially staining cell populations (Figure 4.5 *c, e, g, j*). The occurrence of heterogeneously staining colonies in each tested cell line mirrored their cellular heterogeneity that was observed under high density cell culture conditions (Figure 4.4b), a first sign of their capability to generate multiple hepatic progeny, rather than multiclonality. The percent distribution of different colony types generated by each cell line was then calculated (Table 4.4). ALB/CK19 double staining indicated that all three cell lines produced ALB-positive, CK19-negative ($ALB^+/CK19^-$) colonies as the most abundant type (53-80%). Second most frequent colony population was $ALB^+/CK19^+$. All three cell lines also produced $ALB^-/CK19^-$ colonies at low ratios. The capacity to produce $ALB^-/CK19^+$ colonies was poor, since only Hep3B generated such a colony at a very low frequency. These cell lines also formed colonies with mixed cell populations composed of heterogeneously staining cells. ALB/AFP double staining indicated that Huh7 and Hep40 generated primarily ALB^+/AFP^+, secondarily ALB^+/AFP^- colonies, the latter being the most abundant type in Hep3B. Indeed, these two types of colonies represented 70 to 90% of colonies produced by these cell lines. Hep3B and Hep40, but not Huh7 cell line produced ALB^-/AFP^- colonies at a low frequency. ALB^-/AFP^+ colonies were not observed, but mixed-cell colonies were also evidenced by ALB/AFP staining. A closer examination of the data shown in Table 4.4 indicates that, of the colonies produced by three cell lines, 80-90% are ALB^+, whereas 27-63% and 11-28% are respectively AFP^+ and $CK19^+$. Based on this, we estimated that the dominant cell population in both Huh7 and Hep40 cell lines is $ALB^+/AFP^+/CK19^-$, whereas Hep3B cell line could be rich in both $ALB^+/AFP^+/CK19^-$ and $ALB^+/AFP^+/CK19^+$ cell populations (Table 4.4). Both patterns of marker expression have been previously assigned to liver stem/progenitor cells (Shafritz and Dabeva, 2002, Kubota and Reid, 2000, Allain et al., 2002).

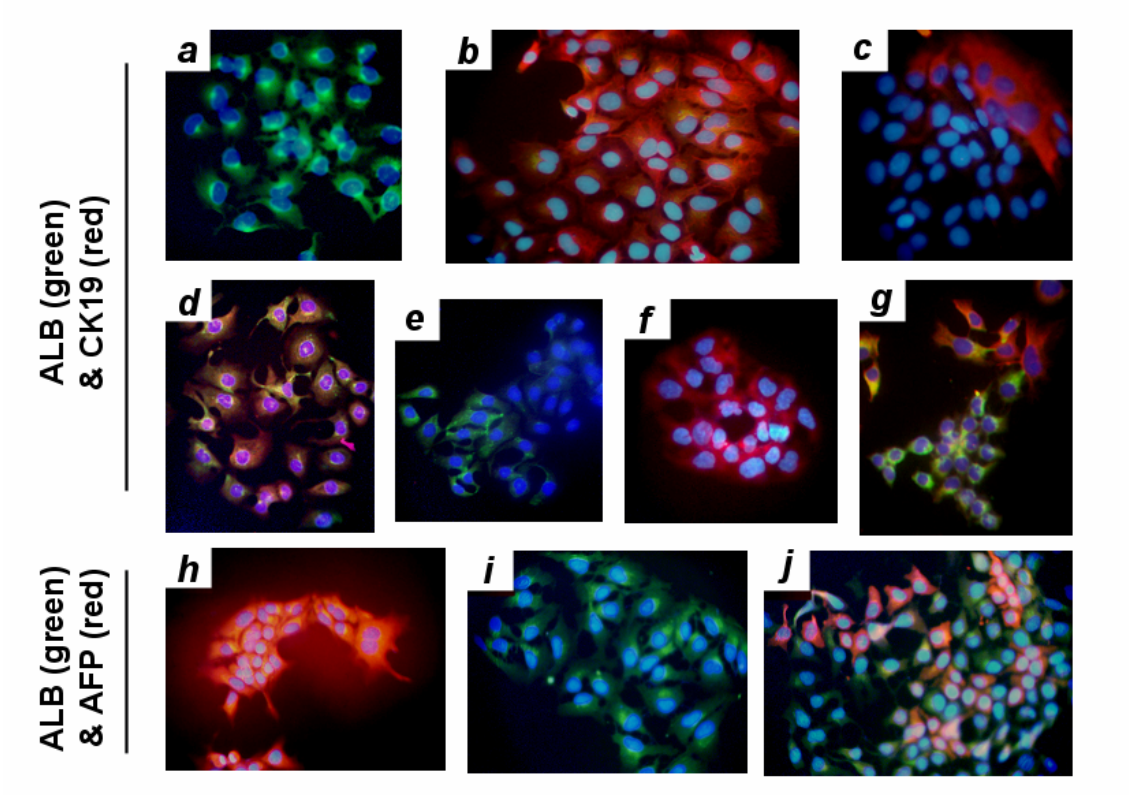

Figure 4.5 Multilineage colony formation from AFP-producing human HCC cell lines. Colonies were stained for ALB/CK19 and ALB/AFP, as described in Figure 4.4b. Representative examples are homogenously (*a, b, d, f, h, i*) and heterogeneously (*c, e, g, j*) staining colonies derived from Huh7 (*a*-c, *h-j*), Hep40 (*d, e*) and Hep3B (*f, g*) cell lines

Table 4.4 Multilineage colony formation from AFP-producing human HCC cell lines[1]

	Percent distribution of colonies				
ALB/CK19	+/-	+/+	-/-	-/+	Het.[2]
Hep3B (*n*=148)	52.7	28.4	12.2	0.7	6.1
Hep40 (*n*=93)	79.6	9.7	9.7	0.0	1.1
Huh7 (*n*=100)	80.0	11.0	2.0	0.0	7.0
Huh7-SC1 (*n*=100)	84.0	13.0	0.0	0.0	3.0
Huh7-SC3 (*n*=104)	81.7	15.4	0.0	0.0	2.9
ALB/AFP	+/-	+/+	-/-	-/+	Het.[2]
Hep3B (*n*=102)	62.8	26.5	8.8	0.0	2.0
Hep40 (*n*=93)	22.6	58.1	6.5	0.0	12.9
Huh7 (*n*=154)	23.4	63.0	0.0	0.0	13.6

[1]Huh7 and Hep40 cell lines were derived from adult HCCs, whereas Hep3B tumour occurred in an 8 year-old male. Hep40 and Hep3B, but not Huh7 have integrated HBV DNA sequences in their genomes (Miyazaki and Namba, 1994, Aden et al., 1979, Nakabayashi et al., 1982, Bouzahzah et al., 1995). Huh7-SC1 and Huh7-SC3 were generated by subcloning from parental cell line. [2]Het.: colonies composed of heterogeneous staining cell populations. Other colonies were composed of homogenously staining cell populations.

Taken together, these findings provided further evidence that AFP-producing cell lines have self-renewing capability and multi-lineage differentiation potential. In order to firmly confirm that AFP-producing HCC cell lines are indeed liver stem - like cells, we generated several single cell-derived clones form Huh7 cells. Both Huh7-SC1 and Huh7-SC3 constituted heterogeneous cell populations at high density cell culture (Figure 4.6*a* and. 4.6*b*, respectively). Both clones also generated heterogeneously (Figure 4.6*c, 4.6d*) and homogenously (Figure 4.6) staining colonies under low density clonogenic culture conditions, similar to their parental cell line Huh7. The percent distribution of ALB/CK19-reactive colonies produced from these clonal cell lines was comparable to that of parental cell line (Table 4.4). Taken together, these results established that AFP-producing HCC cells display multi-

lineage differentiation and self-renewing capabilities, similar to liver stem cells (Lazaro et al., 1998, Spagnoli et al., 1998, Kubota and Reid, 2000, Allain et al., 2002, Dumble et al., 2002, Suzuki et al., 2002).

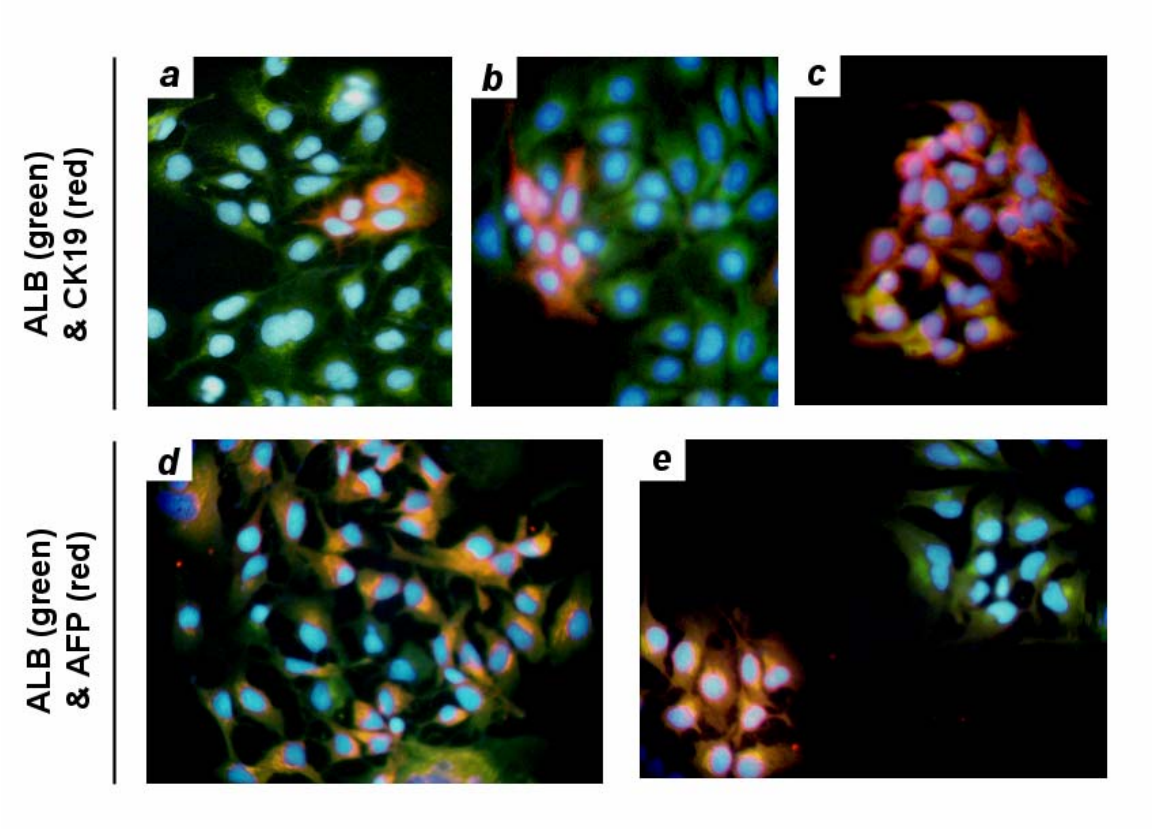

Figure 4.6 Clonal expansion and self-renewal capability of Huh7 cells: Huh7-SC1 and Huh7-SC2 subclones were expanded from single cell-derived clones, and tested for hepatic lineage marker expression. Examples are Huh7-SC1 (*a*) and Huh7-SC3 (*b*) cells forming mixed cell populations at high density cell culture, and Huh7-SC1 cells which generates heterogeneously staining (*c, d*), as well as homogenously staining (*e*) colonies when grown at low density.

4-2.3 *In vitro* modulation of differentiation program of AFP-producing Huh7 cell line

Self-renewal and differentiation fates of stem cells, including liver stem cells are known to be controlled by both extrinsic and intrinsic factors (Shafritz and Dabeva, 2002, Morrison et al., 1997). To investigate whether the differentiation program of AFP-producing HCC cell lines is modulated by external factors *in vitro*, we first tested CK19 levels in Huh7 cells following short-term culture in three different media. The growth of Huh7 cells in FCS-free medium that is

complemented with either selenium or OPI led to an increase in CK19 protein levels at day 6, as compared to cells grown in standard medium. CK18 levels, used as a control, did not change (Figure 4.7*a*). Next, we studied the modulation of cell differentiation following long-term culture (16 days) on diluted Matrigel extracellular matrix. Immunostaining for ALB/CK19 indicated that cell culture in selenium-complemented FCS-free medium favors the emergence of $CK19^{+}$ biliary lineage cell populations forming bile duct-like structures (Figure 4.7*b*). In contrast, cell culture in standard culture medium under the same conditions favored the expansion of ALB^{+} hepatocytic lineage cells, some of which becoming ALB-rich bi-nuclear cells, like mature hepatocyes (Figure 4.7*c*).

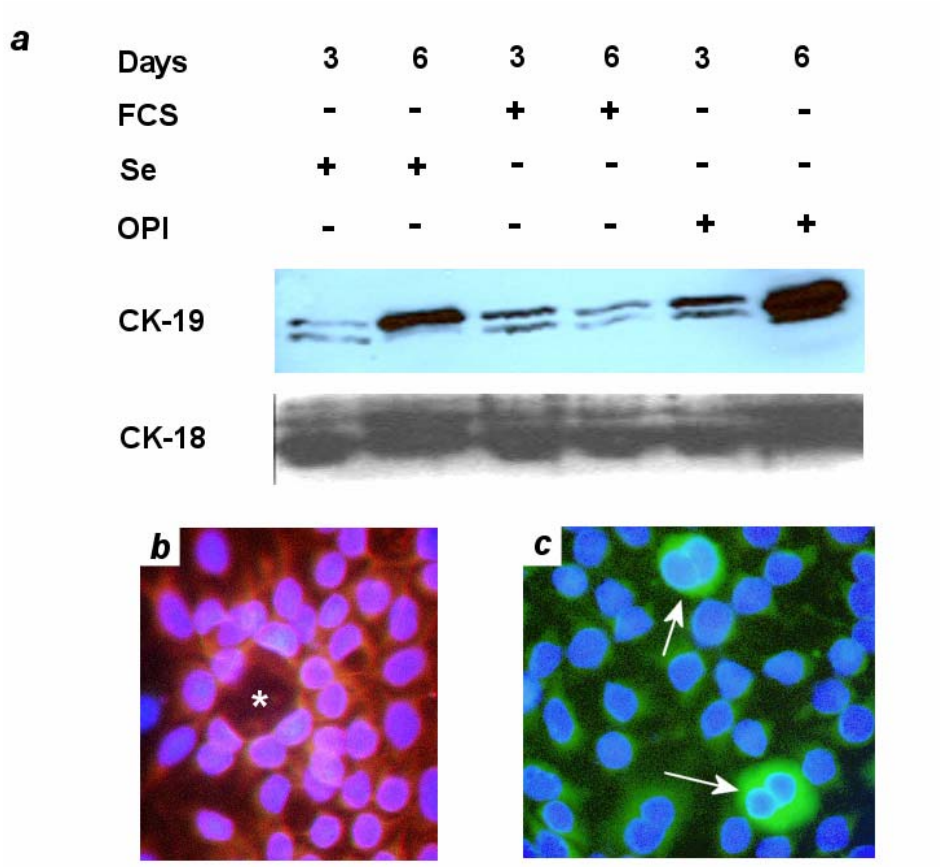

Figure 4.7 External modulation of Huh7 differentiation fates: **(*a*)** Modulation of bile-duct lineage marker CK19 expression. Western blot assay shows that the growth of Huh7 cells in selenium (Se)- or OPI-complemented FCS-free medium leads to an increase in CK19 protein levels at day 6, as compared to cells grown in standard medium (FCS). CK18 levels, used as a control, did not change. **(*b, c*)** Modulation of cell differentiation during long-term growth (16 days) on diluted *Matrigel* extracellular matrix. Double-labeling immunofluorescence staining for ALB/CK19 indicates that cell growth in selenium-complemented FCS-free medium favors the emergence of an $ALB^{-}/CK19^{+}$ cell population forming bile duct-like structures (white $*$ in *b*), as opposed to standard culture medium that favors the growth of $ALB^{+}/CK19^{-}$ cells, some of which becoming ALB-rich hepatocyte-like cells with double nuclei (white arrows in *c*).

4-2.4 Discussion about AFP-producing HCC cells

One of the major implications of our investigations is that human HCC cell lines form two distinct groups, one producing AFP and displaying liver stem cell-like properties, the other not. AFP-producing HCC cell lines express HNF3α. HNF-3β, HNF-4α, and HNF-1α, as well as hepatic lineage markers AFP, ALB and CK19, albeit heterogeneously. Detailed analysis of clones produced by Huh7, Hep40 and Hep3B cell lines (Table 4.4) provides evidence that stem cell-like phenotype of AFP-producing cell lines is associated with a marker expression pattern of $ALB^+/AFP^+/CK19^-$ and/or $ALB^+/AFP^+/CK19^+$, that mimic early and late hepatoblast marker expressions, respectively (Fausto, 1994, Shafritz and Dabeva, 2002, Kubota, and Reid, 2000, Allain et al., 2002, Thorgeirsson, 1996). However, AFP-producing cell lines studied here were derived from adult HCCs (Figure 4.3, Table 4.4). This raises some questions about their cellular origin. They could be originated from mature hepatocytes to become liver stem/progenitor-like cells, according to the "dedifferentiation" theory (Aterman, 1992). However, recent data show that, during chemically induced HCC in rat, mature hepatocytes do not "dedifferentiate" into "oval" cells (Gournay, et al., 2002) putative progenitors of HCC (Sell, and Dunsford, 1989, Sell, 2001). This leaves us with the possibility that AFP-producing HCC cells originate from liver stem cells, like chronic myeloid leukemia cells originating from haematopoietic stem cells (Taipale, and Beachy, 2001, Reya et al., 2001). Although adult human liver stem/progenitor cells have not been isolated yet, rodent "oval" cells share some similarities with AFP-producing HCC cell lines described here. The expression of HNF3 appears be critical for "oval" cell activation, while HNF4 is involved in differentiation of "oval" cells into hepatocytes (Nagy et al., 1994). However, "oval" cells are $ALB^+/AFP^+/CK19^+$ (Thorgeirsson, 1996), in contrast to dominant cell populations in HCC cell lines which are $ALB^+/AFP^+/CK19^-$. If the putative precursors of "oval" cells are similar to $ALB^+/AFP^+/CK19^-$ early hepatoblasts, then it is highly likely that AFP-positive HCC cells originate from putative precursors of "oval" cells, if not directly from "oval" cells, in line with the "stem cell origin" theory (Sell and Dunsford, 1989, Sell, 2001).

Although we can not firmly define their cellular origin, AFP-producing HCC lines act *in vitro* as liver stem-like cells, since they have an unlimited ability to self-renewal and they differentiate towards hepatocytic and biliary lineages, although they fail to form morphologically discernible mature hepatocyte and bile duct cell populations. These cell lines produce solid tumors in immunodeficient mice (data not shown for Huh7, see Ref.(Miyazaki, and Namba,1994) for additional examples), in contrast to normal liver stem cells that generate normal liver tissue (Shafritz and Dabeva, 2002). These features qualify AFP-producing HCC cells as "mis-specified" liver stem cell cancer lines whose cellular programs are deviated from normal tissue repopulation to malignant tumor formation. It was proposed that mutations affecting genes involved in *wnt*, *hedgehog* and TGF-β signaling pathways contribute to the "mis-specification" of stem cells (Taipale, and Beachy, 2001, Reya et al., 2001). In this regard, it is noteworthy that HCC cells display mutations in genes involved in *wnt* and TGF-β signaling pathways. β-catenin and Axin1 gene mutations that are observed in these cancers lead to an aberrant accumulation of β-catenin, and constitutively activate the *wnt* pathway (Ozturk, 1999, Buendia, 2000, Tannapfel and Wittekin, 2002). The p53 mutations that are frequently observed in HCC may also activate *wnt* pathway because of aberrant β-catenin accumulation, as demonstrated in HCC cell lines including those described in this study (Cagatay and Ozturk, 2002). Several genes involved in TGF-β pathway including M6P/IGF2R, TGF-β1RII, SMAD2 and SMAD4 are also mutated in some HCCs (Ozturk, 1999, Buendia, 2000, Tannapfel and Wittekin, 2002)

AFP expression has been closely connected to cells acting as liver stem cells both *in vitro* and *in vivo* (Shafritz and Dabeva, 2002, Sell, 2001). Moreover, AFP has been in use for many decades as a standard HCC tumor marker because 50-70% HCC tumors produce it (Johnson, 1999). Now, this report establishes AFP expression in HCC cells as an indication of liver stem cell cancer phenotype. Although this phenotype was not demonstrated *in situ* with primary tumor cells, our results, together with previous knowledge on AFP expression in liver stem cells (Shafritz and Dabeva, 2002, Sell, 2001, Kojiro, 2002) offers persuasive evidence that AFP-producing HCCs are formed, if not sustained by "mis-specified" liver stem cells. This may provide an impetus for further investigations on stem cell-HCC

connection. Future research could focus on molecular dissection of liver stem cell-like properties of AFP-producing HCC cells. As demonstrated here, these cells have the ability to respond to extrinsic factors and modify their differentiation program accordingly, at least *in vitro*. Through a targeted research for identification of biomodulators (ligands, cell surface receptors, chemicals etc.), it would be possible to identify molecules that are able to modulate stem cell-like properties of HCC cells, in order to limit their self-renewal capacity and survival or to initiate differentiation processes towards non-proliferating mature cells. Such molecules would be promising drug candidates for chemoprevention of HCC in risk groups such as chronic hepatitis and cirrhotic patients, as well as for chemotherapy. Another interesting direction would be the investigation of possible implications of HBV and HCV in liver stem cell biology, in the context of virally induced hepatocellular carcinogenesis.

CHAPTER 5. CONCLUSION AND FUTURE PERSPECTIVES

During this thesis work, we achieved two major tasks. Firstly, we identified of subtype 1b as the major etiological virus type of hepatitis C in Turkey, and we cloned a nearly complete genome of a Turkish HCV 1b isolate. Secondly, we identified a subgroup of HCC cell lines as liver-stem-cell-like cells. This group is characterized by a common "differentiated" phenotype associated with AFP production. AFP-negative cell lines were mostly undifferentiated and lacked stem cell phenotype. Interestingly, the cell lines of the first group (Huh7, HepG2, Hep3B) are used commonly to study the role of both HBV and HCV proteins in hepatocellular carcinogenesis. Since all these cell lines act as "misspecified" liver stem cells, one may claim that all these previous studies on HBV and HCV were done on liver stem cells, but not mature hepatocyte-like cells. Hepatocytes are natural hosts of both viruses, XXX acute infections. Now, it will be possible to set up a whole serious of experiments to address the role of HCV (or HBV) in terms of their implications in hepatocarcinogenesis via hepatic stem or stem-like cells. Now that we have a cloned HCV genome, as well as characterized liver stem-like cells, we believe that furtherstudies may focus on the effects of viral proteins on the phenotype (self-renewal and differentiation) of these cell lines. This newline of investigations may reveal interesting and instructive interactions of viral proteins with hepatic stem cell program.

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Molecular Characterization of a Full Genome Turkish Hepatitis C Virus 1b Isolate (HCV-TR1): A Predominant Viral Form in Turkey

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Abstract. Based on direct sequencing information from 5′UTR and NS5B regions, we identified subtype 1b as a predominant hepatitis C virus genome in Turkey, which affected more than 91% of 79 patients studied. Next, the full genome sequence of a Turkish 1b isolate was obtained by the cloning of polypeptide-encoding region into 7 overlapping fragments. Turkish 1b isolate, which was named HCV-TR1, comprises 9361 nucleotides, including 306 nucleotides of 5′UTR, a single long open reading frame of 9033 nucleotides, and 22 nucleotides of 3′UTR. When compared to HCV 1b polypeptide sequences available at GenBank, the predicted polypeptide displayed a total of 36 amino acid substitutions, of which 16 was specific for HCV-TR1 isolate. Despite these changes, major structural and functional motifs of HCV proteins were maintained in HCV-TR1. In contrast, HCV-TR1 displayed amino acid substitutions in 6 out of 9 major cytotoxic T-cell epitopes. These data suggest that HCV-TR1 encodes functionally intact viral proteins, but it also encodes altered viral epitopes, which may affect host immune-response.

Key words: hepatitis C virus, subtype 1b, HCV-TR1, Turkey, viral epitope

Introduction

Hepatitis C is a major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer. An estimated 175 millions of persons are chronically infected with HCV worldwide and 3–4 million persons are newly infected each year [1]. The striking genetic heterogeneity of Hepatitis C virus is well recognized. This genetic diversity includes six major genotypes, with numerous subtypes (over 80) and minor variants called 'quasispecies' [2]. The geographical distribution of different genotypes and subtypes differs greatly from one region to the other. The reasons for this differential distribution are not well known, but the profile of geographical distribution could reflect the different modes of viral transmission as well as the host immune-response variations. For example, HCV 1a subtype, which is seen frequently in North America, could have been transmitted to other regions of the world, especially to Europe, by contaminated blood-derived products [3]. In contrast, subtype 1b appears to be dominant in Japan and Southern Europe. This particular subtype has been related to more severe liver disease, resistance to interferon treatment and increased risk for hepatocellular carcinoma [4,5].

Turkey with a population of over 65 million people is located between southern Europe and the Middle Eastern countries, which differ from each other by the distribution of major genotypes. In Europe, HCV 1

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is the major genotype and there is a south-north gradient for 1a and 1b subtypes, the prevalence of subtype 1b being increasingly higher in southern Europe [4–7]. The data on HCV subtypes in the Middle Eastern countries is limited. In Egypt, Hepatitis C is an endemic disease that is associated with genotype 4, almost exclusively [8]. Similarly, genotype 4 is also predominant in the Gaza region, but not in Israel where subtype 1b is predominant [9]. The distribution of HCV genotypes in liver disease patients living in Syria, Iraq and Iran is unknown. In one study performed in hemodialysis patients from Syria, genotypes 4a, 1b and 1a were identified in respectively 30%, 27% and 19% of patients, and 30% of sequences were unmatched [10]. On the other hand, in countries located on the northern frontiers of Turkey, subtype 1b appears to be the dominant form [11–13].

The HCV genotype distribution of patients living in Turkey is not well known. To our knowledge, there are only two published reports concerning Turkish patients, which indicated a high frequency (75–87%) of subtype 1b [14,15]. The regional distribution of HCV genotypes in Turkey has not been reported yet. In this study, we report our observations on HCV subtypes in southern Turkey. This region shares its frontiers with Middle Eastern countries displaying heterogenous distribution of subtypes 1a, 1b and 4a of HCV, as stated earlier [8–10,16]. Here, we report that the subtype 1b is the predominant genotype in Hepatitis C patients living in southern Turkey. This confirms that Turkish patients are infected mostly with this particular subtype of HCV. In contrast, subtype 1a was rare, others including subtype 4 being exceptional. Based on this information, we obtained sequence data for the 5′UTR and entire polyprotein coding regions of a Turkish 1b isolate, termed HCV-TR1, following cloning of viral genome into 7 overlapping fragments. We present here the main features of the predicted viral polyprotein sequence of TR1, in comparison with that of other HCV 1b isolates from different geographical regions in the world.

Material and Methods

Patients

A total of 79 HCV-positive patients from the Gastroenterology Department of Çukurova University in Turkey were investigated for HCV genotyping. The great majority of these patients lived in South Anatolia and Southeast Anatolia regions at the time of diagnosis. All patients were positive for anti-HCV antibodies, which were determined using the second-generation ELISA test. Among this group of patients, a serum sample from a 59-year old woman suffering from chronic Hepatitis C, which was collected prior to any treatment for her disease, was used for HCV cloning studies.

Viral RNA Extraction and cDNA Synthesis

We used a modified and optimized RNA extraction protocol derived from a previously published procedure [17]. RNA was extracted from 300 µl of serum with the freshly made lysis buffer containing guanidine-HSCN and mercaptoethanol in the presence of an RNA carrier. The RNA was then recovered by isopropanol precipitation and resuspended in 10 µl DEPC–ddH2O. First strand cDNA synthesis was performed using a commercial kit (MBI). Briefly, 10 µl resuspended RNA was treated in a 20 µl reaction volume with 0.2 µg of random primers, 40 U of M-MuLV Reverse Transcriptase, 20 U of Ribonuclease inhibitor, and 1 mM (each) deoxyribonucleotides at 37°C for 1 h after brief denaturing at 90°C.

PCR Amplification of 5′UTR and NS5B Regions for Genotyping Studies

Initially we used sequence information at 5′UTR for genotyping studies. Sequence information derived from NS5B region was used for confirmation studies. A 285 bp fragment from the 5′UTR of the HCV genome was generated with PCR amplification of one-fourth of cDNA. The first round of 'nested' PCR was performed using outer primers F1 (5′-ATCACTCCCCTGTGAGGAAC-3′) and R1 (5′-TGCTCATGGTGCACGGTCTAC-3′), after denaturation at 94°C for 5 min, with 25 cycles, each containing 45 s at 94°C, 45 s at 55°C and 45 s at 72°C, followed by a final extension for 10 min at 72°C. For the second amplification, 8 µl of the first reaction mixture was further amplified with inner primers F2 (5′-GAGGAACTACTGTCTTCACGC-3′) and R2 (5′-TCTACGAGACCTCCCGGGGCA-3′) under the previous conditions, except the annealing temperature which became 60°C to generate a fragment of 285 bp. For PCR amplification of NS5B region, two-step PCR

with the same primer set was established to generate a 400 bp DNA fragment covering region 7904–8304 (Position of 5′ base relative to HCV genomic sequence in Choo et al.; 1991 [33]) by using NS5B Forward (5′-TGGG-GATCCCGTATGATACCCGCTGCTT-TGA-3′) and NS5B Reverse (5′-GGCGGAA-TACCTGGTCATA-GCCTCCGTGAA-3′) primers. PCR amplification was done after denaturation at 94°C for 4 min, with 30 cycles, each containing 40 s at 94°C, 40 s at 58°C and 40 s at 72°C, followed by a final extension for 10 min at 72°C. For the second amplification, 4 µl of the first reaction mixture was further amplified with the same set of primers in a total volume of 50 µl, using Taq *Polymerase* (MBI). Specific PCR amplification of correct-sized DNA fragments was confirmed by agarose gel electrophoresis and the appropriate amount of PCR products (usually 100 ng) were subjected to automated nucleic acid sequencing using the same sets of primers and cycle sequencing kits from Perkin Elmer and Amersham following the manufacturer's instructions. Sequencing reaction products were analyzed on ABI-377 DNA sequencer (Perkin Elmer).

HCV Genotype Identification and Phylogenetic Sequence Analysis

The 5′UTR and NS5B regions of all available genotypes at NCBI Taxonomy Homepage were aligned using MULTIALIN multiple alignment program to reveal the subtype-specific consensus sequences (http: //npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?-page=/NPSA/npsa_multalinan.html). The same groups of sequences were also used to construct the phylogenetic tree by using the PHYLIP program to determine the genotypic distribution of all samples. In order to compute the distance matrix, we used DNADIST module from PHYLIP software package with Kimura 2-parameter model (http://sdmc.krdl.org.sg:8080/~lxzhang/phylip).

Molecular Cloning and Characterization of a Turkish HCV 1b Isolate (HCV-TR1)

A nearly full length HCV isolate from a 59-year old female Turkish patient was amplified in 7 overlapping PCR fragments. Primer sets for each fragment, which were designed from the most conserved regions in the desired area, are given in Table 1. Fragments smaller than 1 kb were amplified with *Pfu* DNA *Polymerase* in the first round in order to decrease the PCR-mediated mutation risk and in the second round *Expand High fidelity PCR System* (Boehringer Mannheim) was used to obtain PCR products with adenine overhangs. Fragments larger than 1 kb were amplified with *Expand High Fidelity System*. PCR products were cloned into pGEM®-T Easy vector (Promega), which is a linearized vector with T overhangs in the multiple cloning site. Selected plasmids containing desired inserts were purified by using QIAfilter plasmid midi kit (Qiagen), and used for automated DNA sequencing as described. For sequencing,

Table 1. Sequences of primers used for PCR amplification of overlapping cDNA regions of the genome of HCV isolate HCV-TR1

Primer	Sequence (5′ → 3′)	Position*	Amplified Fragment
F1	ATCACTCCCCTGTGAGGAAC	−306	5′UTR and Core
CoreR	(G/A)GAGCA(G/A)TCGTTCGTGACAT	964	
E1F	CCCGGTTGCTCTTTCTCTATC	850	E1, E2, and p7
E2R	ATGC(A/G)GCCATCTCCCGGTC	2791	
NS2F	T(C/T)CT(G/A)(C/T)TG(G/T)C(G/A)TTACCACC	2738	NS2
NS2R	GT(C/T)TG(C/T)TG(G/A/T)G(A/C)GTAGGCCGT	3449	
NS3F	CCGAAGGGGGA(A/G)GGAGAT	3354	NS3
NS3R	GCACCCA(G/A)GTGCT(A/C/G)GT(G/A)ACGAC	5326	
NS4F	ATGCATGTCGGC(C/T)GACCT	5283	NS4A and NS4B
NS4R	TG(G/A)AGCCA(G/A)GTCTTGAAGTC	6329	
NS5AF	TATGTGCCTGAGAGCGACG	6142	NS5A
NS5AR	(A/G)CG(C/T)AGCAAAGAGTTGCTCA	7695	
NS5BF	AGCGACGGGTC(C/T)TGGTCTAC	7543	NS5B and 3′ UTR
NS5BR	CCTGGAGTG(G/T)TT(A/G)GCTCCC	9397	

*Nucleotide numbers according to Ref. 33.

plasmid-derived primers were used initially, followed by HCV sequence-derived sense and anti-sense primers. Sequence data obtained from overlapping fragments was assembled manually to construct a full length HCV genome sequence.

Results

Hepatitis C Virus Genotyping in Turkish Patients

A total of 79 HCV RNA-positive sera from patients living in southern Turkey were used for genotyping. The amplified 5′UTR region was analyzed by automated DNA sequencing. The sequence data was compared with HCV subtype-specific consensus sequence data as described in the section 'Materials and Methods'. A phylogenetic tree for HCV subtypes in our sample population was constructed in comparison with consensus HCV genotypes. For the analysis, a 100-base region located between nucleotides −172 and −72 of HCV was selected, since this region was variable enough between HCV subtypes. Out of 79, 70 samples displayed unambiguous nucleotide sequence at this region. The sequence similarities between our sample population ranged from 84 and 100%. In order to build the phylogenetic tree, we included 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b and 4c subtype consensus sequences into our population data, and we examined the distances between sequence clusters. Prior to this analysis, we calculated sequence similarities between subtype consensus sequences, which ranged from 75 (between 2a and 3b) to 99% (between 1a and 1b). The phylogenetic tree indicated that out of 70 nucleic acid sequences, 3 were clustered with 1a subtype and displayed 0–0.0102 evolutionary distances. Sixty-five sequences were grouped together with 1b subtype showing a distance rate of 0–0.0219. One sequence was grouped with 2a subtype with a zero evolutionary distance, while another sequence grouped with subtype 4c consensus with zero evolutionary distance. There was no sequence clustered with 2b, 3a, 3b and 4a subtypes (Fig. 1). This phylogenetic tree analysis showed that 5′UTR data can be used for subtype identification. Accordingly, the remaining 9 samples were genotyped by manual alignment with subtype-specific consensus sequences. To confirm HCV genotyping results obtained by 5′UTR sequence data, 19 randomly selected samples were genotyped using NS5B region sequence data and all samples displayed the expected genotype (data not shown). When combined, these analyses indicated that, 72 of 79 patients (91%) displayed 1b genotype of HCV, and 5/79 (6%) had genotype 1a. Thus, all but 2 of 79 HCV genotypes analyzed had genotype 1. The other two samples displayed subtypes 2a and 4c, respectively.

Molecular Characteristics of Turkish Isolate of HCV 1b (HCV-TR1)

Following our identification of subtype 1b as the main HCV genotype affecting Turkish patients, we decided to obtain full genome sequence information from a Turkish HCV 1b isolate. Using a single serum sample obtained from a patient prior to any treatment, we cloned the major portion of HCV genome into 7 overlapping fragments. When combined together, these clones covered the entire sequence for HCV polyprotein, as well as most of the nucleotides of the 5′UTR and 3′UTR regions. The overlapping regions all contained the identical sequences. The Turkish 1b isolate, which we named HCV-TR1, comprises 9361 nucleotides, including 306 nucleotides of 5′UTR, a single long open reading frame of 9033 nucleotides, and 22 nucleotides of 3′UTR (data submitted to GenBank nucleotide sequence database and assigned the accession number AF483269). This genomic sequence showed highest homology (91% identity) to a reported HCV 1b isotype (strain HCV-1b, clone HCV-K1-R2) from Japan [18], when tested by BLAST using GenBank database. The HCV-TR1 displayed a single open reading frame encoding a 3010-amino acid polyprotein that showed 93% identity to clone HCV-K1-R2.

Proteolytic processing of HCV polypeptide between C-E1, E1-E2, E2-p7 and p7-NS2 is performed by host cell proteases, whereas viral proteases cleave between the non-structural proteins NS2-NS3, NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B [19]. HCV-TR1 polypeptide displayed no major amino acid change in boundary regions at proteolytic cleavage sites (data on cleavage sites of non-structural proteins are shown in Table 2), suggesting that it can be correctly processed into HCV proteins, including core (1–191; 191 aa), E1 (192–383; 192 aa), E2 (384–746; 363 aa), p7 (747–809; 63 aa), NS2 (810–1026; 217 aa), NS3 (1027–1657; 631 aa), NS4A (1658–1711; 54 aa), NS4B (1712–1972; 261 aa), NS5A (1973–2419; 447 aa), NS5B (2420–3010; 591 aa) proteins. Because of nucleic acid

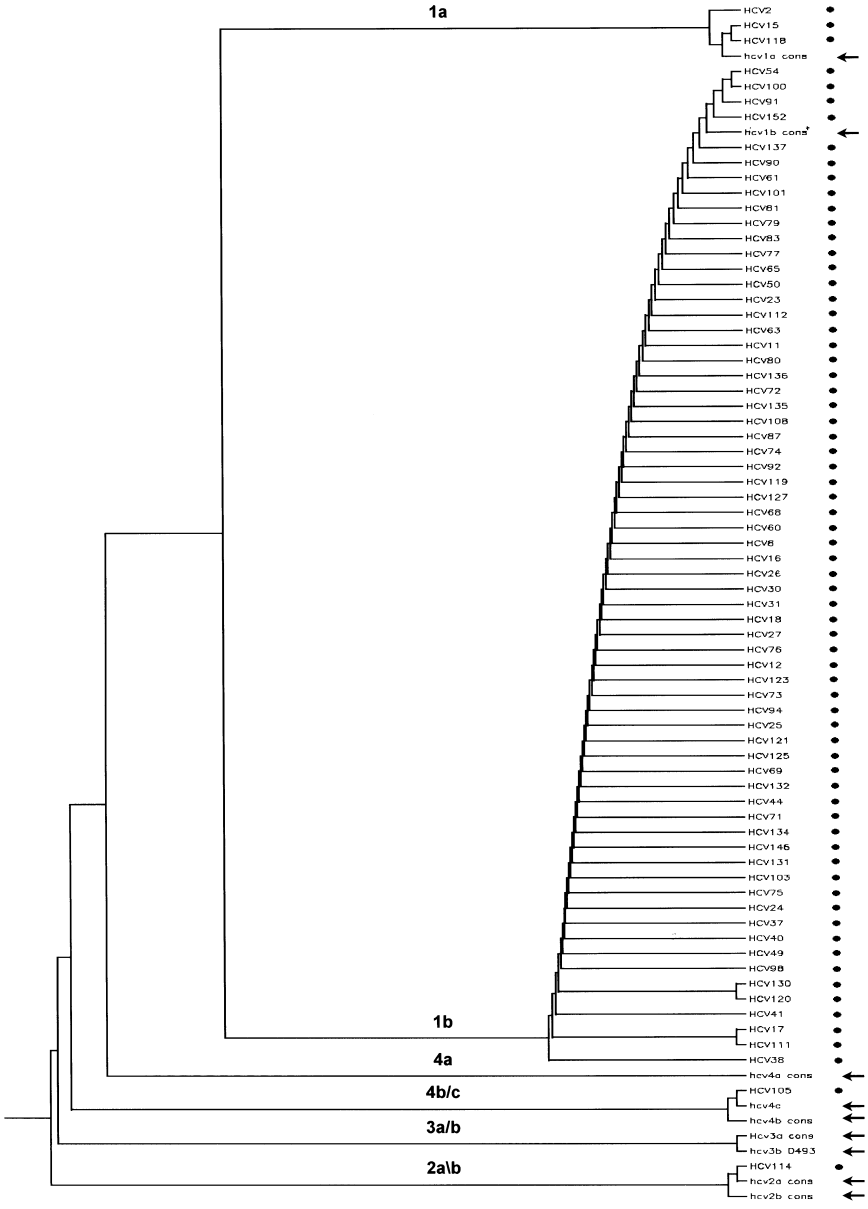

Fig. 1. Phylogenetic tree of the 5′UTR sequences from 70 isolates (filled circles) of HCV from Turkey. The consensus sequence data for subtypes 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b and 4c (arrows) were included as reference sequences. The genetic distances were calculated with the DNADIST module from PHYLIP software package and were based on a Kimura 2-parameter matrix with a transition to transversion ratio of 2 (see text).

sequence ambiguities, we were not able to determine the amino acid residue in 3 positions. The alignment of this polyprotein sequence with that of other 48 HCV-1b isolates available at the GenBank database revealed that there was overall 36 amino acid substitutions in the Turkish isolate. Twenty of these substitutions occurred at amino acid residues that showed variations among different 1b isolates. In contrast, the remaining 16 amino acid changes of HCV-TR1 occurred at conserved amino acid residues.

Table 2. Comparison of inferred amino acids at proteolytic cleavage sites between HCV-TR1, HCV-J and HCV-BK

Genotype	NS2/NS3
HCV-TR1	ADSFKGQGWRLL ↓ APITAY
HCV-J	ADSFGEQGWRLL ↓ APITAY
HCV-BK	ADSLEGRGLRLL ↓ APITAY
	NS4A/NS4B
HCV-TR1	ALYQAFDEMEEC ↓ ASHLPY
HCV-J	VLYQEFDEMEEC ↓ ASHPLY
HCV-BK	LLYQEFDEMEEC ↓ ASHPLY
	NS5A/NS5B
HCV-TRI	SEEASEDVCCC ↓ SMSYTW
HCV-J	SGEAGEDVVCC ↓ SMSYTW
HCV-BK	SEEASEDVVCC ↓ SMSYTW
	NS3/NS4A
HCV-TRI	MACMSADLEVVT ↓ STWVLV
HCV-J	MACMSADLEVVT ↓ STMVLV
HCV-BK	MACMSADLEVVT ↓ STMVLV
	N4SB/NS5A
HCV-TRI	HQWINEDCSPTC ↓ SGSWLR
HCV-J	HQWINEDCSTPC ↓ SGWLK
HCV-BK	HQWINEDCSPTC ↓ SGSWLR

↓, denotes cleavage sites. Amino acid residues of HCV-TR1 that are different from that of two other HCV-1b isolates [34,35] are underlined.

Out of 16 unique amino acid substitutions, half were conserved, the other half not being conserved (Table 3).

Main Features of HCV-TR1 Polyprotein Primary Structure

When compared to consensus HCV 1b polypeptide sequence, ten conserved cystein residues (14, 89, 170, 223, 243, 274, 279, 295, 366, 521) of HCV [20], five N-glycosylation motifs (NXS) of E1 protein [21], the catalytic triad (His_{57}, Asp_{81}, Ser_{139}), AX_4GKS motif involved in ATP binding, as well as Cys_{97}-Cys_{99}-Cys_{145}-His_{149} metal binding site of NS3/NS4A heterodimeric serine protease [22–24] were conserved. In addition, motifs I (207-GXGKS/T-211), II (290-DECH-293), III (322-TATPP-326), V (410-ATDALMTGFTGDFD-423), VI (460-QRXGRXGR-467) and putative Motif IV (370-SK-371) of NS3 helicase were maintained in HCV-TR1 [23–25]. The ISDR (Interferon Sensitivity Determining Region) of NS5A (2209-PSLKATCTTHHDSPDADLIEANLL-WRQEMGGNITRVESEN-2248) was also conserved [26]. The HVRI region, which is the most prone to mutation in HCV, was also maintained for 25 of 27 residues in HCV-TR1 [27]. These comparative data indicates that viral proteins encoded by HCV-TR1 share the main structural and functional features with other HCV isolates.

Table 3. Summary of amino acid differences between the HCV-TR1 Turkish isolate and other characterized HCV-1b genomes

Region	Amino Acid Position	Other HCV 1b isolates[a]	HCV-TR1	Type of Substitution[b]
E2	591	E	D	C
E2	728	D	Y	NC
E2	750	N	I	NC
NS3	1075	N	S	C
NS3	1539	L	V	C
NS3	1628	L	M	C
NS4A	1704	E	A	NC
NS5A	2170	V	T	NC
NS5A	2176	T	I	NC
NS5B	2493	K	E	NC
NS5B	2556	T	V	NC
NS5B	2567	Q	K	C
NS5B	2570	K	M	NC
NS5B	2838	L	I	C
NS5B	2897	S	P	C
NS5B	2996	L	F	C
Core	187	I, T, V	M	
E2	397	F, G, H, I, L, M, Q, R, S, Y	A	
E2	478	D, G, H, N, Q, R, S, V	E	
E2	493	P, Q, R	K	
E2	580	I, T	L	
E2	626	I, L	V	
NS2	828	F, L	I	
NS2	857	L, M	V	
NS2	861	I, V	T	
NS2	949	I, V	L	
NS3	1290	P, S	G	
NS3	1382	I, L	V	
NS3	1636	I, T, V	N	
NS5A	2030	P, Q	S	
NS5A	2079	S, F	T	
NS5A	2302	K, R	E	
NS5B	2554	D, N, Q	E	
NS5B	2617	G, K	A	
NS5B	2665	A, S, V	T	
NS5B	2736	D, N	S	

[a]Data obtained from 48 HCV 1b isolate amino acid sequences available at the GenBank database.

[b]Conserved (C) and non-conserved (NC) amino acid substitutions at residues conserved in all published sequences were based on PAM250 amino acid scoring matrix [36].

Table 4. Comparisons of immunodominant Cytotoxic T cell epitopes of HCV with corresponding amino acid residues in Turkish isolate HCV-TR1

Viral Protein	Amino Acid No.	Epitope[a]	HCV-TR1[*]
Core	178–187	LLALLSCLTV	LLALLSCLT**M**
E2	402–412	SLLAPGAKQNV	SL**F**A**S**G**PT**Q**RI**
NS3	1073–1081	CINGVCWTV	C**VS**G**A**CWTV
	1406–1415	KLVALGINAV	KL*S*G*L*G*L*NAV
NS4B	1671–1680	VLAALAAYCL	VLAALAAYCL
NS5A	1992–2000	VLSDFKTWL	VLSDFKTWL
	2145–2154	LLREEVSFRV	LLREEVSFRV
	2221–2231	SPDAELIEANL	SPDA**D**LIEANL
NS5B	2594–2602	ALYDVVTKL	ALYDVV**S**TL

[a]From Refs 28 and 29.
[*]Amino acid residues of HCV-TR1 that are different from the known epitope are underlined.

The HCV virus was shown to harbor several Cyotoxic T-Cell (CTL) and T-Helper Cell-specific dominant epitopes that may play a major role in host immunity toward viral infection [28,29]. Table 4 shows immunodominant CTL epitopes of HCV, in comparison with corresponding amino acid residues of HCV-TR1. There are 8 known major CTL epitopes of HCV. Among these, one epitope located on NS4B protein (aa 1671–1680), and 2 epitopes located on NS5A protein (aa 1992–2000 and 2145–2154) were fully conserved in HCV-TR1. In contrast, HCV-TR1 displayed amino acid substitutions in the five remaining epitopes located on core, E2, NS3, NS5A and NS5B proteins, respectively. Of particular interest, 6 out of 11 (55%) amino acid residues of an E2 epitope (aa 402–412) were different in HCV-TR1. Similarly, two NS3 epitopes (aa 1073–1081 and 1406–1415, respectively) displayed amino acid changes in three positions (30–33% difference). In contrast to high rate of mutations in CTL epitopes, the dominant T-Helper Cell-specific epitope located on NS3 protein (aa 1251–1259; VLVLNPSVA) was conserved in HCV-TR1 [28].

Discussion

The first observation of this study was that a 100 bp sequence region (−172 to −72) of HCV 5′UTR carries enough sequence variations for differential analysis of 9 subtypes of genotypes 1 through 4, as shown in Fig. 1. The phylogenetic analysis also showed that the HCV isolates identified in Turkish patients did not diverge from other known and commonly found HCV isolates. Indeed, the great majority of these isolates (91%) were identified as subtype 1b with a maximum evolutionary distance of 0.0219 within this group. The subtype 1a was rare (6%), while genotypes 2 and 4 were exceptional (1/79 for each case). Thus, more than 90% of HCV infections in Turkish patients living in the southern region are caused by a single subtype, namely 1b. Our results confirm earlier reports for Turkish patients that showed 1b as a predominant subtype in other regions of Turkey [14,15]. Thus, it appears that HCV infections in Turkey are due almost exclusively to a single subtype, namely subtype 1b. The predominance of 1b subtype in Turkey correlates with the north-south gradient of increased 1b subtype occurrence in Europe. For example, a similarly high frequency of 1b subtype (91%) was reported for Sicilian patients, while only 8% of HCV infections in Finland are due to the same subtype [30]. It was hypothesized that HCV infections with 1a subtype in Europe are due to the use of blood products originating from the USA. The low prevalence of 1a subtype in Turkey may support this hypothesis and indicates that HCV infections in Turkey are due to the local propagation of a 1b subtype. The exceptional occurrence of genotype 4 in southern Turkey is in favor of such a hypothesis. This particular genotype is endemic in Egypt and highly prevalent in the other Middle East countries with the exception of Israel [8,9]. It appears that the genotype 4 did not propagate from these countries towards Turkey.

After identification of subtype 1b as the predominant genotype, whole genome sequencing of a Turkish HCV-1b isolate from a single human carrier was performed. When compared to other variants of 1b subtype, the Turkish HCV-1b isolate displayed highest homology to a Japanese 1b strain. The reasons for this close relationship between Turkish and Japanese HCV isolates are presently unknown. As reported by Smith et al. [31], the average time of divergence of variants of subtype 1b was about 70–80 years ago. In addition, the absence of country-specific groupings by phylogenetic analysis of subtype 1b sequences suggested that the spread of this genotype occurred on a worldwide basis at a similar time [32]. Thus, the high homology of a Turkish isolate (HCV-TR1) with a Japanese isolate provides further evidence for this prediction. The HCV-TR1 polyprotein displayed amino acid substitutions at 36 positions when compared to other 1b variants. More than 50% of these substitutions occurred at residues that were hetero-

genous among different isolates. However, HCV-TR1 displayed specific changes in 16 positions and 8 of them were non-conserved amino acid substitutions (Table 3). Presently, it is unknown whether such changes affect functions of concerned viral proteins, however none of them appear to affect previously known functional motifs of structural and non-structural HCV proteins. Therefore, it appears that the Turkish HCV-TR1 share similar features with other HCV isolates in terms of amino acid residues directly involved in protein function.

In contrast to the conservation of functional characteristics of viral proteins, a high number of immunodominant epitopes of HCV-TR1 displayed structural changes. Of particular interest, three CTL epitopes, one located on E2 and two on NS3 viral proteins displayed a high rate of amino acid substitutions (30–55%). Based on the fact that major functional features of HCV proteins are conserved, but many CTL-epitopes displayed substitutions at several amino acid residues, we believe that mutations affecting immunodominant viral epitopes in HCV-TR1 are not due to experimental errors and represent true changes in the immunogenicity of this strain.

HCV-specific CD8 + cytotoxic T lymphocytes are believed to play an important role in the pathogenesis of liver cell injury and viral clearance in HCV infection [28]. The efficacy of anti-viral cytotoxic immune response relies on the availability of viral epitopes to be recognized by specific CTLs. The fact that 6 out of 9 major CTL epitopes of HCV-TR1 are different from the consensus epitope sequence (Table 4), strongly suggests that the host immune response to this viral strain is defective or deficient. Further studies are needed to know whether mutations affecting immunodominant CTL epitopes may serve as a basis for unusually high frequency of HCV infections with 1b subtype in Turkish patients. More importantly, immunodominant CTL epitopes are considered as candidates for design of therapeutic vaccines for HCV [29]. Such vaccines may not be efficient against strains such as HCV-TR1 since they display major amino acid changes at candidate vaccine epitopes.

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