New Approaches to Hepatitis C Virus Infection

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Hepatitis C virus (HCV) is closely related to pestiviruses and flaviviruses. It was cloned from copy DNA (cDNA) extracted from infectious chimpanzee plasma in 1988. HCV is now recognized as the major causative agent for non- A, non-B hepatitis (NANBH). Based upon nucleic acid sequence analysis, six major HCV genotypes have been identifed worldwide. Enzyme-linked immunosorbant assays (ELISA), recombinant immunoblot assays (RIBA), synthetic peptide assays, polymerase chain reaction (PCR) are utilized for diagnosis. HCV may cause acute and chronic hepatitis, cirrhosis (CIR), hepatocellular carcinoma (HCC). The main route of transmission is parenteral, and most HCV-infected individuals are either IV drug users or recipients of blood products that in the past had not been screened for anti-HCV. Mother-to-infant transmission occurs in about 10% of HCV RNA positive mothers, mainly when maternal virus titers are high. Sexual transmission of HCV is probably absent or rare. There has not been HCV vaccine yet. Interferon is the only agent of proven efficacy in treatment of hepatitis C.The aim of this review is to revise new classification, genotypes, new diagnostic approaches and new treatment protocols about HCV.

Key words: *Hepatitis C virus, genotypes, PCR, diagnosis, treatment, review*

Hepatit C virus infeksiyonunda yeni yaklaşımlar

Hepatit C virusu (HCV) pestiviruslar ve flaviviruslar ile yakından ilişkilidir. 1988'de infekte şempanzelerin plazmasından elde edilen copy DNA (cDNA)'dan klonlandı. HCV non-A, non-B hepatit grubunda major etkendir.Nükleik asit sequence analizleri temel alınarak tüm dünyada 6 büyük HCV genotipi tanımlanmıştır. Tanıda enzim-linked immunosarbant assays (ELISA), rekombinant immunoblot assays (RIBA), sentetik peptid assays, polimeraz zincir reaksiyonu (PCR) kullanılır. HCV akut ve kronik hepatit, siroz (CIR), hepatoselüler karsinom (HCC) meydana getirebilir.Ana bulaşma yolu parenteraldir ve diğer HCV infekte kişilerin çoğu İV ilaç kullananlar veya geçmişte anti-HCV antikorları bakılmadan kan ürünlerini kullananlardır. HCV pozitif annede virus titresi yüksek ise anneden fetusa geçiş oranı %10 civarındadır.Seksüel geçiş nadir veya yoktur. HCV aşısı henüz bulunamamıştır. İnterferon tedavide kullanılan tek ilaçtır. Bu derlemeninin amacı HCV hakkında yeni sınıflandırma, genotipler, yeni tanı yaklaşımları ve tedavi protokollerini gözden geçirmektir.

Anahtar kelimeler: *Hepatit C virusu, genotipler, PCR, tanı, tedavi, derleme*

Hepatitis C virus was cloned in 1988 from cDNA extracted from infectious chimpanzee plasma (1). HCV was shown to be cause of NANBH in over 90% of cases (2). Studies have brought us characterisation of the complete virus, its genetic variability, diagnostic antibody tests, viral nucleic acid detection, epidemiology and antiviral treatment for seven years.

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Virology

HCV is a small-single- stranded RNA virus, 30-38 nm. in diameter with a lipoid envelope. The genome consists of one large open-reading frame of 9379-9481nucleotides (figure:1) (2).

The conserved 5' terminal regions show the putative core and NS 3 regions are relatively well conserved, and antigens from these regions are used in anti-HCV antibody assays (3). Hypervariable domains have been described at the N-terminal part of the E2 envelope region. This mutations in this region probably have a role in viral escape from the host immune response (4).

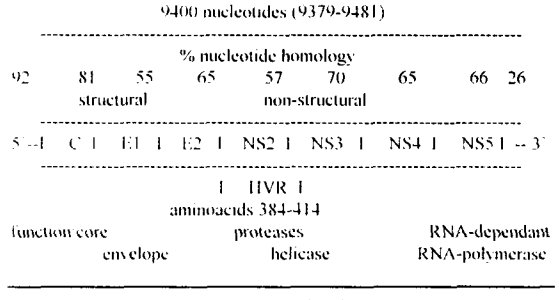

Figure 1: HCV genome organisation

Putative core (C), envelope (E), and non-structural (NS) regions. Nucleotide homology between most divergent isolates is given in percentages among the different HCV regions. Hypervariable region (HVR) is found at the N-terminus of the envelope E2 region.

HCV is classified as a separate genus to the flaviviridae. There are at least six HCV genotypes, according to one proposed classification system based on 5' terminal region and NS5 sequence analysis (5).

HCV infection persists in about 80% cases (6). The reason of this hypervariable envelope domains, other potential factors in immune escape include defective viral particles and extrahepatic virus replication (7). AntiHCV antibodies with different specificities can be detected. However, these antibodies don't show the clearance of the virus or immunity (6,8).

DIAGNOSIS

a) Antibody srceening test:

The first-generation anti-HCV antybody testing with an enzyme-linked immunosorbent assay (ELISA), and second-third generation HCV assays developped. Third-generation anti-HCV ELISAs (ELISA) include antigens from the putative core. and from NS3, NS4, and NS5 regions. ELISA-3 systems are widely used in blood donor screening and are more sensitive and more specific than earlier generation tests (9, 10).

ELISA-3 blood donor screeninig is virtually 100% effective in preventing transmission of HCV to recipients. However, there are two situations in which antibody tests may not detect HCV infection. First, it may take as long as 6 months after primory infection for an anti-HCV response to develop, the mean period between infection and detectable antibody being 12 weeks. Second; immunosupressed patients (eg renal transplant recipients) occasionally have HCV infection without detectable antibodies, in such cases HCV cDNA polymerase chain reaction (PCR) is needed to detect infection. And HCV ELISA-3 is high specific (99.7%), but false-positive results are common among blood donors (10). Anti-HCV ELISA reactivity should be confimed with supplemental assays.

b) Confirmatory tests:

1- Recombinant immunoblot assays (RIBA, Chiron)

2- Synthetic peptide assays (Inno-Lia, Innogenetics) have been developed. In third-generation RIBA system (RIBA- 3) widely used in Europa, there are synthetic peptides from the core and NS4 regions and recombinant antigens from the NS3 and NS5 regions.

Preparation of HCV antigens differs among manufacturers of supplemental assays, which in the case of NS3 antigens is important to reconstitue the conformational epitope. Optimum reconstitution of a (NS3 derived) C3 antigen enhances the sensitivity of the RIBA-3 system compared with the previous generation. RIBA results are generally interpreted as positive when antibodies to antigens of more than one HCV region are detected.

A high proportion (75-80%) of such HCV RIBA-3 positive individuals have viremia, as detected by HCV cDNA-PCR. RIBA-3 positive but HCV cDNA-PCR negative individuals may have cleared the virus from the circulation after previous infection, may be viraemic below the the PCR detection level, or may merely represent false-positive anti-HCV RIBA positive individuals correlate with absence of inflamation in liver biopsy specimens: this observation probably indicates clearence of the virus (11).

c) Virus detection

A standart protocol is PCR after a reverse transcriptase (RT) step, thereby transcribing the viral RNA into cDNA-HCV. cDNA-PCR has developed from research tool into widely used diagnostic test. The most frequent applications are:

1- Confirmation of HCV infection in individuals with RIBA positive or indeterminate anti-HCV antibody results,

2- Early diagnosis in patients with acute hepatitis,

3- Monitoring of perinatal tranmission,

4- Follow-up of antiviral drug treatment (12, 13).

For quantitative HCV RNA detection two adaptations of HCV cDNA-PCR are used:

1- Limited dilution assays,

2- Measurement of the HCV concentration by coamplification of synthetically mutated target RNA. A more precise method is based on signal amplification in a hybridization assay with branched DNA (bDNA). bDNA is less sensitive than cDNA-PCR. By HCV bDNA assay, 70% of HCV cDNA-PCR positive samples are detected.

A commercially available HCV PCR assay, with a single enzyme for reverse transcription and DNA polymerisation, has been recently introduced (14). Table 1 shows amplification methods for HCV RNA detection (15-26).Other methods include ligase chain reaction, and the self-sustaining isothermal nucleic acid based amplification assay.

d) Aminotransferase results

Chronic C hepatitis is characterized by raised or fluctuating concentrations of alanine aminotransferase (ALT) or long-term marginal increases in ALT. 60% of HCV patients have normal ALT values. Therefore, it has limited diagnostic value (2).

Table 1: Amplification methods for HCV RNA detection

Method	Amplifies	Detection	Description
Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)	Target	Qualitative	RNA is extracted, cDNA is synthesized by RT. Single or ' nested set ' primers are used to amplify target sequence(s) over a million-fold during thermal cycling. Target is detected in amplification product by hybridization with oligonucleotide probe or ethidium bromide staining/ gel electrophoresis.
Endpoint Dilution RT-PCR	Target	Semi-quantitative	Serial dilutions are made from patient plasma or RNA extract. HCV RNA titer calculated from highest dilution giving positive signal.
Competitive RT-PCR	Target	Semi-quantitative	A series of known concentrations of synthetic HCV mutant DNA is co-amplified with patient samples. Amplification products show distinct bands upon electrophoresis. Comparison of patient sample band with known concentration bands enables semi-quantitation.
Multycyclic RT-PCR	Target	Qualitative, semi-quantitative	Standards containing known concentrations of HCV RNA are co-amplified with unknowns. Amplification cycles are stopped periodically for sampling of products. Products are dot-blot hybridizied with oligonucleotides probes. Patient samples are compared to standard series for semi-quantitation.
NASBA (Isothermal nucleic acid amplification)	Target	Qualitative, semi-quantitative	Multiple enzymes and primers are used to amplify target RNA sequence over one billion-fold. Detection is by means of gel electrophoresis or oligonucleotide probe hybridization.
Branched DNA(Quantiplex)	Signal	Quantitative	HCV RNA is captured on microwell by synthetic oligonucleotide target probes. Second set of target probes hybridizes to the 5'NCR and core regions of the RNA, and also to bDNA amplifiers. Alkaline phosphatase-linked probe is hybridized to complex. Light emission from reaction with chemiluminescent substrate is directly proportional to amount of RNA.

HCV GENOTYPES

Phylogenetic analysis of NS5 and E1 nucleotide sequences from samples obtained worldwide indicate that there are six major HCV types (19, 27, 28).

Distinction of HCV genotypes is important, since outcome of HCV disease and response to antiviral therapy with interferon correlates with HCV type. Genotype 1, especially subtype 1b, has been associated with more severe chronic liver disease and poor response to interferon therapy (2, 27).

Genotypes 1, 2, and 3 have a brad distribution, with varying frequencies in particular geographic regions. Blood donors from western Europe and Australia are primarily infected with type 1, 2, or 3. In the Far East 1, 2, or 3, 6 have been reported. In the USA, subtypes 1a and 1b are common. Type 4 may have in Africa. Type 5 is prevalent in South Africa. Type 1 and 4 is found in asymptomatic blood donors (27).HCV genotyping methods is shown in Table 2 (16-25).

PCR product followed by multiple probe hybridization (Inno-Lipa) Genotype-specific oligonucleotide probes are immobilized on a membrane strip. amplification product from nested PCR is hybridized to the strip. The pattern of positive and negative probe lines is used to differentiate HCV types and subtypes

Table 2: HCV genotyping methods

Method	Brief Description
Direct sequencing of PCR	Primers from any variable region of the genome (the product core region being preferred) are used to amplify cDNA for direct sequencing
PCR amplification using type-specific primers	Type-specific primers from core and NS5 regions are used to amplify cDNA
PCR followed by probe hybridization	Type-specific oligonucleotide probes for sequences in core and E1 regions are used after non-type-specific PCR amplification
Restriction fragment length polymorphism analysis (RFLP)	cDNA from the 5'NCR region is amplified; amplicons are digested by restriction enzymes and electrophoresed. Distinct bands differentiate types
Serotyping	Type-specific synthetic peptides from the NS4 region are used as capture antigens in ELISA assay. Blocking antibody or competing peptide is used to overcome serological cross-reactivity

CLINICAL MANIFESTATIONS

The natural history of HCV infection represents a balance between viral virulance and host defense and is measured by the disease outcome of this interplay. Hence the natural history of the infection must be describied in terms of

1- the natural history of the virus and particularly its penchant for persistent infection,

2- the natural history of the host's immune response inability to completely neutralize this offending organism,

3- the natural history of disease, hepatitis C, particularly its chronic manifestations (29).

Classically, chronic HCV infection has been thought to occur in 50% of infected individuals measurement of HCV RNA in serum and liver suggest that the extent of persistent infection is higher than the estimate of chronic hepatitis and may be in the range of 80%- 90% . Perhaps there is no more efficient in the viral kingdom. It is demonstrated ,that this agent chronically infects approximately 1% of the world's population (29).

The fact that 80% or more of HCV-infected individuals have persistent infection despite the presence of multiple HCV-directed antibodies suggests that antibodies have minimal role in viral clearance.

The neutralizing responses are highly strain specific and that the quasispecies nature of virus allows other strains to emerge when the predominant strain is under immun pressure, perpetuating the infection in the face of measurable neutralization. HCV persists because of its quasispecies nature and the generation of escape mutants and that host immune responses are generally unable to contain the emergence of neutralization-resistant variants (28, 29).

Acute hepatitis C is generally a asymptomatic diseases. In the transfusion setting, where the acute onset is best documented, 70% - 80% of cases are anicteric and asymptomatic. In a study

series of 86 consecutive post transfusion hepatitis cases, only 30% had a bilirubin greater than 2.5 mg/dL and the mean peak ALT was 708 U/L. In the acute HCV infection, where cases are identified and defined by other presentation with overt clinical illness, 70% were ichteric, 4% had an ALT level between 2.5 and 5 times the upper limit of normal (ULN), 22% had an ALT level 6- 15 times the ULN and 74% had ALTs that exceed 15 times the ULN (29).

Documented fulminant hepatitis C is extremely rare. In HCV infection, the mechanism of liver cell destruction in either acute or chronic infection is largely unknown and it is still unclear if the virus is directly cytopathic (30, 31).

More severe manifestations of HCV disease have been observed in several studies. In Spain, it was reported that there were both transfusion-associated and sporadic hepatitis (29). It was found that only 6%- 10% had apparent remission, while 30%- 39% developed cirrhosis, 13%- 15% experienced hepatic decompensation and 2%-7% developed hepatocelluler carsinoma (HCC). The overall rate of liver-related mortality during the course of the study was 4%- 9% . Ther was no significant difference in disease outcome between transfusion-acquired and sporadic hepatitis.

Chronic hepatitis C is now also the leading indication for treatment with alpha interferon (IFN). Again, this represents a selection bias because IFN is primarily used for viral-induced liver disease and because patients with hepatitis C respond better and with lower dosages than do patients with hepatitis B. Despite this bias, the multitude of patients being treated with IFN attests to the large number of HCV-infected persons demonstrating serious or potentially serious liver lesions. In the U.S.A. multicenter trial of IFN, 27% of enrolles had chronic active hepatitis (CAH) and 55% had active cirrhosis (29).

In Division of Molecular Virology, Baylor College of Medicine (Houston, TX, USA); in the study that I joined , HCV RNA was found to be positive in the sera of 114 hemophiliac patients out of 232 (49.13 %). This high rate depends on factor-VIII. Administration in the other study which is not published yet, I searched HCV RNA by PCR on blood center samples. I found HCV RNA positive in 76 of 372 samples (20.4%). In 100 of total samples (26%), there were anti-HCV positive and high ALT levels.This situation may be related to the high member of drug users and homosexual persons in USA.

In Italy were asked to enroll in a study, to determine the prevalence of chronic liver disease. and 6917 of 10 150 (69%) citiziens enrolled. Analysis revealed that 17.5% of the population had persistent evidence of chronic liver disease including 1.1% with cirrhosis and 0.07 % with HCC. The prevalence of anti-HCV in the entire population was 3.2%, threefold higher than the prevalence of HBsAg. HCV infection was the second leading cause of chronic liver disease in this population, accounting for 16% of cases. Although alcohol was the leading cause of chronic liver disease (CLD), the combination of HCV and alcohol resulted in a tenfold increase in cirrhosis (CIR) and a six-fold increase in HCC compared to alcoholic with no evidence of viral hepatitis. Among the 78 patients that had CIR, 28% were related to HCV, 26% to alcohol and 8% to alcohol in combination with HCV (29).

The acute clinical presentation of hepatitis C has mainly been documented in transfusion-associated cases in which the mean incubation period from transfusion to first increase in ALT is 6-8 weeks (range 2-26 weeks). Acute hepatitis C leads to symptoms in a minority of cases, and as a rule the cinical course is mild. Only 10% of become jaundiced and, although fulminant hepatitis C was previously reported in NANBH patients, most of these cases were later shown not to be related to HCV (2).

The infection persists in about 80% of cases and usually leads to chronic persistent or chronic active hepatitis (32). Progression toward CIR in HCV-infected individuals is probably enhaced in the presence of other risk factors such as co-infection with hepatitis B virus or HIV virus , or hepatotoxic agents such as alcohol.

HCV RNA testing and histological evaluation of liver biopsy specimens showed that the normal ALT concentrations concealed persistent HCV infection with ongoing hepatitis and progression to CIR. HCV infection can also be accompained by autoimmune phenomena; eg , antibodies reacting with epitopes of liver/kidney microsomal (LKM) fractions. Anti-LKM reactivity of

autoimmune hepatitis type 2 is associated with HCV infection (33)

TREATMENT

Interferon is the only agent of proven efficacy in the treatment of hepatitis C. Standart treatment is interferon alpha 2b (INFa2b) at a dose of 3 million units three times per week. The initial course of treatment is 6 months, but nearly all patients relaps and require retreatment. The goal of INF treatment is suppression of active disease, this usually requires long-term therapy. Eradication of virus does not appear to be realistic goal in most patients (34).

Treatment of chronic hepatitis C :

Standart therapy:

Standart initial therapy for chronic hepatitis C infection is recombinant INTa2b at a dosage of 3 million units administered subcutaneously, 3 times per week for 6 months.

Prior trials indicated that 41% of patients normalized the serum ALT level during treatment and 70% of responders had histologic improvement. Response to treatment is greatest in those patients without advanced inflammation or CIR , high HCV RNA levels, or genotypes 1a and 1b. Almost all responders (normal ALT) lose detectable HCV RNA by reverse transcription PCR (RT-PCR) by the end of therapy. However, relaps occurs in 50-70% of patients after the end of the initial course and is associated with return of detectable HCV RNA (34).

In a INF study that I did with F. B. Hollinger, M. D. (at Baylor College of Medicine , Houston,TX, USA), on 36 of 144 patients with chronic hepatitis C, detectable HCV RNA loosed by RT-PCR and high ALT levels decreased normal values, after 6 mounthly standart therapy.

Alternative regimens: Higher doses, longer duration:

The efficacy of INF treatment is to improve the initial response and its durability. Controlled trials shown that the alternate regimens including higher doses, daily dosing or longer durations of therapy have not shown that these schedules improve the response rate (35, 36).

Adjunct to INF therapy and combination therapy:

Ursodeoxycholic acid has been proposed as either a single agent or adjunct to IFN, but its effects on viral replication and inflammation have been incompletely examined. N-acetyl cysteine (NAC) an antioxidant and glutathione source has been shown in one pilot study to induce response to INF when patients had previously failed to respond.

Ribavirin has not proven to significantly increase response to IFN in a single published study. This agent role has only begun to be explored. No scientific rationale exists for prednisone pretreatment in chronic hepatitis C. Corticosteroids increase HCV replication (37, 38, 39).

Alternatives to IFN:

Thymosin is not effective. Ribavirin shows some promise as a single agent in the treatment of chronic hepatitis C, but its effects are difficult to interpret. It is possible that the agent acts through inhibition of some effector to tissue damage. Clarification of the mechanism of action of this agent will help to define the pathogenesis of hepatic injury in HCV infection (40).

Treatment of acute hepatitis C:

There is a growing consensus that INF treatment of acute hepatitis C reduces the risk of chronicity. Trials have shown that a reduction in the proportion of patients with either abnormal ALT levels or detectable HCV RNA following a 4-12 week course of IFN. Although most "responders " maintain their response, some early responders have evidence of infection at a later follow-up (41, 42).

New classes of therapeutic agents such as proteinase inhibitors, antisense compounds, and therapeutic vaccines will eventually find their way to clinical trials.

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