CLONING, PHYLOGENETIC AND EXPRESSION ANALYSIS OF NS2 GENE FROM HCV INFECTED PATIENTS IN PESHAWAR

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ABSTRACT

Hepatitis is liver-specific blood borne disease, which may be acute and chronic. Hepatitis C virus is one of the fundamental cause of liver inflammation and could result in cirrhosis and hepatocellular carcinoma. The prevalence of HCV in Pakistan has been reported to be 4.7% and genotype 3a has been found to be the leading cause of infection in Pakistan. HCV is RNA virus, single stranded and belongs to the family flaviviridae. The RNA genome is 9.6kb in length. NS2, one of the important antiviral drug targets is a transmembrane protein. It contains an autoprotease mechanism causing cleavage at NS2/NS3 junction. NS2 interacts with E1, E2, P7, NS3-4A, NS5A and NS5B thus maintaining co-ordination between different kinds of proteins. In this study a total of 50 patients having positive HCV RNA in their serum were selected from Peshawar district. Extraction of viral RNA followed by cDNA synthesis was performed and then we PCR amplify NS2 gene and was confirmed through agarose gel electrophoresis and subsequently sequenced through Sanger sequencing. Next NS2 gene was cloned into TA vector and sequenced. Phylogenetic analysis was performed on the sequences reported in this study with sequences reported from different regions of the world. NS2 gene was cloned into expression vector and expression analysis was performed in Huh-7 cell line. Cell culture model system developed in this study could be used in future for screening antiviral drugs, as alternative treatment options are needed. In Khyber Pakhtunkhwa few large scale studies have been conducted in different districts and cities but no detailed data is available concerning NS2 gene. This study is a starting step towards the development of novel alternative treatment options that are needed in order to prevent growing HCV infections.

Key words: PCR, Cloning, HCV

INTRODUCTION

Hepatitis C is a transmittable disease that mainly affects the liver. It is triggered by hepatitis C virus (HCV). The virus belongs to a family called Flaviviridae. Choo and co-workers reported in 1989 that hepatitis C is a novel viral agent and it initiate non-A, non-B hepatitis[1]. Humans are the only natural hosts for HCV. Since its discovery, HCV is a foremost health threat to the people globally[2]. Hepatitis C virus is from genus Hepacivirus and it is a prototype member of this genus. Hepatitis C virus is further categorized into seven main genotypes and it differs in their nucleotide sequence by about 30 percent. These 7 different genotypes differs in the way they transmit, its disease evolution and its universal dispersal. Hepatitis C virus is generally spread by contact with blood of infected person and blood products[3].

Due to ease of accessibility of drugs and injectable therapies ratio of HCV has greatly increased. Detection of HCV in initial stages is very difficult because it is often asymptomatic[4]. This makes its treatment very difficult and it is mostly known as "silent disease". Hepatitis C virus causes both acute and chronic infection. 60--80% of patients will develop chronic HCV infection when liver is not able to clean the virus[5]. Currentlyno vaccine is available against the virus. In more than 50 percent of the patients liver steatosis occurs[6]. Non-specific indications such as generalized body aches, weakness, fatigue, nausea, vomiting and pain in the right upper quadrant occur in patients with Chronic Hepatitis C (CHC).

It is reported by current research that "many stem loop structures are present in the negative strand 3'-NTR.This region is known by the viral polymerase as the initiation place for plus-strand creation of the HCV genome"[7]. MicroRNA, miR-122 is found to be in excess in human liver and is precisely expressed[8]. Hepatitis C virus encodes a single polyprotein of about 3010 amino acids. The proteins which are involved in structure are (core, E1 and E2 and P7 proteins. HCV core which is a multi-functional protein and is greatly essential[9]. It helps in the formation of structural element of the virus particle.

The core protein which is expressed in transgenic mice develops Hepatocellular carcinoma which shows that core protein plays a direct role in this process[10]. Enveloped glycoproteins are the E1 and E2. They are structural components of the virion. Host-cell entrance through receptor binding requires both E1 and E2.NS-2, NS-3, NS-4A, NS-4B, NS-5A and NS-5B which are

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non-structural protein are assumed to be essential for replication of the viral genome. P-7 is placed at intersection of the structural and nonstructural protein.P-7 is from viroporins family of viral proteins and it helps in formation of ion channels. NS2 is a non-structural protein and its molecular weight is 23 kDa, 217 amino acids. It has hydrophobic aminoterminal sub-domain having 3 putative trans-membrane parts and a carboxylterminal cytoplasmic domain. NS2 is from H77 strain (genotype 1a) and JFH1 NS2 which is genotype 2a is more stable[11]. NS2-3 protease is formed by the Cterminal domain (residue 94-217) of NS-2 and residues of 1-181 of NS3[12].

For full length RNA replication of HCV replicons cleavage at the NS2 and NS3 intersection is essential. Pegylatedinterferone (INF-alpha) is the drug of choice for the treatment of HCV infections.When INF is activated in body it is known to activate many other defense mechanisms against viruses. But mechanism of INF in vivo is still not clear. Ribavirin (antiviral) which is also used against HCV in combination with IFN. It was first used against respiratory virus infection in children. Ribavirin has broad spectrum antiviral activity[13].When it is used in blend with PEG-INF-alfa-2a it results in improved sustain viral response (SVR) rate. But its mechanism against HCV is not fully clear but some ideas about its mechanism include: change in host immune response, straight inhibition of RNA polymerase of virus, and fatal mutation of HCV-RNA genomes by ribavirin triphosphate by NS5B[14].

Ribavirin is not effective in mono-therapy but it can be a substrate for HCV RNA polymerase[15]. So for handling of HCV ribavirin and (PEG-INF) combination is the present gold standard treatment. But unluckily both ribavirin and (PEG-INF) are harmful and several side effects like fever, depression, myalgia and haemolyticanemias can be faced. Further charges of the treatment are very high. For obliteration of Chronic stage of HCV a lot of time is required up to several months, despite which SVR is mostly not attained. For virologist it is a task to develop an improved and healthier method for treatment of HCV. It is very hard to treat genotype 1 and 4 of HCV[16] and percentage of patients achieving SVR with 1 genotype in only 40-50 percent.

Discovery of PCR make a great contribution in history of science as now it is very easy to amplify a piece of required segment of DNA or RNA in a very limited time. Previous method of amplification was very difficult and time consuming. There are several procedures now for cloning of amplified DNA molecules which include ligation-independent cloning[17], blunted cloning TA cloning etc. Taq DNA polymerase is also proficient in adding additional non template nucleotide to the blunt-ended DNA. PCR Products mostly which are amplified by PCR has a 3'-A overhangs at both ends. Linearized "T vector" having 3'-T at each end can be used straightly to amplify PCR products having 3'-A overhangs[18]. This process is known as "TA cloning". For cloning of products of PCR TA cloning method is modest and more effective than blunt-ended ligation. TA cloning method can be simply transformed into a universal cloning method with slight changes[19]. DNA fragments are made blunt by incubation with T4 DNA polymerase and then Taq polymerase is added in the situation where DNA fragments have protruding 3'-ends as defined above. With this method up to 90 % of cloning efficiency is accomplished[20].

For proper research and study on replication and life cycle of HCV there are many limitations like absence of replication models for better understanding of its life cycle. Many in vitro cell culture models have been studied in chimpanzee and zebra fish models but these models have their boundaries. Intra model cell lines for example Huh-cell and heptoblastoma cells are competent in reduced infection. Replicons are those HCV fragments which are cloned and transfected. Using western and northern blotting these replicons can be easily noticed[21].

Wakita and Zong and Yei et in 2005 disclosed that in Huh 7 cell line cloned HCV2a JFH1 was effectively transected and infectious particles were produced[22]. Current research shows that HCV molecular mechanism can be examined more effectively through cell lines of replication of 3a genotype. Silencing of core protein of genotype 3a of HCV is achieved by RNA intermediated (RNAi). In current research, a serum of HCV genotype 3 was infested and natural environment was given to the cells in order to progress a reliable in vitro infection centered culture model in Huh-7 cell lines. In our model HCV genotype 3a replication was silenced with siRNA directing much conserved core region.

On the basis of phylogenetic analysis there are 7 major genotypes of HCV which is further classified into subtypes.3a is the commonest genotype in Pakistan and in Khyber Pakhtunkhwa.This study deepens the molecular characterization of hepatitis C virus genome by studying the NS2 protein different aspects like its cloning, phylogenetic and expression analysis. There has been a little data concerning in vitro cell culture model system of NS2 and no detailed study has been done in Khyber Pakhtunkhwa.

METHODOLOGY

Thisdescriptive study was carried out in Institute of Basic Medical Sciences (IBMS), KMU during one year. A total number of 50 serum sampleswere collected from HCV-infected patients in Hayatabad Medical Complex (HMC) Peshawar.Both male and female patients who were ELISA positive for HCV antibodies, Real Time PCR positive samples with High viral loadand patients from different districts of KP were included in this study. PCR negative samples or samples with Low viral load, incomplete sequences with incomplete gene segments, recombinant and synthetic sequences, patients co-in-

fected with HBV and HCV were excluded from the study.

HCV positive patients were brought to the microbiology laboratory for genomic studies. Aseptically, 3ml whole blood was taken from each patient. All the specimens were stored at -80°C. For the confirmation of synthesis of cDNA, PCR was carried out by using already published set of primers designed from 5'UTR region of HCV genome (forward and reverse) with optimized conditions. PCR amplification of NS2gene was done by usingprimer3 software. After confirmation of HCV infection, the previously synthesized cDNA was used as template for amplification of NS2gene. The amplified NS2 fragment was cloned into pTZ57R/T cloning vector through the optimized Protocol.

Samples were Sanger sequenced. The eluted DNA was sequenced. Then, expression of the plasmid was made through RT-PCR. After BLAST analysis scanned sequences having maximum identity were subjected to phylogenetic analysis to find out the phylogenetic relationship with sequences obtained in this study. Phylogenetic tree was developed using Mega 6 software with Neighbour Joining and Maximum Likelihood algorithms.

RESULTS

HCV 5'UTR region confirmation

PCR for cDNA synthesis confirmation was run using 5UTR region of HCV genome. 170bp target region was amplified using primers.

Amplification of NS2gene

The NS2 gene of HCV was amplified through PCR at specific conditions of the primers. The amplified product was resolved on 2%agarose gel along with 100bp DNA size marker. Figure 4shows the amplification of desired fragment of 675 bp.

Confirmation of TA Cloning through Colony PCR

The cloning was confirmed through colony PCR using the gene specific primers. The amplified product was resolved on 2% agarose gel along with 1kbp DNA size marker. Figure 3 shows the amplification of desired fragment of 675 bp.

Sequencing

PCR amplification of NS2 gene was confirmed through Sanger sequencing.

NS2 gene sequence:

GGTGAAGACAGCGCAACCCTAGGCCCATGGCTGG-
GGTCTGGTCTCTTCGGCTTTTTTACCTTGTCACCTTG-
GTATAAGCATTGGATCGGCCGCCTCATGTGGTG-
GAACCAGTACACCATATGTAGATGCGAGTCCGC-
CCTTCAAGTGTGGGTCCCCCCTTTGCTTGCGCGT-

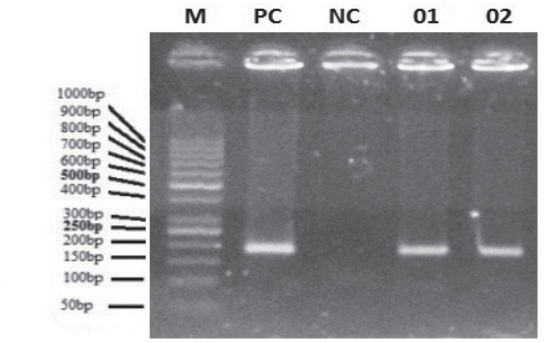

Figure 1: Lane 1 DNA size marker (50 bp), Lane 2 Positive Control, Lane 3 Negative Control and Lane 4-5 PCR product (170bp).

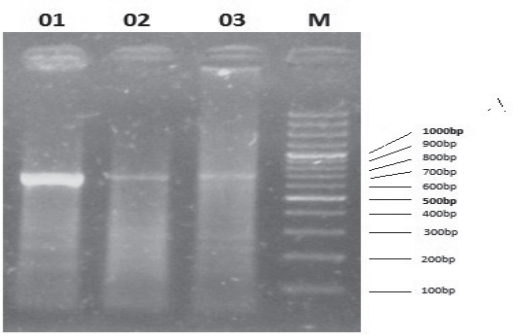

Figure 2: Lane 1-3: amplified gene NS2 Lane 4 DNA Marker (100b)

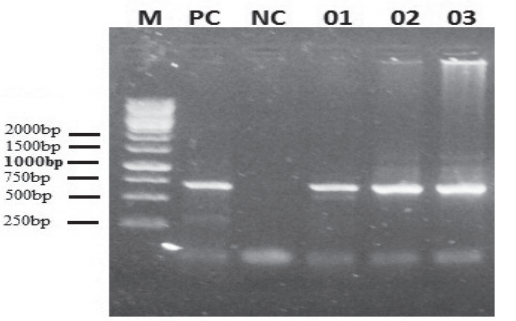

Figure 3: Lane 1 DNA size marker (1kbp), Lane 2 Positive Control, Line 3 Negative Control and Line 4-6PCR product (675bp)

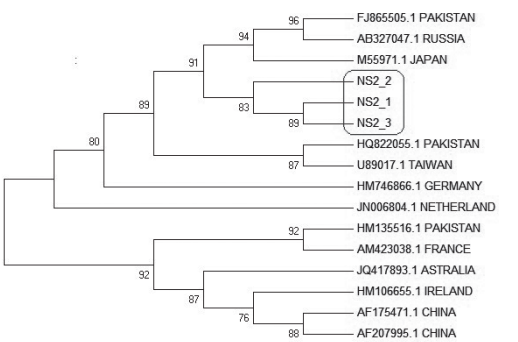

Figure 4: Evolutionary relationship of NS2 genes by Maximum Likelihood method.

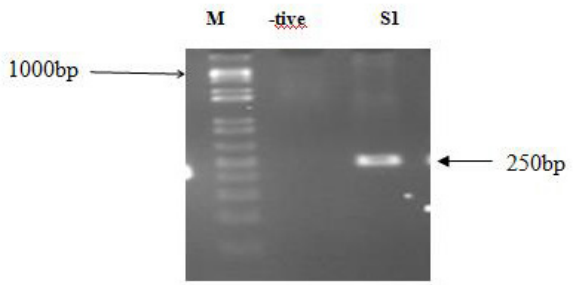

Figure 5: Expression of the NS2 gene through RT-PCR. Lane 1: DNA size marker (50bp), Lane 2: negative control. Lane 3: NS2 amplified from cDNA.

GGGGGTAGGGACGGTGTCATCCTGCTAACAAGCCTGCTTTATCCATCCTTAATTTTTGACATCACTAAGCTGCTGCTAGCAATACTGGGCCCATTATACCTAATACAGGCTGCCATCACTGCTACTCCCTACTTTGTGCGTGCGCATGTACTGGTCCGCCTTTGCATGCTCGTGCGTTCCGTGGTGGGGGGAAAATACTTCCAGATGGTCATACTGCACATTGGCAGATGGTTCAACACCTACCTGTACGACCACCTAGCGCCAATGCAACACTGGGCCGCAGCCGGCCTCAGAGACTTAGCAGTGGCCACTGAACCTGTAATTTTTAGTCCCATGGAGATTAAGGTTATCACCTGGGGCGCAGACACAGCGGCTTGCGGAGATATTCTGTGCGGGCTGCCCGTCTCTGCGCGATTAGGCCGTGAGGTATTGTTGGGACCTGCTGATGACTATCGGAAGATGGGCGGGGGCTTTATGAAA

Phylogenetic analysis

Phylogenetic analysis reveals that whether the sample sequences clustered with previously reported isolates. Evolutionary analysis based on 3 partial NS2 genes of HCV having higher % nucleotide identity with reference sequences retrieved from NCBI.

Expression of NS2 gene

NS2-mRNA expression was investigated, prepared plasmid was transfected into huh-7 cells and mRNA expression level was assayed by RT-PCR. As described in materials and methods the cDNA was prepared first using RT-PCR and RT primers. 1.8% agarose gel with 50 bp DNA size marker was used. Figure 5 shows the results of the NS2 gene expression by RT-PCR.

DISCUSSION

Hepatitis is an infectious transmissible disease affecting millions of the people around the world. It can be acute and chronic. Among the viruses the leading cause of the liver inflammation is hepatitis B and C viruses. Hepatitis C is initiated by the Hepatitis C virus. In chronic infections with hepatitis C it can result in the deadly cirrhosis and hepatocellular carcinoma. Hepatitis C virus isa single stranded RNA virus, belongs to the family Flaviviridae. The HCV genome is a long polypeptide chain of 3010 amino acids which is processed into 10 proteins by cellular and viral proteases. Among these proteins, (core protein, envelop protein E1 and E2)are structural proteins and 7 are nonstructural proteins (P-7, NS-2, NS-3, NS-4A, NS-4B, NS-5A and NS-5B). There are 7 major genotypes of HCV which is further classified into subtypes.3a is the commonest genotype in Pakistan. The genotype which is most prevalentin Khyber Pakhtunkhwa is 3a.

This study deepens the molecular characterization of hepatitis C virus genome by studying the NS2 protein different aspects like its cloning, phylogenetic and expression analysis. There has been a little data concerning in vitro cell culture model system of NS2 and no detailed study has been done in Khyber Pakhtunkhwa.

In the present research work HCV positive patients were selected from native population. Extraction of RNA from hepatitis C virus was done using commercially available kit. For cDNA synthesis MMLV reverse transcriptase along with antisense primers were used using RT-PCR. cDNA synthesis was confirmed through 5'UTR PCR and for confirmation agarose gel electrophoresis was performed. The open reading frame of amino acid was obtained to be further considered for Expression analysis. After BLAST analysis scanned sequences having maximum identity were subjected to phylogenetic analysis to find out the phylogenetic relationship with sequences obtained in this study. Phylogenetic tree was developed using Mega 6 software with Neighbour Joining and Maximum Likelihood algorithms. From phylogenetic analysis it could be observed that Evolutionary analysis based on 3 partial NS2 genes of HCV has greater % nucleotide similarity with reference sequences retrieved from NCBI.

NS2 region of HCV has many viral and cellular proteins which supports in many viral actions. Outcomes of this research in this manuscript demonstrate that individual HCV gene can be expressed in human hepatoma cell line. Sufficient research is not done on genotype 3a and to combat the problems of resistance presented by HCV. Genotype 3a is very common in South-east Asia.

This research demonstrated the molecular aspects of HCV3a NS2 gene such as isolation, amplification, sequencing, cloning, phylogenetic and expression analysis and will play important role in developing sequence- specific drug targets and small interfering (siRNA). Due to the non-availability of vaccine this research study could be of great help to the local population.

This study ofHCV3a NS2 gene has been performed for the first time from Khyber Pakhtunkhwa population. Screening for the development of drugs particular to the sequence can be performed for native population. Our study is a huge step for treatment of HCV and can help researchers to disrupt the code for

this deadly virus.

CONCLUSION

In conclusion, this study defines the isolation, amplification, sequencing, cloning, phylogenetic and expression analysis of HCV3a NS2 gene. In Khyber Pakhtunkhwa this study has been done for the first time.NS2 expression study could be used in future for screening and the development of novel anti-HCV therapeutic agents specifically for the native population. In future this study of NS2 genome will generate important information to the scientific world of medicine and will help in the development of novel therapies against the deadly HCV affecting millions of the people around the globe.

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