ORIGINAL ARTICLE

PCR AMPLIFICATION AND EXPRESSION ANALYSIS OF P7 GENE OF HCV IN Huh-7 CELL LINE

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ABSTRACT

Objectives: To know the PCR amplification and expression analysis of p7 gene of HCV in Huh-7 cell line.

Material & Methods: In this study, blood samples were collected from HCV positive patients from Peshawar Khyber Pakhtunkhwa, Pakistan. RNA was extracted and then converted into cDNA using gene specific primer. P7 gene of HCV was amplified using PCR and subsequently sequenced through Sanger sequencing. P7 gene was then cloned into mammalian expression vector pCR3.1and expressed in Huh-7 cell line. RNA was extracted from transfected cells and then converted into cDNA with the oligo (dt) primers, expression of p7 gene was studied.

Results: For the first ever time in KP, Pakistan, p7 gene was amplified, sequenced, cloned and expressed in Huh 7 cell line.

Conclusion: Expression of p7 gene of HCV can be used for comparative analysis of another strain of HCV of different location and this provides an opportunity for the development of novel anti-HCV drugs with good efficacy and minimal side-effects.

Key Words: RT (reverse transcriptase), Hepatitis c virus, Untranslated regions, human hepatocellular7- cell line.

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INTRODUCTION

Hepatitis Cvirus infection results into chronic hepatitis, cirrhosis and hepatocellular carcinoma. In the western world, liver transplant is very costly and a financial burden[1,2,3]. In Pakistan, it affects 5% or around 12 million people annually, out of which 1 million develop hepatocellular carcinoma while four million, develop chronic hepatitis[4].

Prevalence of HCV infection is more in the developing countries because of two facts: one is the usage of contaminated blood products not following the standard protocols regarding the screening of blood transfusion[5]. Second, there is no proper cell culture model to fully study the HCV virus pathogenesis immune control at molecular level despite of increasing knowledge of genome structure and individual viral proteins[7].

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Hepatitis C virus is a virus of flaviviridae family and its length is 9.6kb. It has been identified as an enveloped positive sense single stranded RNA virus. It has a long reading frame which consists of 3010 amino acids[9]. Hepatitis C virus has 5` and 3`untranslated regions. This 5`UTR site contains internal ribosome entry site (IRES), This site mediates the translation of HCV RNA by viral and cellular proteases. 5UTR plays an important role in initiation of HCV replication[10].

HCV genome consists of structural and nonstructural genes. Of structural genes are Core protein, E1, E2glycoproteins which has hypervariable regions and p7protein[10,11]. Nonstructural proteins are NS2, NS3, NS4A, NS4B, NS5A and NS5B[7,12]. Virion constitutes the core protein, envelope proteins E1, E2and p7 protein which is essential for production of infectious particles[13]. A core protein forms a nucleocapsid which consists of HCV nucleic acid surrounded by lipid membranes. The core protein is involved in hepatocarcinogenesis[14]. P7 is a structural protein situated between E2 and NS. It is important for virus particle assembly and its egression. P7 also has an ion channel activity which is sensitive to small structured molecules and it modifies the membrane permeability[15].

Development of cell line for expressing structural and non-structural proteins of HCV plays an important role in identifying the important genes and their replication process and their role in the development of cirrhosis and hepatocellular carcinomas. These stable expressing cell lines also have direct effect on the prognosis of the disease. In this study isolation, cloning, sequencing and expression analysis of p7 gene HCV isolates from KP population has been done for the first time.

Non-structural proteins are cleaved from the rest of HCV polyproteins by HCV proteases. NS2 is transmembrane protein, causes the NS2 and NS3 cleavage[16]. NS2 and NS3 are important for the synthesis of structural viral protein and NS3 also has serine protease activity and helicase-ATPase activity and causes cleavage of NS3-4A,4A-4B,4B-5A,5A-5B[17]. NS3 mediate viral replication in the host cells[18]. NS4A is essential for HCV RNA replication. NS4B forms the membranous web site for viral RNA replication[19]. Finally NS5A has ISDR which contain interferon alfa stimulated genes which usually indicates the sensitivity or resistance of HCV patient to the interferon treatment[20]. This NS5A protein is associated with apoptotic signaling pathway and has a role in transformation of hepatocytes in hepatocellular carcinoma. NS5B is the RNA-dependent RNA polymerase (RdRp) which is also being actively targeted for antiviral drug development. NS5 is among the hypervariable regions while others include, E1, E2, and core region of HCV[21].

This study was carried out especially involving p7 protein of HCV. In this p7 gene of HCV was amplified through PCR, sequenced, cloned and expressed in expression vector. In general population where HCV frequency rate is up to 10% such studies are helpful for therapeutic purposes.

MATERIAL AND METHODS

Blood samples were taken from HCV positive patients of tertiary hospitals of Peshawar, Pakistan from January 2016 to June 2016. In this study, serum samples used were exclusively of those patients for whom quantification results were obtainable and were confirmed to be infected by HCV subtype 3a. RNA was extracted using silica membrane technology. RNA was purified. Purified RNA was placed in hydration solution for cDNA conversion. cDNA was synthesized using the reverse transcription with Moloney Murine Leukemia transcriptase (kit name, M-MLV RTase). Then, thermal cycling was performed. This cDNA was confirmed by 5UTR PCR. Next p7 gene of HCV was amplified by using specific gene primer using Primer 3 software http://bioinformatics.weizmann.il/cgi-.bin/primer/primer3.cgi.

Sequence of the primers should be same with target sequence. Program blast is used to compare the sequence with Genbank. This blast is available at the National Centre for Biotechnology Information website www.ncbi.nlm.nih.gov. When both primer sequences showed homology to a geneoptimization of primers' reaction conditions were performed. The primers were optimized in order to obtain suitable PCR product. In the reaction with Taq DNA polymerase, the PCR product of a specific gene was confirmed by Agarose Gel. The PCR product was of 240 base product sizes. After PCR amplification, the PCR product was purified through gel elution for further sequencing and cloning. For this purpose PCR product was run on 1.5% low melting TBE Agarose gel and eluted and samples were sent to CAMB for Sanger sequencing. After sequencing the amplified gene with added restriction sites, it was cloned in pCR3.1/FlagTAGexpression vector**.** To investigate the p7-mRNA expression, prepared plasmid was transfected into huh-7 cells and assayed the mRNA expression level by RT-PCR. The sequence was submitted in Genbank.

RESULTS

HCV cDNA synthesis was confirmed by using the 5UTR region of HCV genome. Target region was amplified through PCR using arbitrary primers. The PCR amplification of p7 gene was resolved on1.8%TBE agarose gel along with 100bp DNA size marker. The amplified product was send to CAMB at Lahore for Sanger sequencing. Figure 1,2show5UTR confirming the cDNA and figure 2 shows the amplified p7 gene of HCV of exact size. The amplified sequenced gene was used in

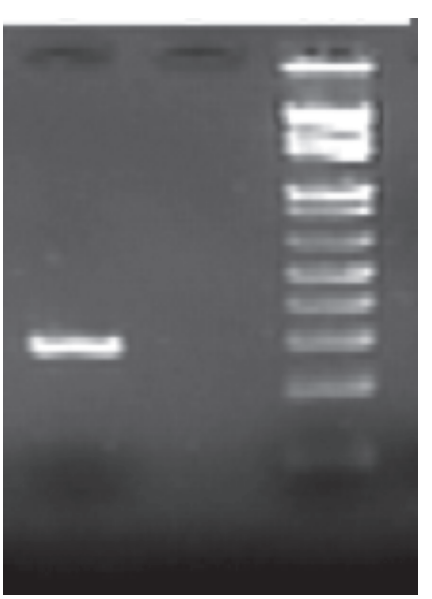

Figure 1: Lane 1:5-5UTR PCR product (278 bp). Lane 2: -tive control. Lane 3 DNA size marker (100 bp)

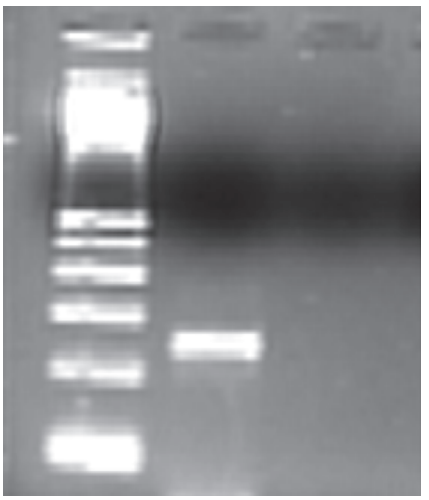

Figure 2: The p7 region amplification through PCR (241 bp). Lane 1: DNA Size Marker (100bp). Lane 2: amplified gene p7, Lane 3: negative control.

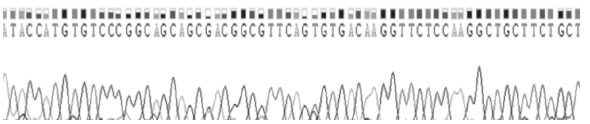

Figure 3: Sequencing chromatogram

the construction of expression vector and then cloned in mammalian expression vector pCR3.1 (FlagTAG/p7). This cloning was confirmed through sequencing as shown in figure 3. The resulted sequence of p7 gene was matched to other HCV3a genotype isolates in GenBank database.

This expression vector has CMV promoter which has ability to transduce eukaryotic cells for expression analysis. This expression cloning of the p7 gene was confirmed using designed cloning primers with added restriction sites. Not1 enzymes were used for restriction digestion. To investigate the p7-mRNA expression, prepared plasmid was transfected into huh-7 cells and assayed the mRNA expression level by RT-PCR. The resulted sequence of p7 gene was matched to other HCV3a genotype isolates in GenBank database.

P7 gene sequence:

CGCGTGTGCGTTGCCCTTTGGCTGATGTTGATGA-TATCACAAGCAGAAGCAGCCTTGGAGAACCTTGT-CACACTGAACGCCGTCGCTGCTGCCGGGACACAT-GGTATCGGTTGGTACCTAGTAGTGTTCTGCGCGGC-GTGGCACGTGCGGGGCAAACTTGTCCCGCTGGT-GACCTACGGCCTGACAGGT

DISCUSSION

With the help of established cell culture model, life cycle of HCV can be studied at molecular level. My study was on p7 protein of HCV which is established as an infectious particle, and play important function in virion morphogenesis[24]. Up to 20% of chronically HCV infected patients develop hepatocellular carcinoma[25]. This leads into socioeconomic burden of HCV world-wide. Therefore proper cell culture model is essential for amplification of viral proteins which up-to-date is not available[26]. In other parts of the world where other genotypes are founded, different mini-genomics models have been recognized for the replication of HCV[7].InPakistan predominant genotype is 3a[22]. For the first time stable cell lines are established in Pakistan which can be used for expressing different structural, nonstructural proteins of HCV and play an important role in amplification of those proteins which are involved in hepatic fibrosis and hepatocellular carcinoma. Further studies can be helpful in designing new anti HCV drugs[27].

In 2013, for the first time in Pakistan a complete genome sequence of HCV-1a was deduced[28]. This complete HCV sequence was phylogenetic ally analyzed and compared with HCV isolates which showed that the sequence phylogenetically similar to German strain as compared to the rest of countries like USA, UK and Japan[28]. My study was on the amplification and cloning of p7 gene of HCV positive patients of Khyber Pakhtunkhwa. Previously it was thought that it might be membrane permeabilizing protein involved in release of infectious particles[29]. Through in vitro studies it was found that HCV-p7 protein has ion-channel activity for small molecules[30-35]. Other studies demonstrated that p7 of HCV, amplified and cloned from Japanese patient suffering from fulminant hepatitis, played crucial role in production and release of infectious particles[36,37]. Another study demonstrated the production of infectious particle and its release in vivo by using chimeric HCV genomes.

The main objective of my research was to study the p7 gene of HCV at molecular level. In this study, HCV RNA positive patients were selected from the local population. For the very first time in KP, the p7 gene was amplified, cloned and expressed in Huh 7 cell line. Using the invitrogen RNA extraction kit (CatalogNo.K2100-12) viral RNA was extracted from the sera of the patients in accordance with the manufacturer's procedures with certain modifications. MMLV reverse transcriptase with antisense primers were used to synthesis cDNA fragments by RT-PCR. PCR with Tm 60C and 240bp fragments containing p7 gene was used to amplify gene-specific primers. Agarose gel electrophoresis was used to confirm this. QIAquick gel extraction kit protocol was used to elute the PCR product from agarose gel slices. The QIAquick provides an appropriate and reliable method to attain highly pure DNA fragments. Following this, sequence analysis of plasmid DNA was done. BLAST (Basic Local Alignment Search Tool) software was used to study the homology

of the sequenced gene as well as the already present sequences in the gene data bank. The design of the p7expression vector (pCR3.1/Flag TAG/p7) includes: a CMV promoter in order to direct the transcription of DNA, Flag TAG for checking of the expression of constructed vector in the transfected cells (due to the availability of antibodies against FLAG TAG) and the p7 segment of HCV genome (3a). Cloning of the p7-encoding sequence segment, which was amplified from eluted product, using PCR was done. Not1sites were created in p7 primer's sequence in order to make cloning of p7encoding sequences into the vector possible. Behaving as reporter assays reverse transcriptase PCR (RT-PCR) was used to determine whether the pCR3.1/ Flag TAG/p7can proficiently express the p7 gene. Using Lipofactamine transfection reagent, the expression plasmid was transfected into the Huh-7 cells using. After the transfection, cells were lysed and RT-PCR was used to measure the RNA level.

CONCLUSION

Expression of p7 gene of HCV can be used for comparative analysis of another strain of HCV of different location and this provides an opportunity for the development of novel anti-HCV drugs with good efficacy and minimal side-effects.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

Tariq S:	Main idea
Sarwar T:	Over supervision
Ahmed J:	Over supervision
Afridi G:	Data Collection
Bukhtiar S:	Data Analysis
Arif S:	Data Collection
Ain QU:	Typing
Hayyan:	Bibliography

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.