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Reverse Genetic Analysis of NS2 Mutational Diversity in Hepatitis C Virus

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Abstract: Hepatitis C Virus (HCV) is a main etiological and causative agent for acute and chronic liver disease, leading to liver cancer. The NS2 protein of HCV has been recently implicated in virus particle assembly. Objective of the study is to evaluate the mutation variations in the NS2 Gene of HCV and map epitopes in the variable NS2 region After clinical evaluation, 40 serum samples from HCV patients were obtained under sterile conditions. RNA was extracted from stored serum samples. Primers were designed, and amplicons were amplified**.** The mean age of the patients was 47.6 years. The RNA was extracted from all 40 serum samples and was detected through agarose gel electrophoresis. A single band of 470bp with maximum concentration was obtained at 61ºC. SNPs were detected in the amplified regions**. A** complex network of interactions involving NS2 and other viral structural and nonstructural proteins was observed during virus assembly.

Keywords: hepatitis C virus, NS2 nonstructural proteins, single nucleotide polymorphism.

丙型肝炎病毒非结构蛋白2突变多样性的反向遗传分析

摘要：丙型肝炎病毒是导致肝癌的急慢性肝病的主要病原体和病原体。丙型肝炎病毒的非结构蛋白 2 蛋白最近与病毒颗粒组装有关。本研究的目的是评估丙型肝炎病毒非结构蛋白2基因的突变变异，并在可变的非结构蛋白2区域绘制表位图。临床评估后，在无菌条件下获得了来自丙型肝炎病毒患者的40份血清样本。从储存的血清样品中提取核糖核酸。设计了引物，扩增了扩增子。患者的平均年龄为 47.6 岁。从所有 40 个血清样品中提取核糖核酸，并通过琼脂糖凝胶电泳检测。在 61ºC 时获得最大浓度的 470bp 单条带。在扩增区域中检测到单核苷酸多态性。在病毒组装过程中观察到涉及非结构蛋白 2 和其他病毒结构和非结构蛋白的复杂相互作用网络。

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关键词：丙型肝炎病毒，非结构蛋白 2，单核苷酸多态性。

1. Introduction

Hepatitis C virus (HCV) causes the development of hepatocellular carcinoma [1]. HCV belongs to the genus Hepaciviruses in the family *Flaviviridae* is an enveloped positive single-stranded RNA [2]. HCV is classified into seven major genotypes and vary in subtypes [3].

HCV has a heterogenic genome of 9.6 kb in size and is responsible for encoding a single open reading frame with almost 3,011 codons [4]. Genomic Translation generates a long polyprotein into 10 different products. These precursor proteins, the core, E1, p7, E2, nonstructural (NS)2, NS3, NS4B, NS5A, NS4A, & NS5B, help in the viral replication process but also disturb many cellular events [5, 6]. About 30 genotypes of HCV have been identified worldwide [7].

HCV encodes NS3-NS2 cysteine protease and NS4A-NS3 serine protease, and this enzyme is encoded by the NS2 C-terminal composed of 121 amino acids [8]. The NS2 N-terminal that is hydrophobic mediates interaction with cellular membranes, although the NS2 membrane topology is not fully recognized [9]. The cytosolic C terminal Cleavage suggests one or three transmembrane domains are present in NS2 [10]. Recent evidence suggests the involvement of NS2, NS3, and NS4A in viral particles assemblage [11]. Previous literature explained the NS2 as unnecessary from subgenomic replication that replicon is engineered to express NS3 over NS5B [12, 13].

The study conducted by Pietschmann et al. reported that the Transmembrane domains of NS2 elevate the virus titters to the virus than in J6/JFH chimera [14, 15]. In addition, NS2 and NS3 have adaptive mutations that provoke virus production [16]. NS2 and NS3 are independently expressed by cysteine protease, required for virus production [17]. Inadequate mutations in NS2 have been reported involved in the inhibition of virus assembly [6].Regardless of these facts, NS2 role in virus assemblage is leftover because of the high number of rapid mutations in the virus that do not get caught by the immune system. Due to this fact, vaccinations against such variants are less successful. NS2 is important in virus particle assembly by combining the viral structural and nonstructural proteins. Likewise, NS2 is important in mediating the E2 and NS3 interactions. Several reported mutations inhibited NS2 protein complex formation that also inhibited virus assembly. Objective of the study is to map out mutational diversity in NS2 Gene focusing on epitopes of a variable region.

2. Methods

2.1. Sample Collection and RNA Extraction

Forty (40) HCV patients diagnosed on routine criteria and who had already undergone all diagnostic tests were selected from different hospitals in Lahore, Pakistan. Quantitative and qualitative PCR was performed to assess the clinical state peripheral blood sample of about 3 ml was collected in a clotted vial. For separating the serum, the samples were centrifuged for 15minutes at 4000 rpm at 4 °C. Before extraction, the separated serums were stored at -20°C. After thawing the frozen samples, the AJ roboscreen innuPREP RNA extraction kit (AJ RoboscreenGmBH, Leipzig, Germany) was used to extract the maximum quantity of RNA in the study. For polymerase chain reaction (PCR), optimal template concentration in ng/µl and 260/280 ratio were measured for each serum sample using the Nanodrop 2000 spectrophotometer (Thermoscientific, Dubuque, USA).

2.2. HCV Nonstructural Protein NS2 Amplification and Sequencing

Primers for amplification of the sequence of the HCV nonstructural protein NS2 were designed using Primer 3 web software [18], and NCBI genome databases were used to retrieve the reported genomic sequence of HCV. The primer set was again designed with MEGA 6 because of non-specificity. The 57 selected HCV genomic sequences from different databases were aligned; their reference numbers are listed in the table 1 (Supplementary data).

Table 1 Supplementary table of Genome Reference No.

Reference no.					
AB691596.1	JN588558.1	KJ470612.1	GU294484.1	HW121730.1	JX227955.1
AB691595.1	JN180458.1	HQ852462.1	D17763.1	HW121729.1	JX227954.1
JQ717260.1	JN180457.1	JF343784.2	GQ275355.1	HW121728.1	JX227954.1
JQ717259.1	JN180456.1	HQ852457.1	AB792683.1	D11168.1	KJ470619.1
JQ717258.1	JN180453.1	JN180460.1	D28917.1	JF735125.1	KJ470618.1
JQ717257.1	EU204645.1	JN180459.1	JX826592.1	JF735124.1	KJ470617.1
JQ717256.1	KJ470615.1	JN180455.1	NC_009824.1	JF735123.1	KJ470616.1
JQ717255.1	KJ470614.1	JN180454.1	AF046866.1	JF735122.1	D49374.1
JQ717254.1	KJ470613.1	JN180452.1	JN588558.1	JF735121.1	

Conserved regions were selected from these aligned sequences for primer design (Table 2). Maloney murine leukemia virus reverses transcriptase (MMLV-RTase) (Fermentas, USA), and gene-specific anti-sense primers were used to synthesize the cDNA. For amplification of N2S from RNA samples by polymerase chain reaction (PCR), the different annealing temperatures (61-55°C) were tested with gradient PCR on control samples. The fragments were amplified in the thermocycler (BIO-RAD, T100TM) To analyze the polymorphic sites of the HCV NS2 gene. PCR amplifications were performed to detect the mutations at these sites, and the amplified products were electrophoresed on a 1.2% agarose gel. Invitrogen 1Kb plus RNA ladder (catalog number 10787018) was used to quantify the RNA. After gel elution, PCR amplicons were sequenced using the Sanger chain termination method. Chromas lite2.1 software was used to read the sequencing results of the NS2 gene. The software CHROMAS analyzed the sequencing results after conversion to FASTA format. These sequences in FASTA format were aligned using the online software BLAST.

Table 2 Primers used to amplify the HCV coding region for sequencing

NS2 Reported Primer Sequence (5_–3_)
NS2 forward primer CGTTGAAATGGGAATTTGTCATCC
NS2 reverse primer ATCTCCCGATAGTCATCAGCAG
New Synthesized primers Sequence (5_–3_)
NS2 forward primer CACCTCCACCAAAACATCGTGG
NS2 reverse primer TGCGGAGATATTCTTTGCGGGCT

3. Results

A total of 40 patients with clinically diagnosed HCV were recruited from the Service Institute of Medical Science, Lahore, Pakistan. The selection criteria were based on clinical symptoms and laboratory investigation in male and female patients. Control individuals were healthy normal volunteers. The patients had a mean age of 47.6 years. HCV Real-time reverse transcription (RT)-PCR [19] was done on all 40 serum samples to quantify viral RNA and was identified through agarose gel electrophoresis. The annealing conditions for the primers were optimized using temperature gradient thermocycler. A single band of 470bp with maximum concentration was obtained at 61ºC. Therefore, the temperature was selected for amplification for patient samples. Table 3 shows the optimized cyclic conditions used to amplify the HCV nonstructural protein NS2 gene, while Fig. 1 shows the gel diagram of RNA and the amplified product. The amplified NS2 gene was eluted from the agarose gel and sequenced. The sequences were converted to FASTA format and submitted to the NCBI BLAST database for single nucleotide polymorphism (SNPs). The NS2 mutant's details are in Table 4. ClustalW2 web server was used to construct the sequence alignment of the NS2 protein. 14 sequences in NS2 depicting all the 7 main genotypes were used in the alignment.

Table 3 Cyclic condition of PCR

	Initial Denaturation	**Cyclic Denaturation**	**Annealing**	**Extension**	**Cyclic Denaturation**	**Annealing**	**Extension**	**Final Extension**
Cycles	1X	20X			25X			1X
Temp	95 ℃	94 ℃	61 ℃	72 ℃	94 ℃	61 ℃	72 ℃	72 ℃
Time	5 min	30 s	30 s	40 s	30 s	30 s	40 s	10 min

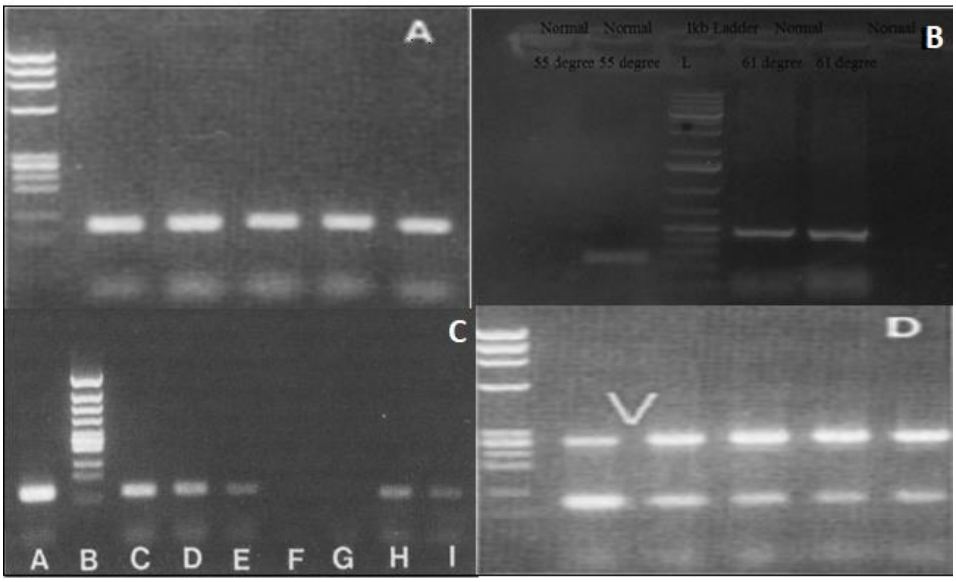

Fig. 1 A) Synthesized cDNA confirmation by specific core primers for HCV; B) Gel electrophoresis; C) Visualization of results by gel electrophoresis obtained through PCR amplification of cDNA using old primers; D) Reconfirmation of PCR products after optimizing conditions

Table 4 NS2 site-directed mutagenesis; mutation sites are underlined

Primer sequence (5_–3_)
TCTGTGCATGGCCAGATAGCCGCGGCTCTGCTGGTAATG
CACTCTCTTTACTCTCACCGCCGGGTATAAGACCCT
CTCTCACCCCCGGGTATGCCACCCTCCTCGGCCAGTGT
TCCTCGGCCAGTGTCTGGCCTGGTTGTGCTATCTCCTG
TCCTCGGCCAGTGTCTGTGGGCCTTGTGCTATCTCCTG
TGTCTGTGGTGGTTGTGCGCCCTCCTGACCCTGGGGGAA
TCTCCTGACCCTGGGGGCCGCCATGATTCAGGAGT
TCTCCTGACCCTGGGGAAGGCCATGATTCAGGAGT
ATGATTCAGGAGTGGGTAGCCCCCATGCAGGTGCG
TACCACCCATGCAGGTGGCCGGCGGCCGCGATGGCAT
ATGCAGGTGCGCGGCGGCGCCGATGGCATCGCGTGG
AGGTGCGCGGCGGCCGCGCCGGCATCGCGTGGGCCGT
CCGTCACTATATTCTGCGCCGGTGTGGTGTTTGACATT
TCTGCCCGGGTGTGGTGGCCGACATTACCAAATGGCTT
TGCCCGGGTGTGGTGTTTGCCATTACCAAATGGCTTT
TGCCCGGGTGTGGTGTTTAAGATTACCAAATGGCTTT
TGGTGTTTGACATTACCGCCTGGCTTTTGGCGTTGCT
TGGTGTTTGACATTACCGAATGGCTTTTGGCGTTGCT
CTTTTGGCGTTGCTTGGGGCCGCTTACCTCTTAAGGGCCGCTT
ACTGGGCCGCTAGCGGCCTGCGCGCCTTAGCGGTCGCCGT
GAGCTCACGCTCTGATAGCCGTATGCGCTTTGGTGAA
AAGCAGCTCGCGGGGGGTAGGGCCGTTCAGGTGGCGCTATT
ACTGGGCCGCTAGCGGCCTGGCCGACTTAGCGGTCGCCGT
TGACACATGTGCCGTACGCCGTCAGAGCTCACGCTCTG
TGGAACCCATCATCTTCGCCCCGATGGAGAAGAAGGT
TGGAACCCATCATCTTCGACCCGATGGAGAAGAAGGT
TGGAACCCATCATCTTCGAGCCGATGGAGAAGAAGGT
TCATCTTCAGTCCGATGGCCAAGAAGGTCATCGTCTG
ACTTCCCGTGTCCGCCGCCCTCGGCCAGGAGATCCTC
ATCGTCTGGGGAGCGGAGGCCGCTGCATGTGGGGACA
TCATCGTCTGGGGAGCGGCCACGGCTGCATGTGGGGA
TCAGTCCGATGGAGAAGGCCGTCATCGTCTGGGGAGC

4. Discussion

New therapeutic strategies against HCV focused on inhibiting specific steps of the viral life cycle [20]. The sensitivity and dynamic range of RNA reduce viral load resulting in increased volume of patients with SVR [21]. We performed reverse genetic analysis of NS2 genotype 2a replicates efficiently and produces high titters of infection. Pietschmann and colleagues reported NS2 being involved in a higher titter of virus infection than J6/JFH1 chimera [15]. Currently, this reported study is the suitable genetic background for NS2which we hypothesized. Recently X-ray crystallography reported 94 to 217 NS2 cysteine protease domain [22].

In flaviviruses, NS3/ NS2B are serine proteases [23]. However, HCV NS2 does not resemble the NS2A proteins of the yellow fever virus and Kunjin virus [24]. Further genetic analysis confirmed that the packaging function of YFV NS3 is different from the NS2 of HCV. It is delivered sufficiently in trans of its serine protease and RNA helicase activity [25]. The previously reported genetic alterations justify our results. The found G10A mutation in NS2 had only a moderate effect on virus assemblage, as previously Jirasko et al. explained G10A mutation involved in severe assembly defects in JFH1 [26]. In addition, literature support that weak cleavage of signal peptidase occurs at the junction of NS2/P7 in the G10A mutant [6]. No notable difference in the NS2 G10A mutant was found in the current study. The report by Zhou et al. revealed that NS2 quickly degraded by genotype 1 viruses dependent on proteasome degradation, resulting in the phosphorylation of S168 by casein kinase II [27]. The reported NS2 mutants like S168A and S168D indicated severe defects in virus assembly. Our results are consistent with the recently reported finding explaining NS2 S168A mutation inhibition in virus assemblage [24]. Any alterations in the stability of mutant NS2 proteins, both with or without proteasome inhibitors, were not identified.

5. Conclusion

Our research explained the NS2 mutational spectrum of the disease and the pathogenesis at

molecular levels. Although the role of NS2 in virus assembly yet remains unclear, this data explained the viral assembly interaction network of NS2 with viral structural and nonstructural proteins.

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