RESEARCH ARTICLE

IN SILICO ANALYSIS OF NS5B GENE OF HCV GENOTYPE 4 IN NORTH EGYPT

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Abstract

Background: Hepatitis C virus (HCV) genotype and subtype are related to response to antiviral therapy. Our purpose was to investigate the current HCV genotypes and subtypes collected from Menoufia Egypt distributions in hepatitis C patients in Egypt.

Methods: Serum of 5 HCV samples was subjected to reverse-transcription polymerase chain reaction, followed by direct DNA sequencing and phylogenetic analysis of the NS5B region to determine HCV genotypes/subtypes. The drug resistance mutations of HCV genotypes/subtypes were analyzed.

Results: the isolates were classified as genotype 4a except for isolate 3 was genotype 4c.

Conclusions: Genotype 4a is still the most prevalent HCV subtypes in Egypt. Detection of resistance mutations could help in clinical decisions.

Introduction:

Hepatitis C virus (HCV) is a leading cause of chronic liver disease and presents a major threat to global public health. Worldwide, more than 185 million people have been infected [Groeger et al., 2013], and these individuals face an increased risk of developing liver cirrhosis and hepatocellular carcinoma. Egypt has the highest prevalence of HCV worldwide; where 6% to 20% of the Egyptian population are HCV positive with an average of 13.8% [Farag et al., 2010].

Hepatitis C infection is a progressing and devastating live threatening health care problem in developing and developed countries worldwide caused by hepatitis C virus. HCV can be classified into seven genotypes and at least 67 confirmed subtypes, 20 provisionally assigned subtypes, and 21 unassigned subtypes [Farag et al., 2010]. HCV genotypes are geographically distributed worldwide, with HCV genotypes 1, 2 and 3 widely distributed and subtypes 1a, 1b and 2a specifically called epidemic subtypes [Magiorkinis et al., 2009, Cochrane et al., 2005]. Genotype 3 circulates mainly in south Asia, genotype 4 in central Africa and Middle East, genotype 5 in Southern Africa, genotype 6 in South East Asia and genotype 7 in Congo [Barnes et al., 2009, Sablonnet al ., 2015].

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In Egypt, genotype 4 is the most predominant genotype [The Polaris Observatory HCV Collaborators, 2017] and 4a is the dominant subtype [Fakhret et al., 2013, Ray et al., 2000 and Yousef et al., 2009].

HCV genotyping is clinically important in predicting the efficiency of antiviral therapy, in determining the duration of treatment [Lyra et al., 2015]. NS5B is one of the six nonstructural proteins encoded on the HCV genome. It is an RNA-dependent RNA polymerase responsible for replicating the HCV-RNA genome, which is a vital step in the HCV life cycle. The RNA-dependent RNA polymerase exhibits a classic fingers, palm, and thumb structure in which interactions between the finger and thumb subdomains create the catalytic site that ensures the synthesis of positive and negative strands of HCV RNA [Pawlotsky et al., 2006].

In this study we will determine the prevalent of genotypes and subtypes and detection of resistance mutations in HCV patients from Menufia-Egypt as this will have influence on management of patients and expected therapeutic response because response to treatment varies according to genotype and treatment may prevent progression of infection to hepatocellular carcinoma.

Materials And Methods:

Viral RNA extraction from plasma and RT-PCR:
RNA Extraction and RT-PCR was done according to Demetriou et al., 2009. Viral RNA was extracted from 140 ml plasma using the QiAmp1 UltraSens1 Virus kit (Qiagen, Venlo, The Netherlands) and 15 µl of the RNA was used in a one-step Reverse transcriptase-PCR HiSenScript RH(-) RT PreMix kit (intron Biotechnology, Korea), following a heat-shock step at 70°C for 20 sec to denature the RNA secondary structure. The RT-PCR was performed in a 50 µl reaction with 20 pmol each of the outer sense and antisense degenerate primers derived from the NS5B region of the HCV genome, designed to amplify all HCV genotypes. A nested PCR was performed using 3 µl of the RT-PCR product with 40 pmol each of the inner PCR primers, using 2x PCR master mix (I-Taq) ((intron Biotechnology, Korea) in a 50 µl reaction. PCR amplification was confirmed by visualization with ethidium bromide staining of a 2% agarose gel. DNA sequencing was carried out with the ABI 310 capillarity sequencer.

Sequence analysis:
Sequence analysis was done using BLAST programs from National Center for Biotechnology Information (NCBI), USA, (http://www.ncbi.nlm.nih.gov/Blast). HCV isolates correspond to different genotypes and subtypes were obtained from GenBank and were used to construct Neighbor-Joining tree between HCV isolates.

GenBank Submission:
NS5B sequences were submitted to GenBank under the accession numbers MN068031-MN068035.

Evolutionary relationships:
The evolutionary history was inferred using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Prediction of sites:
Amino acid sequence of our isolates was analyzed using the PROSITE program [Bucher and Hofmann, 1997], from the Predict Protein Server [Yachdav and Liu, 2004]. Prosite is a pattern data bank, based on scientific publications or research describing the function of determined protein groups [Bucher and Hofmann, 1997].

Results:

Amplification of partial HCV NS5B gene and sequence analysis:
A total of 5 serum samples of HCV infected patients collected in 2018 from Menufia governorate-Egypt were analyzed. PCR amplification of partial NS5B resulted in a product of 500b. After sequencing and sequence editing our isolates were aligned with GenBank database using BLAST and showed similarity to HCV NS5B gene of 88.59%-95.11% and 91.80%-99.18% for nucleotide and amino acid respectively. Also, our isolates were aligned together as shown in Fig.1; divergence between isolates was 0.04-0.11.
Fig. 1: Multiple amino acids sequence alignment of our isolates of HCV NS5B. Amino acids positions correspond to complete genome sequence of genotype C (accession number AB644287).

Effect of mutations on functional properties of the HCV NS5B gene:
A PROSITE (http://ca.expasy.org/prosite/) search indicated that the RDRP-SSR domain of NS5B protein was not affected by the detected mutation (Fig. 1). Prosite recognized the functional RDRP-SSR domain in each NS5B sequence of our isolates as shown in Fig. 2. Scores were 11.66, 11.60, 11.74, 11.31 and 11.54 for isolates 1-5 respectively. The highest scores resulted with isolate Menoufia-3 (Fig. 2).

Fig. 2:- ScanProsite results viewer of isolate 1 showing RDRP-SSR domain. A isolate Menoufia1 and B reference isolate.

Association of hepatitis C virus NS5B variants with resistance to new antiviral drugs among untreated patients:
Mutations located in NS5B are typically associated with resistance to interferon (IFN) and ribavirin (RIB) and to new antiviral drugs. The prevalence of these mutations was examined in all our isolates, genotypes and subtypes
used in this study (Fig. 3 and 4 respectively). Q309R (67) was common in most of the genotypes. The most common mutation was D310N (68), was major in genotype 3 and 6 and present in 2 isolates of genotype 2 (Fig. 3). C316N (74) was detected in one isolates of genotype 2. D244N (2) was detected in most of genotype 3 isolates. We did not detect the S282T mutation in any of the samples analyzed.

Q309R (67) was common in most of the subtypes of genotype 4. D310N and C316N mutations presented in subtype 4f Martinique and Cameron isolates (Fig. 4). D244N was detected only in 2 isolates of subtype 4r and 4i, and one isolate of subtype 4o and 4n. We did not detect the S282T mutations in any of the samples analyzed.

| Genotype | Subtype | Mutation |
|----------|---------|----------|
| 1        |         | Q309R    |
| 2        |         | D310N, C316N, D244N |
| 3        |         | Q309R, D310N, C316N, D244N |
| 4        |         | Q309R, D310N, C316N, D244N |

**Fig. 3:** Multiple amino acids sequence alignment of HBV polymerase gene. Amino acids positions correspond to complete genome sequence of genotype C (accession number AB644287).
Fig. 4: Multiple amino acids sequence alignment of HCV NS5B subtypes of genotype 4. Amino acids positions correspond to complete genome sequence of genotype C (accession number AB644287).

**Phylogenetic classification of HCV genotypes and subtypes:**

To classify our isolates NS5B, 69 sequences correspond to the main 6 NS5B HCV genotypes and 000 correspond to different NS5B subtypes obtained from GenBank database was used to construct a phylogenetic tree (Fig. 5 and 6 respectively). Phylogenetically Our isolates grouped with HCV isolates of genotype 4. Distances within genotypes were 0.1, 0.06, 0.03, 0.08, 0.03, 0.04 and 0.0 for genotypes from 1-7 respectively. Mean divergence between genotypes was 0.022-0.028.

In Fig. 6, isolates 1, 2, 4 and 5 clustered with subtype 4a Egyptian isolates, however isolate 3 clustered with subtype 4c isolates from Central Africa (Gabon) and South Africa.
**Fig 5:** Neighbor-joining phylogenetic analysis of HCV genotypes NS5B using our isolates against GenBank database.
Fig 6: Phylogenetic analysis of HCV subtypes NS5B using our isolates against GenBank database.
Discussion:
It is important to classify Knowledge on HCV genotypes and subtypes, since HCV genotypes respond differently to several treatment regimens [European Association for the Study of the Liver, 2004, Kandaet al., 2012]. In this study, HCV-NS5B gene of five isolates collected from HCV-infected patients from Menufia-Egypt was analyzed in comparison to those of GeneBank database.

It was reported previously that genotype 4a is the most predominant HCV genotype in Egypt [Fakhret al., 2013, Ray et al., 2000 and Youssef et al., 2009]. This was confirmed in the current study, in which genotype 4a was observed in 4/5 of the studied isolates. Isolate Menoufia-3 clustered with subtype 4c from Gabon, this was previously confirmed in a previous study (Mahmoud and Medhat 2013) where an isolate from Sohag was more closely related to an isolate from Gabon. It is possible that subtype 4c circulates originated from South Africa.

Different known drug-resistance mutations were detected in the isolates used in this study. The most common mutation was Q309R, present in 100% of genotype 3 isolates and partially in genotypes 1, 4 and 5. Q309R was not detected of any of the isolates of genotype 2 and 6. Also, D310N was a common mutation detected in 100% of genotype 3 isolates and was found in 80 and 90% of genotype 2 and 6 isolates, respectively. In contrast to Q309R, D310N was not detected of any of the isolates of genotypes 1, 4 and 5. C316N was detected only in genotype 2 (10%). D310N was previously described to be associated with IFN/RBV resistance (Asahinaet al. 2005).

The C316N mutation was previously related to a new non-nucleoside compound, but 24% patients with subtype 1b already carried this primary mutation despite never having been exposed to this new agent (Castilho et al., 2011).

The codon 316 implicated with resistance to new NS5B inhibitors were also within (Dutartre et al. 2006, Shi et al. 2008, McCownet al. 2009). D244N was detected only in genotype 3 (80%). The D244N mutation was mainly found in subtype 3a (Asahina et al. 2005). In genotype 4 subtypes, Q309R was a common mutation in most of subtypes.

In conclusion, the data suggest that genotype 4a is the predominant genotype in Egypt. Furthermore, the study indicates the introduction of isolate 3 4c from South Africa as new subtype of genotype 4 in Egypt. The presence of NS5B drug-resistance mutations in different genotypes could be used as a marker to determine the suitable treatment.

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