



Isolation, Cloning and Co-expression of Hepatitis C Virus Envelope Proteins: As Potential HCV Detecting Antigens

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Authors' contributions

This work was carried out in collaboration between all authors. Author MM designed the study, wrote the protocol, performed the experimental process, and wrote the first draft of the manuscript. Authors GA and MS managed the experimental process and analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2015/16720

Editor(s):

(1) E. Umit Bagriacik, Department of Immunology, Gazi University, Turkey.

Reviewers:

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(6) Julieta Trinks, Instituto de Ciencias Básicas y Medicina Experimental (ICBME), Argentina.

Complete Peer review History: <http://sciencedomain.org/review-history/10271>

Original Research Article

Received 11th February 2015

Accepted 23rd May 2015

Published 23rd July 2015

ABSTRACT

Aim: Approximately 3% of the world population is infected with Hepatitis C virus (HCV) which is the main cause of chronic liver disease. Blood transfusion is thought to be the leading cause of global epidemic of HCV. The envelope proteins E1 and E2 are involved in the early stages of the virus life cycle. These proteins have a major role in binding to receptors on the cell surface, fusion and integration of the virus into the host cell. Considering the potency of E1 and E2 in the development of diagnostic methods, the aim of our present study was co-expression of recombinant envelope proteins in eukaryotic HEK293 (human embryonic kidney) cells.

Methods: The viral genomic RNA was used for cDNA (complementary DNA) synthesis. Isolation

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of HCV envelope proteins coding fragment was performed using cDNA and specific primers. The target gene was cloned into pcDNA3.1 expression vector, and transfected into HEK293 cells, an expression host. Accuracy of the cloning and expression was confirmed using PCR and Western blot analysis.

Results: The isolation and cloning of the gene fragment encoding the E1 and E2 proteins was successful. Co-expression of these proteins was confirmed using monoclonal antibodies specific for each protein.

Conclusion: This study showed that HEK293 host cell is suitable for the expression of hepatitis C virus E1 and E2 coding gene. These proteins can be used in numerous virological studies and detection of HCV infection.

Keywords: Hepatitis C infection; envelope protein; protein expression; HEK293 cell line.

1. INTRODUCTION

Hepatitis C virus (HCV), a member of *Flaviviridae* family, is an enveloped virus containing a positive single stranded genomic RNA which is 9.5 kb in length and a single open reading frame [1-3]. Hepatitis C virus causes approximately 3% of the world's population infection, more than 170 million people, and is the leading cause of chronic liver disease. About 80% of people infected with the virus, show liver cirrhosis causing hepatocellular carcinoma in some cases [4-6]. Various serotypes and genotypes of hepatitis C virus have different distribution in geographical regions of the world. The most prevalent worldwide genotypes of HCV are orderly genotype 1 (46.2%), genotype 3 (30.1%), genotypes 2, 4, and 6 (22.8%), and genotype 5 (<1%) [7].

The infected blood transfusion is believed to be the main responsible for the global epidemic of hepatitis C, and therefore reliable detection of HCV in blood samples is very important. The method which now widely used for the diagnosis of hepatitis C is HCV antibodies detection using recombinant proteins derived from the HCV genome [8]. This method is highly sensitive and specific for the detection of hepatitis C virus infection and can be used along with other complementary methods of detecting viral RNA using RT-PCR [9-11]. Structural envelope glycoproteins E1 and E2 and core antigen are targets for diagnosis of HCV infection in the early stages of infection [12,13]. The main point of serological diagnostic methods is to choose the antigens which are involved in early steps of the virus life cycle.

The hepatitis C virus genome encodes a poly-protein with a length of about 3010 amino acids that processed into at least 10 mature proteins (C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A,

and NS5B) by the viral proteases and host signal peptidases [14]. Capsid protein (C) and envelope glycoproteins E1 and E2 are the structural components of the viral particle. Non-structural proteins are involved in the viral life cycle (e.g. genome replication) but do not have a role in viral particle formation. The two glycoproteins E1 and E2 are released from poly-protein by the host cell signal peptidases. These proteins form a non-covalent heterodimer in the endoplasmic reticulum compartment of the host cells [15]. Envelope proteins are involved in the early stages of the virus life cycle. In addition to their role in attachment to the cell membrane, the viral envelope proteins also play a major role in the fusion and integration into the host cells [16]. It seems likely E1 and E2 trigger binding of the virus, while the virus and the host cell membrane are involved in fusion [17-21]. Moreover, the presence of envelope proteins E1 and E2 is critical for the proper folding and function of the other one [22,23]. Despite the major findings about the structural properties of HCV envelope proteins, the E2 structure is still unknown and more studies need to be done in this field [24].

According to the importance of E1 and E2 antigens in the development of HCV diagnosis, the present study reports co-expression of recombinant proteins in eukaryotic host HEK293 cell line.

2. MATERIALS AND METHODS

2.1 cDNA Synthesis and Gene Isolation

Viral RNA was kindly provided by Dr. Teimoori (department of virology, Tarbiat Modares University). The complementary DNA was synthesized according to the standard method for RT-PCR (reverse transcriptase polymerase chain reaction) using cDNA synthesis kit (BioNEER). The fragment encoding HCV

envelope proteins; E1 and E2 was isolated by a pair of specific primers designed for gene amplification and cloning (Table 1). After amplification using pfu polymerase enzyme, the PCR product was electrophoresed in 1% agarose gel and TAE (tris base, acetic acid and EDTA) buffer followed by purification using high pure PCR product purification kit (Roche).

2.2 Gene Cloning and Colony Screening

Digestion and ligation of the target DNA fragment and the plasmid vector (pcDNA3.1) were performed using *NheI* and *EcoRI* restriction enzymes, and T4 DNA ligase (Roche) according to the manufactures' instructions followed by agarose gel electrophoresis. The *Escherichia coli* strain DH5 α was used for the recombinant DNA cloning. Briefly, a colony of *E. coli* was cultured in Luria-Bertani broth (LB) medium overnight. The following day new bacterial culturing was performed to obtain optical density of 0.5. After three times cold washing with 0.1 M CaCl₂, the recombinant DNA was added to the competent cells and the mixture was incubated on ice for half an hour followed by two minutes heat shock at 42°C. Afterward, 0.5 ml of LB medium was added to the mixture and incubated one hour at 37°C with shaking 200 rpm, the bacterial suspension was spread on LB-agar selective medium containing 100 μ g ampicillin/ml. Colonies were screened for the presence of recombinant plasmids by colony-PCR and plasmid extraction (using high pure plasmid extraction kit, Roche) followed by agarose gel electrophoresis. The accuracy of cloning result was confirmed by enzymatic digestion pattern and sequencing.

2.3 Cell Transfection and Protein Expression

All the culture media and reagents were purchased from Gibco/ Invitrogen. HEK293 (human embryonic kidney) cells were cultured in RPMI 1640 medium (Roswell Park Memorial Institute) containing 10% FBS (fetal bovine serum), 100 I.U. penicillin /ml, and 100 μ g streptomycin /ml and incubated in 37°C with 5% CO₂. Afterward, the proliferated cells were enzymatically detached using 0.25% Trypsin-EDTA, followed by washing in PBS (phosphate-buffered saline). Then the cells were counted and cultured in 6 well plates (3 \times 10⁵ cells/well). Transfection of the attached cells was performed using FuGENE HD Transfection Reagent (Roche) in a ratio of 1: 1 (1 μ g of recombinant vector: 1 μ l of the transfection reagent) according

to the manufacturer's instructions. After 48 hours, the cell extract was prepared using Lysis M (Roche) and collected for the recombinant protein expression analysis.

2.4 Western Blot Analysis

Western blot analysis was performed according to the standard protocol. In brief, the cell lysate was loaded on 10% polyacrylamide gel and the protein bands were separated via SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis). Semi-dry electro-blotting the protein bands were transferred on PVDF (polyvinylidene difluoride) membrane.

The membrane was blocked in 5% skim milk in PBS at 4°C overnight. After washing with PBS, the membrane was incubated in primary Hep C E1 and E2 mouse monoclonal antibodies (Santa Cruz) for two hours with gentle shaking at room temperature. Following washing three times with PBS solution containing 0.1% of Tween 20, the membrane was incubated for two hours in HRP-conjugated (horseradish peroxidase) anti-mouse secondary antibody (abcam). After washing again, the specific protein band got visualized by ECL (enhanced chemiluminescence) substrate.

3. RESULTS

3.1 Isolation of the DNA Fragment Encoding Envelope Proteins

The hepatitis C virus genomic RNA was used as a template for cDNA synthesis. Specific primers for the gene encoding the envelope proteins were designed according to the sequence obtained from GenBank (Table 1). The forward primer contains expression start codon (atg), Kozak sequence to enhance expression in eukaryotic systems (agcatgg considering the importance of nucleotides "a" in -3 and "g" in +4 position of the expression start site), and the *NheI* restriction enzyme cleavage site (gctagc) for the formation of sticky end and cloning into expression vector. The reverse primer includes the reverse complementary of the expression stop codon (cta), and the *EcoRI* restriction enzyme cleavage site (gaattc).

To isolate the target gene, PCR was performed using specific primers and cDNA (as a template) according to the program described in Table 2. The main cycle for amplification includes templates and primers denaturation, primer annealing, and primer elongation was repeated 35 times.

Table 1. The specific primers designed containing the start and stop codons, the Kozak sequence to enhance expression in eukaryotic systems (*Italic*), and the restriction enzyme cleavage sequence of *NheI* and *EcoRI* as cloning site (Underlined). GenBank AF009606.1 was used to primer design

	Primer sequence	Restriction enzyme
Forward primer	5'-ctagctagcatggcctaccaagtgcgcaattc-3'	<i>NheI</i>
Reverse primer	5'-ccggaattcctacgcctccgctgggatatg-3'	<i>EcoRI</i>

Table 2. PCR program

Cycles	Steps	Temperature	Time
1	Initial denaturation	95°C	5 min
35	Denaturation	95°C	30 sec
	Annealing	65°C	30 sec
	Elongation	72°C	1 min, 30 sec
1	Final extension	72°C	5 min

Amplified PCR product was run on 1% agarose gel and 1.6 kb fragment was identified on gel electrophoresis as shown in Fig. 1.

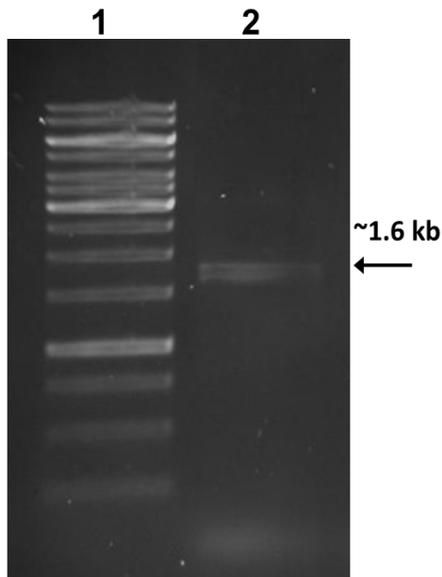


Fig. 1. Isolation of the HCV envelope proteins coding gene; the cDNA sample was used as template in the PCR reaction. Using the specific primers, the expected band of approximately 1.6 kb was obtained. Lane 1: 1 kb DNA ladder, and Lane 2: the 1.6 kb amplified fragment, specific for HCV envelope marked with an arrow

performed using T4DNA ligase. Following bacterial transformation, the presence of the insert in the grown colonies was checked by colony PCR (Fig. 2).

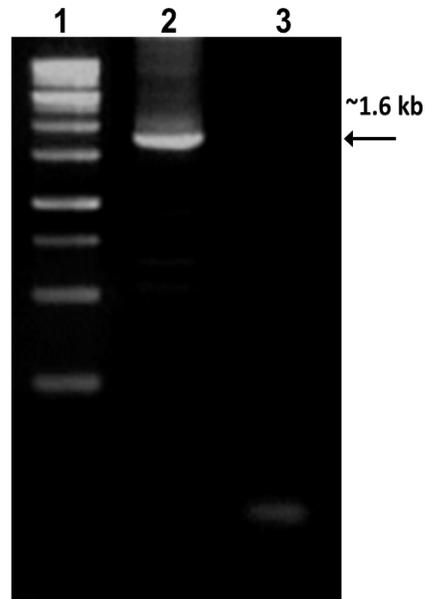


Fig. 2. Colony PCR; the growth colony on the selective medium was used as the template in PCR by the specific primers. Lane 1: 1 kb DNA ladder, Lane 2: 1.6 kb band as expected, Lane 3: Absence of the expected band due to using a plasmid-free *E. coli* colony as a negative control

3.2 Construction of a Recombinant DNA Vector

The isolated fragment and the plasmid vector pcDNA3.1 were enzymatically digested with *NheI* and *EcoRI* and then insertion reaction was

After plasmid extraction, the accuracy of the construct was confirmed by sequencing (data not shown) and digestion pattern (Fig. 3). According to the blast analysis of the sequenced gene fragment, it would be belong to genotype 1a. As

shown clearly, the restriction enzymes cut out a 1.6 kb E1E2 coding fragment from the 5.5 kb pcDNA3.1 vector. The recombinant vector named pcDNA-HCVE is schematically shown in Fig. 4.

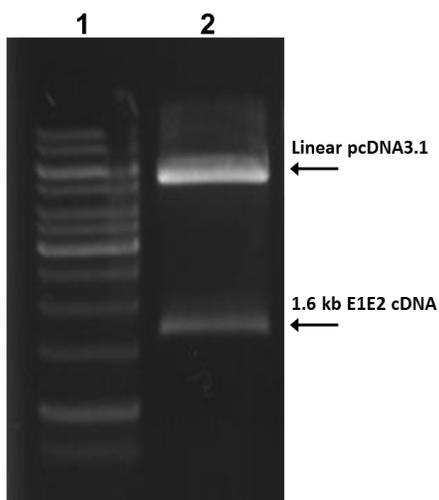


Fig. 3. Digestion pattern of the recombinant pcDNA-HCVE; the recombinant plasmid was double digested by *NheI* and *EcoRI* restriction enzymes. Lane 1: 1 kb DNA ladder, Lane 2: the 1.6 kb envelope proteins cDNA which was cut out from 5.5 kb pcDNA 3.1

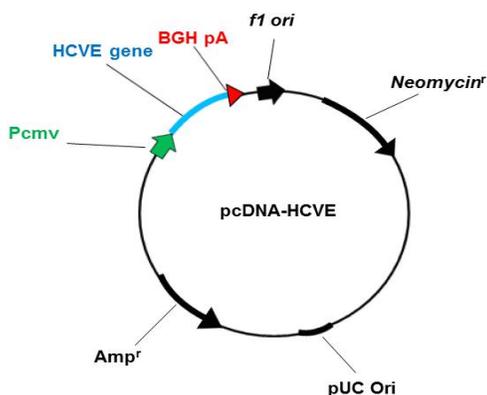


Fig. 4. Schematic scheme of the recombinant plasmid pcDNA-HCVE. Shown elements are HCVE gene: a gene encoding HCV envelope proteins, BGH pA: poly adenylation signal derived from bovine growth hormone gene, f1 ori: replication origin site of f1, Neomycin^r gene responsible for resistance to the antibiotic neomycin, pUC ori: replication origin site derived from the plasmid pUC, Amp^r: ampicillin resistance gene, and Pcmv: strong promoter derived from cytomegalovirus

3.3 Expression of the Recombinant HCV Envelope Proteins

At 48 hours after transfection, cell extract was obtained to analyze the expression of the target proteins. The presence of recombinant HCV envelope proteins was confirmed via western blot using specific monoclonal antibodies. The results of this study are shown in Fig. 5, where the appearance of specific bands in two lower rows of column 2 shows the reactivity of the expressed proteins with hepatitis C envelope proteins E1 and E2 monoclonal antibodies. The house-keeping beta-actin protein shown in first lane was used as a control for western blotting. The 31 and 70 kDa bands of the E1 and E2 proteins confirmed that the virus envelope glycoproteins expected to show higher molecular weight than the estimated weight according to the number of amino acids.

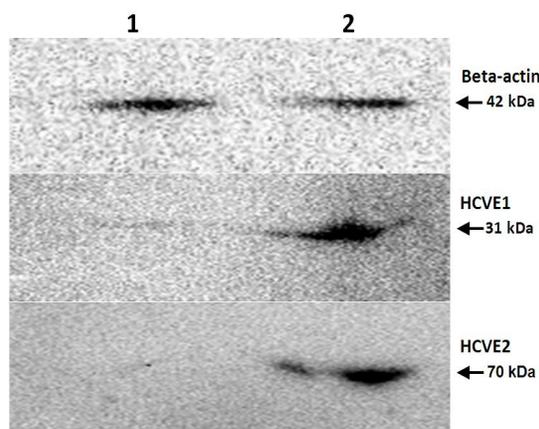


Fig. 5. E1 and E2 proteins expression was confirmed by Western blot analysis; the protein bands of cell extracts visualized using anti Beta-actin antibody (row 1), the specific antibodies against HCV E1 (row 2) and E2 (row 3). Columns 1 and 2 orderly show samples of control cells and transfected cells with recombinant DNA. E1 and E2 proteins expression was confirmed by the appearance of the bands on the 2nd and 3rd row of column 2. Beta-actin house-keeping protein was used as a control

4. DISCUSSION AND CONCLUSION

Hepatitis C virus envelope proteins E1 and E2 play important roles in the early stage of the virus life cycle which include viral attachment and entry into the host cells, that is why the production of recombinant envelope proteins is

important for developing the prevention and detection methods of hepatitis C infection [25,26]. These proteins have been expressed in various expression systems. In most studies, the expression of these proteins in a bacterial host (*E. coli*) leads to form insoluble inclusion bodies [27,28]. Thus, despite the advantages of the prokaryotic expression system such as being inexpensive and easy to scale up, it is not suitable for the expression of envelope glycoproteins because of lacking in glycosylation system, and the importance of glycosylation in HCV envelope proteins folding. According to the preference of eukaryotic hosts for the expression of these proteins, E1 and E2 proteins expression in insect cells was reported [29]. The main problem with the insect system is hyper glycosylation producing proteins which are highly antigenic. Expression of these proteins in the form of virus-like particles (VLPs) and also transient expression has been reported in several studies [30,31].

Comparison with other cells shows mammalian cell lines are more suitable for expression of a recombinant target protein considering the aspects of glycosylation changes result in the proper protein folding [32]. Moreover, it has been found that E1 protein proper folding depends on the presence and simultaneous expression of E2 protein [22]. Chaperone-like role of E1 protein for the correct folding of E2 protein has been noted as well [23]. In fact, these two proteins are involved in the proper folding and function of each other. Hence, the simultaneous expression of proteins E1 and E2 using a single construct is important.

In the present study, cloning and co-expression of the E1 and E2 proteins in eukaryotic HEK293 cells is reported. The recombinant proteins (E1 and E2) showed greater molecular weights (31 and 70 kDa) than the expected sizes estimated by the number of amino acids. This phenomenon has also been reported in another similar study [29]. Expression of both E1 and E2 proteins in *cis* was separately confirmed via western blot analysis using two monoclonal antibodies specific for each protein. The reactivity of the recombinant proteins with monoclonal antibodies indicates proper expression and preservation of their antigenic properties.

The results of this study suggest that the isolation, cloning and expression of the gene encoding the E1 and E2 proteins of the hepatitis C virus in the eukaryotic host HEK293 cells has

been successfully performed. These proteins can be used in numerous virological studies for detection of hepatitis C infection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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