

Exposition of hepatitis B surface antigen (HBsAg) on the surface of HEK293T cell and evaluation of its expression

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Abstract

Hepatitis B virus (HBV) is considered as a global health concern and hepatitis B surface antigen (HBsAg) is the most immunogenic protein of HBV. The purpose of this study was to evaluate the expression of HBsAg on the cell surface of human embryonic kidney cell line (HEK293T). After transformation of expression vector pcDNA/HBsAg to E.coli TOP10F', plasmid was extracted and digested with BglII. Afterwards, the linearized vector was transfected to cells and treated with hygromycin B for 5 weeks to expand the resulted clones. The permanent expression of HBsAg followed by flow cytometry uptill now about one year. Genomic DNA was extracted from transfected cells and the existence of HBsAg gene was assessed by PCR. Real-time RT-PCR was utilized to measure the expression at the RNA level and flow cytometry was carried out to assess protein expression. Insertion of HBsAg cDNA in HEK293T genome was confirmed by PCR. The results of real-time RT-PCR illustrated that each cell expresses 2275 copies of mRNA molecule. Flow cytometry showed that compared with negative control cells, 99.9% of transfected cells express HBsAg on their surface. In conclusion, stable expression of hepatitis B surface antigen on the membrane of HEK293T provides an accurate post-translational modification, proper structure, and native folding in contrast with purified protein from prokaryotic expression systems. Therefore, these exposing HBsAg cells are practical in therapeutic, pharmaceutical, and biological sets of research.

Keywords: Hepatitis B, HBsAg, Recombinant HEK293T cell

INTRODUCTION

Hepatitis B virus (HBV), a prototype member of the *hepadenaviridae* family (1), is the main etiological agent of acute and chronic hepatitis (2). The World Health Organization (WHO) has recently estimated that more than 350 million suffer from chronic HBV infection which leads to an annually over 780,000 death worldwide from the consequence of this disease, mainly from cirrhosis or hepatocellular carcinoma (3).

HBV is the smallest human enveloped DNA virus with circular and partially double stranded genome having approximately 3200 base pairs (4). The hepatitis B surface antigen (HBsAg) particularly the tiniest part, S antigen, is the most significant viral antigen

causes an immune system response (5). This surface protein is the main immunogenic antigen in serum and the exact target of neutralizing antibodies during natural infection or after vaccination (6,7).

Nowadays, there is no conclusive treatment for HBV chronic infection and treatment protocols are extensively used as protective and symptomatic therapy (8), whereas, these general drugs are not capable of controlling the disease efficiently. According to these considerations, all activities for the prevention, treatment, and carrier detection are valuable and would be regarded as an impressive procedure to prevent the spread of virus infection (9).

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Vaccination is the most drastic tool against the development of HBV chronic infection that is recommended by WHO to all infants as the primary focus of a strategy to eliminate HBV transmission (3). Nowadays, antibodies are used in purification process of recombinant HBsAg production by using immunoaffinity chromatography (IAC) and in diagnostic method to detect carriers and HBV infected patients (10).

Purification of HbsAg via IAC is one of the principal steps that refines the product up to 90-95% purity (6). In this process, the binding affinity of antibodies to an antigen is the basis of separation (11). Despite all the advantages of antibodies some of defects, encourage researchers to seek alternative molecules like aptamers (12,13).

Aptamers are single stranded oligonucleotides, which have high affinity for various targets. These molecules are cost-effective, non-immunogenic and stable compared with monoclonal antibodies which could be applied instead of antibody for purification, carrier detection and diagnostic test. Aptamers are selected by systematic evolution of ligands by exponential enrichment (SELEX) method as a molecular directed evolutionary approach (14,15). Cell SELEX, a kind of SELEX, is a rapid, easy-handled, and reproducible process that uses intact living cells whose surface has displayed molecules as a target (16,17).

Additionally, inability of HBV to infect cells *in vitro* and the lack of an animal model for *in vivo* learning has made a drastic problem in the study of this virus (18). A cell line with the main virus antigens is a useful alternative or stable model for relevant studies of HBV. Consequently, in this study, it is attempted to construct a HEK293T cell line that expresses HBsAg for manufacturing of antibody, for animal immunization and aptamer selection in cell SELEX.

MATERIALS AND METHODS

Plasmid construction

Coding sequence of HBsAg from Adw subtype of HBV (sequence ID: X02763.1), including start to stop codon, with site of

NheI at 5' end and *XhoI* at 3' was ordered to be synthesized and cloned in pUC57 plasmid to Genecust Company (Luxembourg). This purchased plasmid was transformed to chemically competent *E. coli* Top 10F' according to Higa and Mendel protocol. The extracted plasmid of one of the resulted clones and pcDNA 3.1 Hygro (+) plasmid (Invitrogen, USA) were digested by *NheI* and *XhoI* restriction enzymes (Thermoscience, USA) separately.

The digestion products were electrophoresed and purified from agarose gel by Bioneer DNA extraction kit (South Korea). Ligation reaction was done between linear pcDNA 3.1 Hygro (+) plasmid and HBsAg coding fragments with T4 DNA ligase. Ligation product was transformed into competent *E. coli* Top10F'. The resulted colonies were analyzed by colony PCR and their extracted plasmids digested with *XhoI* and *NheI* to find inserted clones. In colony PCR, a mix of Taq DNA polymerase (0.25 μ L, 1.25 U), 10 \times buffer (2.5 μ L), 10 mM dNTPs (0.5 μ L), 50 mM MgCl₂ (1 μ L), ddW (17.75 μ L), and 1 μ L (10 μ M) of each forward (F) pcDNA backbone primer 5'-ACTAGAGAACCCACTGCTTACTG-3' and reverse (R) pcDNA backbone primer 5'-ATGGCTGGCAACTAGAAGG-3' were employed.

The PCR program was initiated with 1 cycle at 94 °C for 4 min, continued by 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and ended with 1 cycle at 72 °C for 5 min in BioRad thermocycler (Bio-Rad Laboratory, USA). As a result, one of these verified colonies was grown and the extracted plasmids were digested with *BglII* enzyme. The linear plasmids were evaluated by agarose gel electrophoresis, as well.

Cell culture studies

Human embryonic kidney (HEK293T) cell line was purchased from Pasteur Institute of Iran (Tehran, Iran) and grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) supplemented with 10% fetal calf serum (Gibco, USA), 100 U/mL penicillin and 100 μ g streptomycin (Gibco, USA) at 37 °C in a 5% CO₂ atmosphere.

In vitro transfection

Transfection was conveyed applying PolyFect (Qiagen, Germany) based on its protocol. HEK293T cells in exponential growth phase were seeded (5×10^5 cells) into each well of 6-well plate the day before the transfection and it was replaced 2 h before beginning new fresh medium. The confluence of adherent cells at the time of transfection was 60-70%. Fifty microliter of linear plasmid with 100 ng/ μ L concentration was added to 700 μ L serum free-DMEM medium, suspended by pipetting and incubated for 1-2 min. About 15 μ L of polyfect reagent was added to the plasmid suspension and incubated for 15-20 min at room temperature. Then the content of the tube was added to the 6-well plate, mixed by swirling and incubated at 37 °C overnight. A day later, culture medium was changed, and after 48 h, the cells were treated with 150 μ g/mL hygromycin B for 5 weeks to expand the resulted clones. The permanent expression of HBsAg was followed by flow cytometry for about one year.

The dose of hygromycin B was continuously increased from 150 μ g/mL to 400 μ g/mL for selection of cells with a large copy number of inserted gene in the genome. Untransfected HEK293T cells were used as negative control.

Polymerase chain reaction (PCR) on HEK293T genomic DNA

To confirm the integration of linear pcDNA/HBsAg into the genome of HEK293T cells, genomic DNA of about 1 million transfected and untransfected (as a negative control) cells were extracted by Genetbio DNA extraction kit (Genetbio, Korea) according to manufacturer's instruction. PCR was done on both genomic DNA with forward and reverse pcDNA primers separately. The PCR process was done as described above, but the DNA template was genomic DNA. PCR reaction on genomic DNA of untransfected HEK cells and a reaction without DNA template was used as a negative control.

Real-time PCR

Total RNA of about 10^6 transfected cells was extracted using RNX kit (Cinagen, Iran).

The quantity and quality of the extracted RNA were measured by gel electrophoresis and nano drop spectrophotometer. The extracted total RNA was treated with DNase I enzyme (Thermo Scientific, USA) according to its protocol and then inactivated by adding the same volume of phenol:CIAA (1:1; CIAA is 24 parts chloroform, 1 part isoamyl alcohol) through the phenol cleanup process. First strand cDNA was synthesized from 2 μ g of the total RNA according to first strand cDNA synthesis kit instruction (Thermo Scientific, USA).

Absolute quantification was based on a standard curve prepared from our recombinant plasmid containing HBsAg fragment with 171 ng/ μ L concentration which could determine the copy number of any unknown sample through insertion of the Ct of unknown sample in standard curve and figuring out the relevant copy number. Eight-point serial dilutions were prepared to ensure this standard curve could cover the expected range of expression within the samples. For preparation of real time RT-PCR reactions, 10 μ L of SYBR Green master, 0.5 μ L forward primer (10 μ M), 0.5 μ L reverse primer (10 μ M), 7 μ L RNase-free water, and 2 μ L of diluted plasmid for each dilution point or cDNA of unknown samples was combined and pipetted gently. In non-template control (NTC) tube, 2 μ L water was added instead of cDNA or plasmid and samples prepared triplicate.

Real-time PCR at the same time was performed on the cDNA and serial diluted plasmid samples in a 20 μ L volume using forward HBsAg primer 5'-ATCAACAACAACAGTACGG-3' and reverse HBsAg primer 5'-GACGATGGGATGGGAATAC-3'. The reactions were carried out by SYBR Green Master Mixes (Ampliqon kit, Denmark) with the subsequent program: 95 °C for 10 min, continued by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All the reactions performed in triplicate. To extract quantification data from real-time RT-PCR amplification curves, the results need to be plotted as a standard curve. The copy number of the mRNAs per cell was calculated by standard curves and it was based on the number of transfected cells and dilution factor.

Flow cytometric studies

About 1×10^5 transfected and untransfected HEK293T cells/100 μ L medium was prepared separately in each tube. The cells of each tube were stained with 5 μ L FITC conjugated polyclonal anti-HBsAg antibody (Abcam, USA), and incubated at 4 $^{\circ}$ C for 45 min in a dark place and the cells were centrifuged at 1700 rpm for 5 min and washed with PBS (Gibco, USA). Finally, the cells of both tubes were resuspended in 200 μ L PBS and detected using flow cytometer (BD bioscience, NJ, USA) by accumulating up to 100,000 events per tube. The obtained data were analyzed by Cell Quest software (BD Biosciences, USA).

RESULTS**Construction of the pcDNA-HBsAg**

After transformation, more than 50 clones were obtained from the plate. The result of PCR reaction with backbone primers on remained clones showed 824 bp bands (backbone of plasmid) in the majority of clones.

Digestion on extracted plasmids that were positive for colony PCR with *NheI* and *XhoI* enzymes revealed a 5501 bp band and a 696 bp band of HBsAg (Fig. 1A). The linearization of plasmid confirmed by *BglII* led to a 6197 bp band (Fig. 1B).

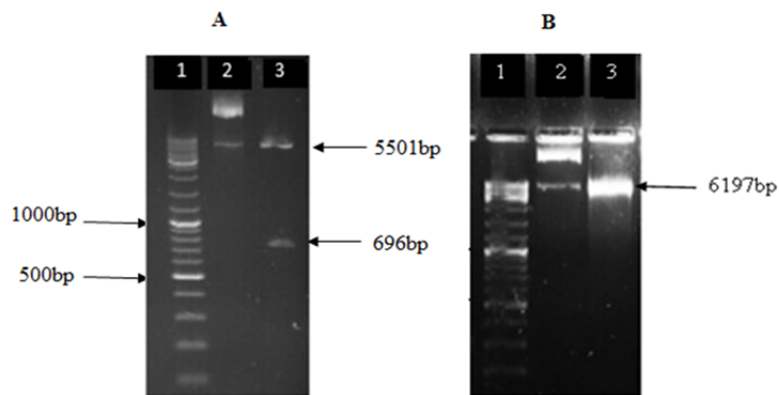


Fig. 1. (A) Digestion on extracted plasmid of one of positive clones with *NheI* and *XhoI* confirmed HBsAg fragment insertion in pcDNA vector. Column 1: Mix DNA ladder (Thermoscientific, USA). Column 2: Undigested circular plasmid. Column 3: 696 bp and 5501 bp bands that confirmed cloning. (B) Digestion of recombinant plasmid with *BglII* enzyme. Column 1: Mix DNA ladder (Thermoscientific, USA). Column 2: Undigested plasmid. Column 3: A 6197 bp linearized plasmid.

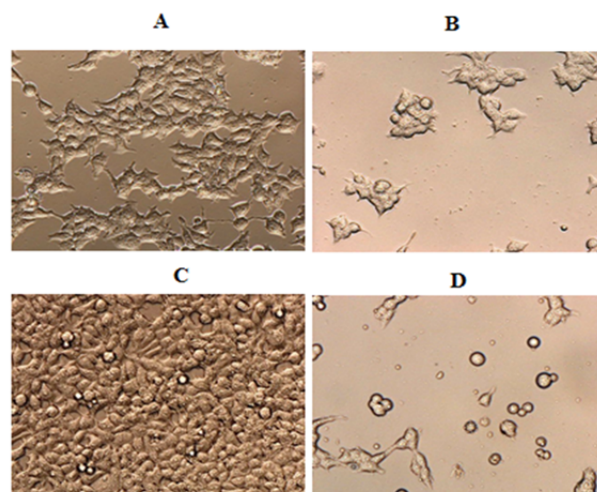


Fig. 2. (A) Untransfected HEK293T cells 3 days after hygromycin treatment. (B) Transfected HEK293T cells 2 weeks after hygromycin treatment. (C) Transfected HEK293T cells 3 weeks after hygromycin treatment. (D) Untransfected HEK293T cells 1 week after hygromycin treatment.

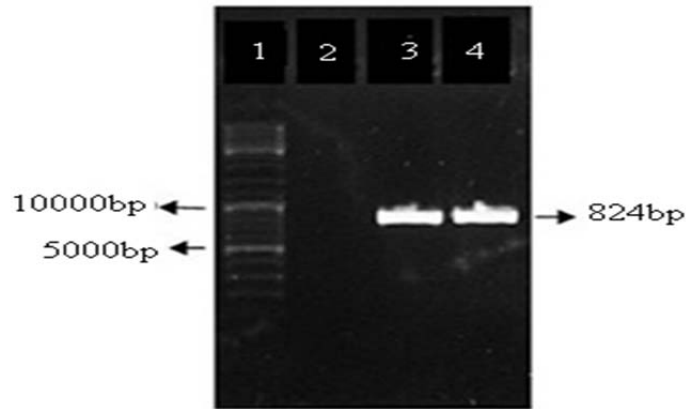


Fig. 3. PCR reaction on genomic DNA of selected HEK293T cells with pcDNA backbone primers. Column 1: Mix DNA ladder (Thermoscientific, USA). Column 2: PCR reaction in untransfected cells. Column 3 and 4: PCR reaction in transfected cells (824 bp band that confirmed the integration of HBsAg in genomic DNA)

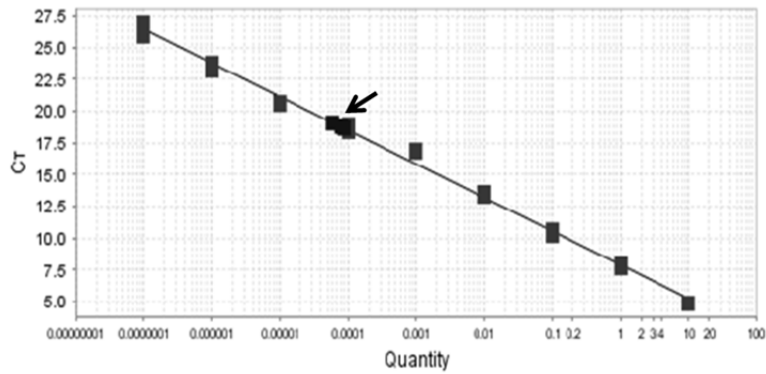


Fig. 4. Standard curve of real-time PCR. Standard samples have 10^{12} - 10^4 copy number of pcDNA-HBsAg. The arrow indicates the cDNA of HEK293T/HBsAg has 9.1×10^6 copy number of HBsAg.

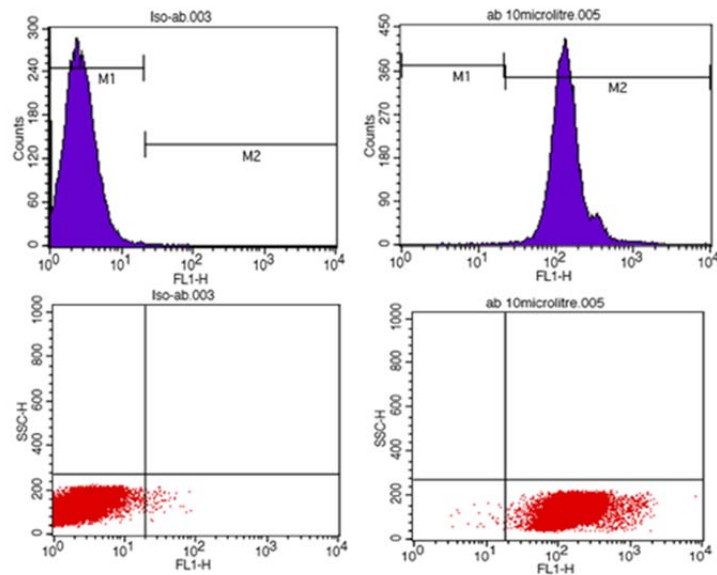


Fig. 5. Flow cytometric analysis after cell staining with Ab conjugated to FITC against HBsAg. (A) Untransfected cells stained with Ab conjugated to FITC showed the auto fluorescent and non-specific binding (M1). (B) Transfected cells showed 99.9% expression on the cell surface compared with untransfected cells (M2).

Transfection and integration results

Treatment of transfected cells by hygromycin B caused to positive selection of some stable transfected HEK293T cells after 3 weeks (Fig. 2). Then, these cell colonies were grown and exposed by higher concentrations of hygromycin B (until 400 µg/L).

PCR reaction on genomic DNA of selected cells with pcDNA backbone primers displayed 824 bp band that verified the integration of HBsAg in genomic DNA (Fig. 3).

Evaluation of the HBsAg cDNA expression with real-time PCR

Real-time RT-PCR results comparing with standard curve displayed average 9.1×10^6 mRNA molecules of HBsAg in 2 µL of cDNA (Fig. 4).

The total number of cells was 200000 and 50 µL RNA purified from the cells and cDNA was synthesized on 10 µL of RNA and only 2 µL of cDNA used in real-time PCR. So to calculate the number of HBsAg in each cell, the result (9.1×10^6) should be multiplied in 10 (2 µL cDNA of 20) and 5 (10µL RNA of 50) and divided by total number of cells (200000). This number was calculated for each cell on average in 2275.

Flow cytometric analysis

Additionally, the results of flow cytometric analysis indicated that approximately 99.9% of cells overexpressed HBsAg on their surface (Fig. 5).

DISCUSSION

Construction of eukaryotic proteins would be applicable in immunization of mouse and camel to produce nanobody, mono, and polyclonal antibody and in SELEX process to select specific aptamers. Improvements in the existing *in vitro* cell line expressing HBsAg provide useful source in the study of HBV immunogenicity and new classes of agent with anti-HBV activity.

In the present study, we have created a stably artificial cell line that expresses HBsAg on its surface. Since eukaryotic proteins could be expressed in prokaryotic expression systems, Elghanam *et al.*, in 2012 reported the

HBsAg encoding gene that was cloned and expressed in *Escherichia coli* for the purpose of production of HBsAg in high amount with low cost (19). In a majority of studies, HBsAg gene has been transfected to yeast cells (20) like *Sacharomyses cerevisia* and this protein has been used as a recombinant vaccine after purification (21). Although prokaryotic and yeast protein expression systems are easier and cheaper and produce abundant yields compared with mammalian cells (22), these systems cannot express proteins with native post-translational modifications (PTMs) and correct folding especially in the case of membrane and viral coat proteins (23). Eukaryotic proteins commonly have PTMs such as glycosylation, phosphorylation, and fatty acid addition, which are important for biochemical, biophysical, and biological activity of relevant proteins. In addition, the majority of diagnostic and therapeutic antibodies target the special epitopes which contain these modification residues (24). Any deprivation in PTM change of a protein could induce undesired variations in protein folding, function, and antigenicity. Therefore, in prokaryotic expression systems, to ensure proper folding, some *in vitro* modification and specific chromatography or dialysis are required to recover proper disulfide bonds and folding (25). On the other hand, protein production in prokaryotic cells occurs in cytoplasm, while in eukaryotic expression systems like mammalian cell lines, a majority of eukaryotic surface or viral proteins express and anchor on membrane similar to their natural form, and even the appearance of protein in comparison with the original purified protein has better spatial folding structure (26,27). Displaying eukaryotic membrane or even secreting and intracellular proteins on surface of mammalian cell lines e.g. HEK293T, would be an alternative way to overcome the above cited obstacles.

Over the last two decades, several stable mammalian cell lines has been produced which express HBV after transfection of viral DNA into the cells (4). In a study by Gholson *et al.*, pre S and core genes of HBV were expressed together on cell surface of a rat fibroblast cell line and their expressions

were evaluated with immunofluorescence microscopy or flow cytometry (23). Additionally, Roossinck *et al.*, confirmed expression of the core ORF of HBV in the NER-41 cell line with northern blot and *s*₁ nuclease mapping technique (28).

HBV whole genome was transfected in previous study (4) and some stably cell line like HepG2.2.15 or HepAD38 was produced but in these kind of cell lines beside HBsAg, other viral antigens are exposed on the cell surface. Therefore, these kind of cell lines would be more suitable for virus life cycle study (29) and could not be used for exact selection of a specific HBs antibody or aptamer in cell SELEX process.

In all accomplished studies, HBsAg produced in eukaryotic cells in order to extract and purify this antigen, but evaluation of HBsAg expression individually on the cell surface of eukaryotic cells has not been addressed. Our results illustrated that transfected HEK293T permanently produced the relevant structural protein on their cell surface. Plasmid insertion into host chromosomes occurs randomly in spontaneous nicks in the cell genome (30). Recombinant cell was named HEK293T/ HBsAg which expresses high levels of HBsAg.

Evaluation of HBsAg gene transcription in RNA level was estimated using real-time RT-PCR based on number of RNA copies in each cell. The results showed that expression vector containing HBsAg was successfully transfected and integrated in target cells. Calculations indicated that 2275 mRNA copy numbers of HBsAg were expressed in each cell which categorized in high level expression proteins, whereas native HEK293T cells do not have any HBsAg coding gene.

The expression of HBsAg at the protein level was assessed and supported by flow cytometry and the mean fluorescent intensity of transfected cells indicated HBsAg overexpression on the surface of 99.9% of transfected HEK293T cells.

CONCLUSION

In this research, a recombinant cell line (HEK293T/HBsAg) over-expressing HBsAg

was constructed. The expression of HBsAg on the cell surface was characterized by flowcytometry. A highly and stably expressed HBsAg cell line could be applied in future animal immunization and cell SELEX projects to produce polyclonal and monoclonal antibodies, nanobodies and aptamer.

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