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ORIGINAL ARTICLE

Evaluation of the eukaryotic expression of *mtb32C-hbha* fusion gene of *Mycobacterium tuberculosis* in Hepatocarcinoma cell line

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ABSTRACT

Background and Objectives: HBHA and Mtb32C have been isolated from culture supernatants of *Mycobacterium tuberculosis (M. tuberculosis)* and *Mycobacterium bovis (M. bovis)* and their immunogenicity previously studies have been confirmed. In this study, capability of constructed vector containing two mycobacterial immunodaminant antigens (Mtb32C-HBHA), in producing new chimeric protein under the *in-vitro* condition was examined.

Materials and Methods: In present study Huh7.5 cells was transfected with Mtb32C-HBHA –pCDNA3.1+ recombinant vector using the calcium phosphate method and expression of chimeric protein was assessed by RT-PCR and Western blot methods.

Results: Results of RT-PCR and Western blot showed expression of 35.5 KD recombinant protein (Mtb32C-HBHA) in this cell line.

Conclusion: The constructed vector can produce two highly immunogenic antigens that fusion of them to gather makes chimeric antigen with new traits. Other attempts are needed to evaluate specific properties of this new antigen such as molecular conformation modeling and immunologic characteristics in future studies.

Keywords: *Mycobacterium tuberculosis*, HBHA , Mtb32C, Huh7.5 cells

INTRODUCTION

Tuberculosis (TB), is an infectious disease caused by *Mycobacterium tuberculosis* (1). This bacterium commonly enter from respiratory tract, involves lung and start to grow in alveolar macrophage however it can attack other parts of the body (2).

According to the latest reports of world health organization (WHO) TB causes solely about 9 million annual deaths and 1.4 new cases in the world (3). Bacillus Calmette–Guérin (BCG) vaccine can prevent disseminated forms of disease such as meningitis but unable to protect against pulmonary tuberculosis and cannot be used in people with immunodeficiency disorders. So the most important goal of WHO is to produce new effective vaccine to deal with this problem. Therefore, many efforts to develop an effective vaccine against tuberculosis are ongoing in the world (3-6).

One of the basic steps of the bacterial pathogenesis is the attachment of the microorganism to the host cells. Identifying adhesins involved in the early stages of the bacterial attachment and colonization followed by restricting these adhesins can prevent

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initiation and spread of infection to other sites.

In a study by Pethe et al. it was shown that the disruption of *hbha* gene in *Mycobacterium tuberculosis* or coating the wild type mycobacteria with anti- HBHA impaired the spread of infection from the nasal route to the spleen

Therefore, these adhesins are suitable targets for vaccines or drugs design. HBHA in *M. tuberculosis* is an adhesion binds to lung epithelial cells and plays a critical role in the spread of infection outside the lungs. Strains of *M. tuberculosis* lacking HBHA are not able to spread from lungs to other organs (7-13).

Mtb32C is a C- terminal fragment of Mtb32A that encoded by *Rv0125* gene. This gene presents only in several mycobacterial pathogens such as *M. tuberculosis, M. leprae* and *M. bovis.* Mtb32A is involved in pathogenicity and is a vaccine candidate especially its own C-terminal region (Mtb32C) (14-18).

In the present study, ability of Mtb32C-HBHA recombinant vector in expressing of fused Mtb32C - HBHA gene was evaluated in Huh7.5 cell line

MATERIALS AND METHODS

In previous study Mtb32C and HBHA genes isolated from *M. tuberculosis* H37Rv genome and cloned into pCDNA3.1+ vector (19). In current study its potency to express chimeric Mtb32C-HBHA protein was tested.

Cell line and cell culture. Huh7.5 cell line was used to investigate the expression of mycobacterial antigens. It is an immortal cell line. Since this cell line is very stable and has a fast growth rate, it suits the studies aiming at evaluating the expression of fusion proteins from recombinant vectors.

For transfection of cells, initially 10^4 of Huh7.5 cells cultured in 6 well plate (Invitrogen, USA) containing Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal bovine serum (Invitrogen, USA), 0.2% sodium bicarbonate (Sigma, USA), 100U/ml penicillin (Invitrogen, USA), and 100µg/ml streptomycin (Invitrogen, USA) and incubated at 37°C and 5% CO_2 until cell confluency reached to 70 %. Huh-7.5 cell line was transfected by calcium phosphate method as described previously (20); briefly, 5mg of the vector containing mycobacterial genes was diluted with 225µl double distilled water (DDW). Then, 25 µl of $CaCl_2$ (2.5M) (21) was added until the final volume reached to 250 µl. Then, 250 µ1 of HEPES buffer (274 mM NaCl, 10mM KCl, 1.4 mM Na_2HPO_4, 15mM D-glucose, 42 mM HEPES at pH= 7.05-7.06) was added and final solution was kept for 20 -30 min at room temperature. At last stage, the obtained composition was added to Huh7.5 cell culture with 70% confluency. Four to six hours after transfection, old medium was replaced with fresh DMEM completed medium. Forty eight hours after transfection, transfected Huh7.5 cells along with untransfected cells as control were treated with trypsin enzyme (Invitrogen, USA) in order to recover cells (22).

RNA extraction. RNA was extracted from transfected and untransfected Huh7.5 cells using RNX plus solution (CinnaGen, Iran) according to the manufacturer's protocols. In this way total RNA was obtained. To remove plasmid DNA, purified RNA was treated with *DNase I* (Fermentas, Germany) enzyme as described previously (23); Briefly, 3µg of RNA with 5 units of DNaseI enzyme and 10 X buffers in 10µl total volume prepared then incubated at 37 °C for 30 min. For inactivation of *DNase I* enzyme, mixture incubated at 65 °C for 10 min. Moreover, the extracted RNA from transfected cells was examined for contamination with transfected recombinant plasmid by PCR method. Briefly, PCR reaction using specific primers for *hbha* and *mtb32C* genes was prepared and extracted RNA was used as template in the mixture. Negative results indicated absence of any plasmid contamination in RNA samples.

The extracted RNA was then used for cDNA synthesis and RT-PCR.

cDNA synthesis and RT-PCR. In order to prepare cDNA for each sample, 5 µg of total RNA, 10pmol of oligo-dT primer, 3 µl DEPC-treated water mixed together and incubated the mixture at 65 °C for 5 min and chilled on ice and added to the mixture containing 5× RT buffer, 5 unit RNase inhibitor, 0.5 mM each dNTP, 5 unit thermo-resistance RT enzyme. The tubes were placed in thermal cycler (Applied Biosystem, USA) programmed as follows: (25 °C 10 min, 47 °C 60 min ,70 °C 10 min). PCR reactions were prepared as follows: 2 µl of RT reaction, 2 µl of 10x PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM each

dNTPs, 10 pmol of each primer specific for *mtb32C* gene fragment and 1 unit *Taq* DNA Polymerase. The PCR cycling program performed at initial denaturation at 95 °C for 5min and then 35 cycles as follows: 95 °C for 60 seconds, 58 °C for 60 seconds, 72 °C for 60 seconds followed by a final extension at 72 °C for 180 seconds. Finally, 5µl of the RT-PCR product was loaded on 1% agarose gel. A distinct approximately 400bp PCR product was visualized after Green Viewer staining (Pars Tous, Iran) in tranfected sample while in control sample and RNA samples no band was found.

Western blot analysis. Presence of recombinant protein was confirmed using Western blot analysis. To prepare samples, transfected cells and non-transfected cells were harvested and washed with ice-cold 0.5 ml phosphate buffer solution (PBS). Phenyl methyl sulfonyl fluorid protease inhibitor (PMSF) (Invitrogen, USA) was added at 1mM final concentration and then samples sonicated at 60Hz for 5 min, 2 times for releasing the proteins from cells. Determination of protein concentration was performed by Bradford assay. Loading buffer (4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 6.8 was added to each sample and boiled at 95-100 °C for 5 minutes. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 20 µg of protein was separated and then transferred to polyvinylidine difluoride (PVDF) (BioRad, USA) membrane in wet condition. In order to prevent any non-specific background binding, the membrane was blocked with blocker solution (5% (w/v) BSA, 0.5M NaCl, 0.02 M Tris pH= 8.5, 0.05% Tween 20) for 2 hours at room temperature. Finally, the membrane stained with mouse anti-HisTag antibody (AbD Serotec, UK) as primary antibody (1:1000 diluted in Tris-buffer saline-Tween (TBS-T) containing 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH=7.6) and peroxidase conjugated rabbit anti-mouse IgG antibody (AbD Serotec, UK) as secondary antibody (1:1000 diluted in TBS-T). Expected band was detected by diamino banzidine (DAB) (Sigma, USA) (19, 24).

RESULTS

Huh7.5 cell line was transfected with purified recombinant vector (Fig. 1) and expression of recombinant protein was confirmed by RT-PCR (Fig.M2).

Using calcium phosphate method, mycobacterial antigens successfully were expressed in Huh7.5 cell line. In transfected cells a protein of interest was detected (Fig. 3).

DISCUSSION

Tuberculosis (TB) is a chronic disease that has

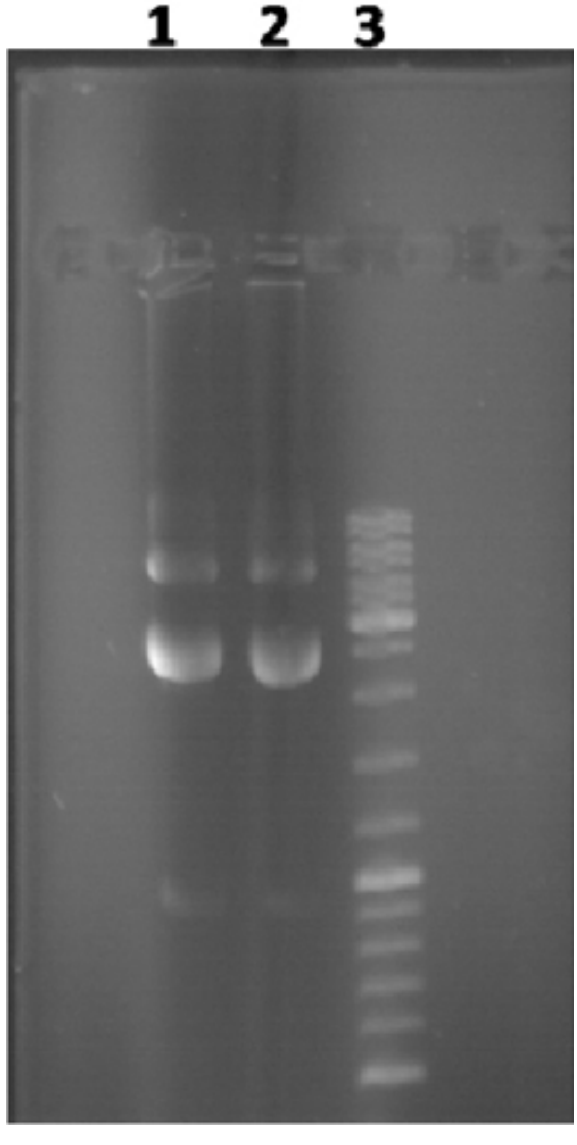

Fig.1. Gel electrophoresis of the recombinant vector encoding *mtb32C-hbha* fusion gene, after purification. Lanes 1 and 2: purified recombinant vector containing *mtb32C-hbha* genes; lane3: 100bp DNA size marker (Fermentas, Germany)

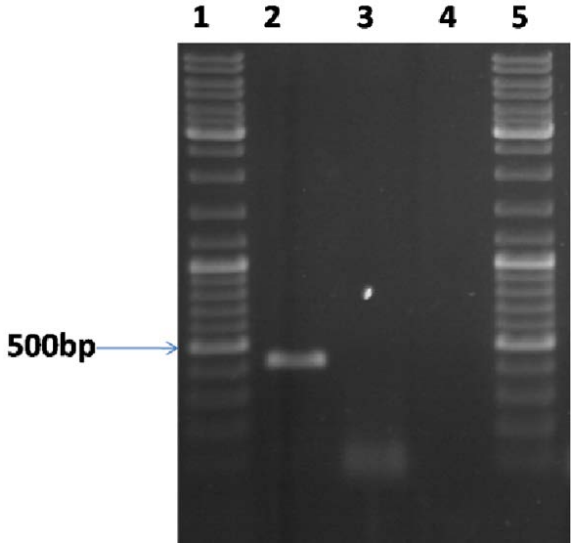

Fig. 2 Identification of Mtb32C mRNA in transfected and non-transfected Huh-7.5 cells by RT-PCR method. A 400bp fragment was amplified by RT-PCR method in transfected cells (lane 2); no amplification was resulted by RT-PCR on extracted RNA treated with DNase I (lane 3) and on synthesized cDNA of non transfected cells (lane 4). Lanes 1 and 5: 100bp DNA size marker (Fermentas, Germany)

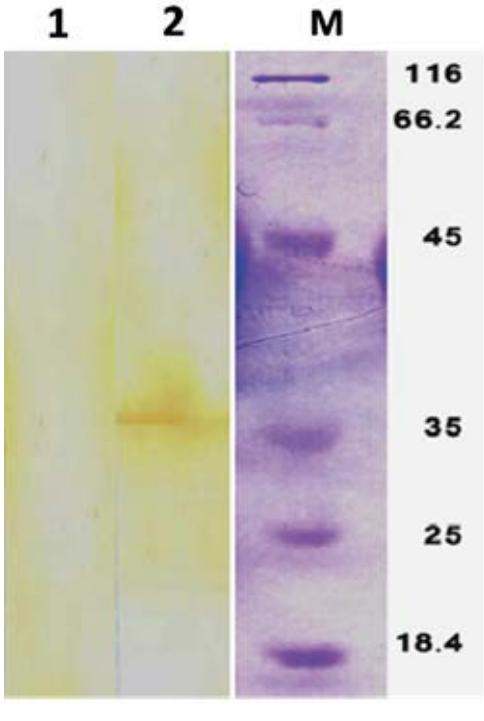

Fig. 3. Recognition of an approximately 35.5 kDa band corresponding to Mtb32C-HBHA fusion protein in transfected cells (Lane 2) but not in non-transfected cells (Lane 1) by Western blot method

affected mankind for many years. Despite many attempts TB remains a leading cause of death from a single agent of infection across the world and it is the first infectious disease announced by the World Health Organization (WHO) as international public health emergency. It is estimated that about 7% of all deaths in developing countries are contributed to TB (25-27).

There are several ways to control TB such as improvement diagnostic methods, expansion and development of new drugs and vaccines but most of researches in these areas were not successful. *M. tuberculosis* is easily transmitted by aerosol so the first host cells that encounter the bacteria are respiratory epithelial cells (13, 28). The result of the interaction between microorganisms with epithelial cells in the early stages of infection play an important role in the pathogenesis of bacterial infection and entering to the epithelial cells; therefore, effective immunity against adhesions can prevent bacterial colonization. Alveolar macrophages are the main target of the tubercle bacillus, but the number of lung epithelial cells in this area is 500 to 1000 times higher than macrophages. Therefore, much more likely these cells become infected than macrophages (29, 30). Evidence shows that mycobacterium can grow in epithelial cells and in infected individuals bacteria can be isolated from these cells. These cells are important to spread the infection outside the lung. In this regard HBHA as bacterial adhesion has significant role so that inactivation of this protein decline evasion of bacterium to the internal organs such as spleen. Several studies have shown that HBHA recognized by T lymphocytes; leading to induction of IFN-γ production in high-level thus HBHA is a proper vaccine candidate and is a good marker for differentiation of both normal individuals who have active tuberculosis from who are infected with *M. tuberculosis* (31-34).

A gene encoding *mtb32a* is found in *M. tuberculosis* complex but absence in non- pathogenic and environmental species. This gene is contributed to metabolism of bacteria and cell survival. C-terminal fragment of Mtb32a (Mtb32C) can strongly motivate TCD8 cells that produce IFN-γ and is a vaccine candidate. In previous study a DNA vaccine encoding Mtb32C and HBHA from *M. tuberculosis* was constructed and then its capacity in making protein investigated in current study (16, 35, 36).

Not more than a decade has passed since the introduction of the DNA vaccine. DNA vaccine is a simple method for introducing antigens to the host that the

first time was presented by Wolf (37). The DNA plasmid enter directly into the cells where transcription and translation genes of interest occur (27). In 1992 Tang and colleagues demonstrated that plasmid DNA can lead to the stimulation of the host's immune system (38). Next year the first report on the impact of protective role of DNA vaccine against infectious diseases such as influenza was announced by Ulmer et al. (39). DNA vaccines have several important advantages to other vaccines such as easy preparation, stability, low cost and safety for people with weakened immune systems. Nowadays DNA vaccine for cancer and malaria are on the clinical stages and promising strategy to develop novel vaccine and drug (40, 41).

In this study calcium phosphate method was used for transfection of Huh7.5 cells. In this method DNA can be introduced into wide variety of cell types via calcium phosphate co-precipitation(24).

DNA vaccine is quite a straightforward and efficient way for the stimulation of humoral and cellular immune responses. In previous studies, the efficiency of mycobacterial DNA vaccines in small rodents have been confirmed (42).

DNA vaccines have several advantages over other types of vaccines. Among the advantages are the relatively simple and cost-effective procedure of their production. This would result in the production of millions of doses of vaccines in a short time. Also, the vaccine is highly stable and does not require refrigeration chains. DNA vaccine is able to simultaneously stimulate the TCD8 + and Th1 CD4+ cells as well as the production of long lasting antibodies. Silva and Lowrie firstly reported that the DNA vaccine encoding Hsp 65 gene can stimulate protective responses and prevent the spread of tuberculosis in mice (42).

The disadvantage of DNA vaccine is its low immunogenicity. In order to enhance DNA vaccine immunogenicity, extensive studies have been conducted such as the usage of genes related to stimulatory molecules, cytokines and using different routes for vaccine administration. Among these efforts are the injection of DNA vaccine along with CPG as an adjuvant which leads to the differentiation and proliferation of B Cells, NK Cells, monocytes, macrophages and lymphocytes (43).

Recently, the prime-boost strategy has been employed to increase the efficiency of the DNA vaccine. In this method, the DNA vaccine has been administered along with an alternative antigen. This alternative antigen can be a subunit protein, a recombinant viral vector such as adenovirus or inactivated microorganisms. This strategy is very effective in increasing the immunogenicity compared to cases in which the vaccine is administered alone (43).

Today, 15 years have passed after the first DNA vaccine entered the clinical trial studies but still no TB- specific DNA vaccine in humans have been commercialized. Nevertheless, many researchers are working on methods such as optimization of codons, recognition of the proper antigens, identification of innate immune signaling molecules and using them as adjuvants, and using prime-boost strategy to increase the quality of DNA vaccines (44).The present work was successful in expression of recombinant Mtb32C-HBHA in Huh7.5 cell line and for confirming of expression in eukaryotic cells, RNA extraction, cDNA synthesis, RT-PCR and Western blot were used.

In conclusion, the prepared vector efficiently can multiply in Huh7.5 cell culture system and produce protein. This construction can be used in another study for evaluation the immunological responses in animal model.

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