Original Article

Isolation, cloning and molecular analysis of ag85a and tb10.4 genes from *Mycobacterium tuberculosis*

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Abstract

Background: Novel tuberculosis (TB) vaccines that aim to boost and/or replace Bacillus Calmette-Guerin (BCG) are currently in development. DNA vaccines can stimulate both humoral and cell-mediated immunity in different animal models of TB and is thought to be a promising strategy in the development of new vaccines against TB. The aim of this study was to design and construct a DNA vaccine encoding ag85a and tb10.4 fusion genes of *Mycobacterium tuberculosis*.

Materials and Methods: tb10.4 fragment was amplified by PCR and the product was digested with restriction enzymes. Next, it was cloned into the pcDNA3.1+ plasmid. The ag85a gene and pcDNA3.1+/tb10.4 plasmid were digested by EcoRI and BamHI restriction enzymes. Constructed vector was sequenced. The molecular analysis was done using bioinformatics software. New chimeric vector containing ag85a-tb10.4 genes were purified. Expression of pcDNA3.1+/tb10.4-ag85a plasmid was confirmed in eukaryotic cells.

Results: Fragments of 297 bp for tb10.4 and 1017 bp for ag85a were observed in agarose gel electrophoresis. Alignment of ag85a-tb10.4 genome sequence with reference genes in GenBank showed exact identities that indicate correction of all cloning procedures. Transfection of eukaryotic cells with pcDNA3.1+/tb10.4-ag85a vector and existence of tb10.4-ag85a fusion gene were both confirmed with RT-PCR.

Conclusion: In this study, tb10.4 and ag85a genes were isolated from Mycobacterium tuberculosis H37Rv strain and cloned into pcDNA3.1+. Also, the capability of constructed vector in producing fusion ag85a-tb10.4 protein was confirmed with RT-PCR. pcDNA3.1+/tb10.4-ag85a vector can be used for further studies in future.

Keywords: *Mycobacterium tuberculosis*; ag85a; tb10.4; pcDNA3.1+; DNA vaccine.

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Introduction

In 1882, Robert Koch, identified *Mycobacterium tuberculosis* (*M. tuberculosis*) as the cause of tuberculosis (TB) (1-3). TB is one of the important causes of worldwide death, with an estimated 9.4 million new cases and 1.7 million deaths in 2008 (4, 5).

Mycobacterium bovis bacillus Calmette–Guérin (BCG), the only licensed TB vaccine, is a live attenuated strain of *M. bovis* which was passaged by Calmette and Guérin almost one hundred years ago. BCG confers resistance to meningitis caused by *M. tuberculosis* and the disseminated form of TB among children (4, 6, 7). On the other hand; it confers a highly variable protection against pulmonary disease in adults. This is the major reason for the high

morbidity and mortality among the TB-infected patients (4, 8).

The limited effectiveness of BCG vaccine against tuberculosis has led scientists to the development of a more effective vaccine such as plasmid DNA vaccines encoding the dominant genes of *M. tuberculosis* (9-11).

DNA vaccines induce protective immunity in several animal models of parasitic, viral and bacterial infections. DNA vaccines have advantages over other vaccines. These include the easy procedure to produce, stability, and safely used for immunocompromised patients (12-15).

An understanding of the nature of protective immunity is required to design an effective vaccine against TB. A cellular immune response is an essential requirement for protective immunity against mycobacterial diseases, and all new TB vaccines are supposed to induce high levels of cellular immunity. CD4+ T cells, together with the Th-1 cytokines, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) are necessary for protective immunity (4, 16). CD8+ T cells are probably also required for optimal protective immunity (4, 17).

Several immunogenic secreted antigens have been identified using advanced molecular technologies (18). An important part of secretory proteins is formed by Ag85 complex; a family of three proteins (Ag85A, Ag85B, Ag85C). All three proteins have mycolyl transferase enzyme activity. These fibronectin-binding proteins are responsible for binding to human macrophages during the early phase of infection (19, 20).

tb10.4 gene belongs to ESAT-6 family that encodes three highly homologous proteins (TB10.4 (Rv0288), TB10.3 (Rv3019c) and TB12.9 (Rv3017c)). These members of TB10.4 family are present only in strains of *M. tuberculosis* complex (1).

The aim of the present study was to clone tb10.4 and ag85a genes in pcDNA3.1+ plasmid and transfection of eukaryotic cells by pcDNA3.1+/tb10.4-ag85a recombinant plasmid.

Methods

DNA extraction. DNA extraction from *Mycobacterium tuberculosis* H37Rv strain was performed as described previously; briefly, *Mycobacterium tuberculosis* H37Rv strain was cultured on Lowenstain medium and then, grown colony diluted in buffer (Tris- Cl (100 mM, pH = 7.5), Tween-20 (0.05%)) and DNA extraction performed based on boiling method as described previously (21).

Amplification and cloning of tb10.4 fragment. Specific primers for isolation of tb10.4 were designed using GeneRunner software. tb10.4 fragment amplification was performed by PCR then, it was cloned into pcDNA3.1+ plasmid as described previously (21).

Ag85a gel purification and enzyme digestion. First, terminal sequence of ag85a was deleted from the end of the gene. In next step, two restriction enzymes (BamHI and EcoRI) which did not have restriction sites inside the gene were selected using gene runner software. Then, full length sequence of ag85a flanked by these two restriction sites and a kozak sequence at the 3´ end was designed and synthesized by GENEray (China). The synthetic gene cloned into pUC57 and the plasmid were digested with BamHI and EcoRI restriction enzymes (Fermentas, Germany). Then, digested plasmid was purified from the gel and ligated into the digested and purified pcDNA3.1+/tb10.4 plasmid.

ag85a cloning confirmation in pcDNA3.1+/tb10.4 vector. pcDNA3.1+/ag85a-tb10.4 construct was used for transformation by heat shock method (22). Transformed bacteria were inoculated on LB agar medium containing 100 μg/ml ampicillin for 16 hour at 37°C. Cloning of ag85a into pcDNA3.1+/tb10.4 was confirmed by colony-PCR method using forward primer of ag85a fragment and reverse primer of tb10.4 fragment and enzymatic digestion with EcoRI and BamHI enzymes.

Colonies were screened by colony-PCR. PCR reaction mixture contained 0.5μl dNTP (0.2mM), 0.3μl Taq DNA polymerase (5U/μl), 1.5μl MgCl2 (1.5mM), 2.5μl Buffer 10X (Fermentas, Germany), 17.2μl DNase free water, 1μl Forward primer of ag85a (5'AATATAGGATCCACCATGGACAGCTTGTT GACAGGGTTCGTGGC–3') (10 pmol) and 1μl Reverse primer of tb10.4 (5'ACTATATCTAGATTACTAACCTCCCCATTTG GCG –3') (10 pmol) (CinnaGen, Iran).

The following Colony-PCR program for ag85a

was used: First cycle at 95°C for 300 seconds followed by 40 subsequent cycles of 95°C for 60 seconds, 52°C for 60 seconds, 72°C for 60 seconds and final extension of 72°C for 420 seconds.

Expression in eukaryotic system. pcDNA3.1+/ag85a-tb10.4 was purified with alkaline method and transfected in eukaryotic HeLa cells that was cultured in DMEM medium containing 10% FBS (Fetal Bovine Serum) and 1% antibiotics (penicillin and streptomycin) using cationic liposome method (23). In this method, the complex contains pcDNA3.1+/ag85a-tb10.4, lipofectamine and DMEM medium without FBS and antibiotics. The culture was incubated for 24h in 37°C with 5% CO2. Then, the medium was exchanged with DMEM containing FBS and antibiotics.

RNA extraction. To confirm tb10.4-ag85a gene expression in eukaryotic cells, the cells were collected 48 hours after transfection. Next, RNA extraction was performed with RNX-PLUS kit (CinnaGen, Iran) as described previously (21).

RT-PCR for confirming the expression. RT-PCR was performed with the RNA extract and oligo dT (used as a primer) and DEPC- treated water free RNA enzyme in an RNase free microtube. After addition of pre-mixed solution containing RNase inhibitor and thermos-resistant RT, the microtube was incubated at 70°C for 10 min.

In the final stage, to confirm the expression of tb10.4-ag85a fragment, we have done PCR. Forward primer of ag85a and reverse primer of tb10.4 were used for this reaction.

Sequencing. For the final confirmation, recombinant vector containing tb10.4-ag85a genes was sequenced with the universal primers T7 and BGH MacroGen, Korea)

Figure 1. Purified PCR product of tb10.4 gene on 1.5% gel: Lane 1: 290 bp band of tb10.4, Lane 2: 1kb DNA size marker (SM0313, Fermentas, Germany).

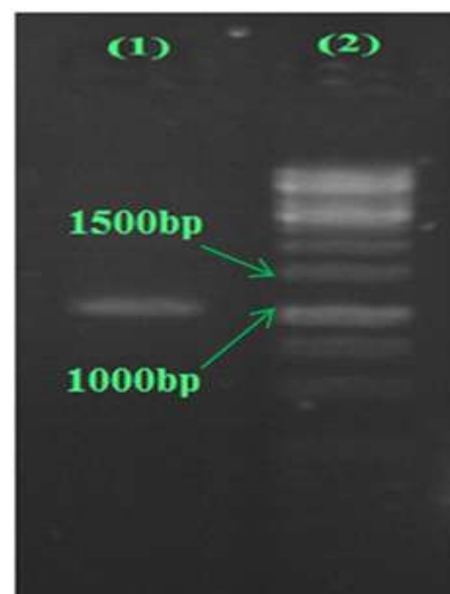

Figure 2. Purified PCR product of ag85a gene on 1.5% gel: Lane 1: 1017 bp band of ag85a , Lane 2: 1kb DNA size marker (SM0313, Fermentas, Germany).

Results

Extracted DNA was used for PCR reactions. Then, the PCR products were electrophoresized on a 1.5% agarose gel. 290 bp amplified fragment of tb10.4 (Figure 1) and 1017 bp fragment of ag85a gene were observed (Figure 2).

After enzyme digestion and purification of ag85a fragment, it was ligated to a pcDNA3.1+/tb10.4 recombinant vector and then pcDNA3.1+/ag85a-tb10.4 recombinant vector was transformed to a competent *E. coli* JM109 strain.

16h after transformation of competent bacteria at 37°C, some colonies were grown on LB agar medium containing ampicillin. The pcDNA3.1+/tb10.4/ag85a was confirmed by colony-PCR using forward primer of ag85a and reverse primer of tb10.4. Colonies with the specific plasmid

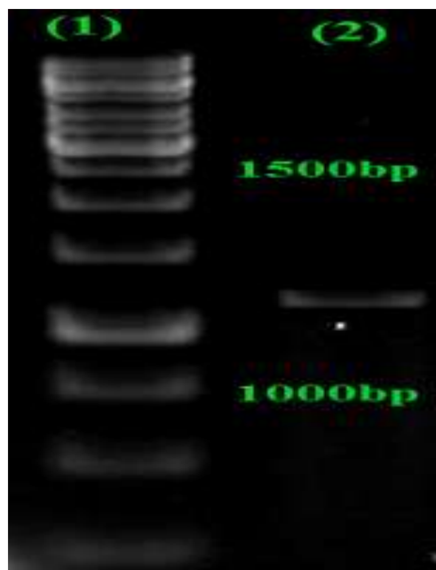

Figure 3. Colony-PCR results. Lane 1: 1kb DNA size marker (SM0313, Fermentas, Germany). Lane 2: 1300 bp band of tb10.4/ag85a.

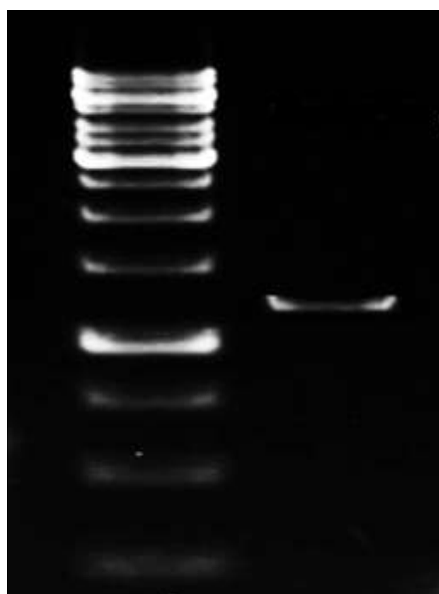

Figure 4. Restriction enzyme digestion of pcDNA3.1+/ag85a-tb10.4. Lane 1: 1kb DNA size marker (SM0313, Fermentas, Germany). Lane 2: 1300 bp band of tb10.4/ag85a.

were positive and showed the corresponding 1300bp lane compared to 1kp DNA size marker (Figure 3).

Digestion was performed using EcoRI and BamHI restriction enzymes. 1300 bp fragment of tb10.4/ag85a was observed (Figure 4).

Sequence analysis of tb10.4-ag85a genes showed that there is 100% identity between acquired sequence and registered sequences in Gene Bank. These data confirm all cloning procedures.

HeLa eukaryotic cells (grown in DMEM culture medium) were transfected with PcDNA3.1+/tb10.4-ag85a recombinant vector and cells for 48h at 37°C. Finally, to confirm the expression of this gene in eukaryotic cells, RNA extraction, cDNA synthesis and RT-PCR (using tb10.4 reverse primer and ag85a forward primer) was performed respectively, and a 1300 bp fragment of tb10.4/ag85a was observed (Figure 5).

Discussion

The strategy of developing vaccines which confer cellular immunity toward the pathogen results in the increase in the number of specific T-cells for the pathogen. In the case of tuberculosis, there are no biomarkers of protective immunity and consequently a better understanding of what constitutes a protective T cell response could more effectively guide vaccine development (24).

Novel TB vaccines that aim to boost and/or replace BCG are currently in development and some have shown promising results in in vitro studies, animal models, and phase I and II clinical trials (25).

DNA vaccines can stimulate both humoral and cell-mediated immunity in different animal models of

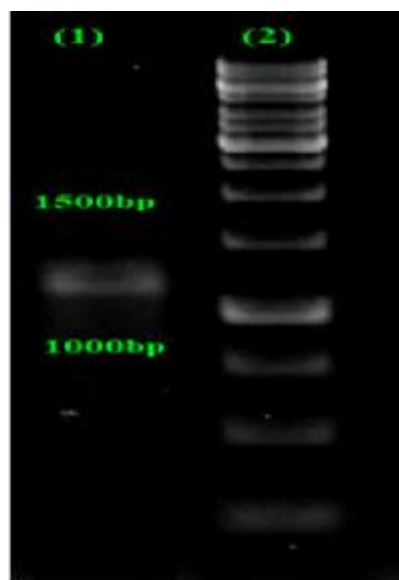

Figure 5. RT-PCR results. Lane 1: 1300 bp band of ag85a/tb10.4; Lane 2: 1kb DNA size marker (SM0313, Fermentas, Germany).

TB and is thought to be a promising strategy in the development of new vaccines against it. DNA vaccine candidates expressing several antigens of *M. tuberculosis* have been shown to provide protective immune responses against TB (26).

When complete genome sequence of *M. tuberculosis* was identified, more than 100 subunit and DNA vaccines have been studied. However, still no protective antigen of *M. tuberculosis* has been found for vaccine development purposes and this has created a major barrier in the development of TB vaccines. To date, only a few DNA vaccine encoding ag85a, ag85b, esat-6, Pst-3 and hsp65 have shown promising degree of protection (27).

Subunit vaccines comprise a small set of secreted antigens, i.e., proteins secreted by metabolically active *M. tuberculosis*. The frequently used Ag85 cognates, ag85a or ag85b, which are most widely used in the current vaccine candidates are shared by BCG and *M. tuberculosis*. Additional antigens that are unique to *M. tuberculosis* are ESAT6 and tb10.4. They (ESAT6 and tb10.4) are encoded within the region of difference 1 (RD-1) gene cluster which is present in *M. tuberculosis* and absent in BCG (28).

In the present study, in order to produce a vaccine against *M. tuberculosis* strain H37Rv, tb10.4 and ag85a antigens were used. The antigen 85 complex is composed of at least three different closely related proteins named 85A, 85B, and 85C and is a major secretion product of several mycobacterial species with a MW range of 30–32000 daltons. These proteins are also found associated with the bacterial cell surface. Their properties, especially the capacity to bind fibronectin, in addition to the surface association, make them an attractive virulence factor candidates (29).

Sushila D'Souza study indicated that vaccination with plasmid DNA encoding *M. tuberculosis* ag85a was immunogenic and could protect mice against an aerosol and *M. tuberculosis* challenge infection (10, 30).

Esat-6 gene family (such as tb10.4), has attracted significant interest and has been demonstrated to encode several immunodominant molecules that are strongly recognized by the immune systems in different animal models of TB, as well as by T cells from human beings exposed to *M. tuberculosis* (1).

In this study, restriction enzymes EcoRI and XbaI were used to clone tb10.4. Transformation was performed using *E. coli* strain JM109. Eukaryotic cells were transfected with PcDNA3.1+ vector. To confirm the expression in eukaryotic cells, RNA extraction, RT-PCR and cDNA synthesis was performed.

Ag85a and tb10.4 are strongly recognized by T-cells isolated from TB patients and have been observed to induce a robust cell mediated immune response (31).

Gilson C. Macedo et al showed the ability of Ag85A, Ag85B, and ESAT-6 to differentiate TB patients from controls (32).

Jia Lu et al showed that two DNA vaccine separately encoding antigen Ag85A and ESAT-6 could induce strong humoral and cell-mediated immunity in vaccinated mice which resulted in some degree of protection in mice challenged with virulent *M. tuberculosis* (26, 27).

Vaccination has a major role in the final goal of global eradication of tuberculosis (33). In this study, ag85a and tb10.4 genes of *M. tuberculosis* with the use of pcDNA3.1+ plasmid were fused. Then, eukaryotic cells were transfected with pcDNA3.1+/tb10.4-ag85a plasmid for confirming expression of tb10.4-ag85a in these cells. This study can be used for development of more new DNA vaccines in future studies.

Conclusion

The risk of increasing spread of TB and development of drug resistance make treatment a matter of greatest concern. In this study, a DNA vaccine encoding ag85a and tb10.4 fragments was prepared. Successful cloning provides a basis for development of new DNA vaccines against tuberculosis. The designed expression vector can be used as a DNA vaccine in future studies. In addition, it can be administered with other TB vaccines in animal models.

Conflicts of Interest

The current study was from a thesis presented for obtaining the MSc degree from Mashhad University of Medical Sciences, Mashhad, Iran (Thesis

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Acknowledgment

There is no conflict of interest.

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