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Designing and Construction of a DNA Vaccine Encoding Tb10.4 Gene of *Mycobacterium tuberculosis*

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KEY WORDS

Mycobacterium tuberculosis

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ABSTRACT

Background: Tuberculosis (TB) remains as a major cause of death. Construction of a new vaccine against tuberculosis is an effective way to control it. Several vaccines against this disease have been developed. The aim of the present study was to cloning of *tb10.4* gene in pcDNA3.1+ plasmid and evaluation of its expression in eukaryotic cells.

Methods: Firstly, *tb10.4* fragment was amplified by PCR and the PCR product was digested with restriction enzymes. Next, it was cloned into pcDNA3.1+ plasmid. Following that, pcDNA3.1+/*tb10.4* recombinant plasmid was transfected into eukaryotic cells.

Results: 5700 bp band for pcDNA3.1+/*tb10.4* recombinant plasmid and 297 bp fragment for *tb10.4* were observed. Cloning and transfection were successful and designed recombinant vector was confirmed by sequencing.

Conclusion: Successful cloning provides a basis for the development of new DNA vaccines against tuberculosis.

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Introduction

In 1882, Robert Koch isolated and identified *Mycobacterium tuberculosis* as the cause of tuberculosis (1). *M. tuberculosis*, the causative agent of TB, is a common pathogen that has not been controlled effectively in many parts of the world (2).

TB is a major cause of mortality with almost 3 million people death each year (3). Our ability to control and in some cases eradicate human disease caused by pathogenic bacteria and viruses has been improved by the capacity to stimulate protective immunity by vaccinating susceptible hosts with attenuated or inactivated bacteria (4).

In 1992, Tang et al. showed that injection of DNA could induce immune responses (5). It was later reported that DNA vaccines induce protective immunity in several animal models of parasitic, viral and bacterial infections. DNA vaccines have advantages over other vaccines (6).

The only vaccine permitted by the WHO for human use in cases of TB is BCG (2, 4, 7). According to the routine immunization program, this vaccine is injected at birth or after the first contact with *M. tuberculosis* (2). While infant vaccination with BCG vaccine has been effective to reduce the severe form of childhood tuberculosis in endemic areas, its protective effects decreases with time (lasts fewer than 15 yr). This results in highly variable efficiency, which seems insufficient to control pulmonary tuberculosis among adults (2, 4, 8, 9).

The efficacy limitation of BCG vaccine is a motivating force for the development new and better vaccines against tuberculosis (2, 4). These include plasmid DNA vaccines encoding dominant genes of *M. tuberculosis*, recombinant BCG vaccine, attenuated *M. tuberculosis*, and recombinant protein antigens subunit vaccines (3).

Among the new vaccine platforms, genetic vectors such as recombinant plasmid DNA vectors have widely been used to deliver microbial antigen-coding genes. These vectors could strongly induce both CD4+ and CD8+ T cell responses, required for effective TB vaccination (10).

The gene encoding TB10.4 belongs to a subfamily of the ESAT-6 family that encoded three homologous proteins including TB10.4 (Rv0288), TB10.3 (Rv3019c) and TB12.9 (Rv3017c). These three members are only present in some strains of *M. tuberculosis* complex including BCG and *M. kansasii* (1).

To extract DNA, *M. tuberculosis* H37Rv strain (Pasteur Institute, Tehran, Iran) was used. Some of the bacteria grown in Middle Brook medium were transferred into Lewen Stein Johnson medium. After that, the medium was incubated at 37 °C until colonies formed. After that, their DNA was extracted with Tris/Tween20 method (11).

Amplification of *tb10.4* fragment

To amplify *tb10.4* fragment with PCR method, two primers were used, 5′-ATATATAGAATTCTCGCAAATCATGTACAAC-3′ as forward primer and 5′-ACTATATCTAGATTACTAACCTCCCCATTTGGCG–3′ as reverse primer (in forward and reverse primers, the underlined letters, respectively, indicates positions of *Eco*RI and *Xba*I restriction enzyme restriction sites.

PCR reaction mixture contains 1μl DNA (100 ng/μl), 0.5μl dNTP (0.2 mM), 0.3μl *Taq DNA polymerase* enzyme (5U/ml), 1.5ml MgCl2 (1.5mM), 2.5μl 10X Buffer (Fermentas, Germany), 17.2μl DNase free water, 1μl Forward primer (10 pmol) and 1μl reverse primer (10 pmol) (CinnaGen, Iran). PCR program was as follows (Table 1).

Table 1
PCR Program for *tb10.4*

Time (second)	Temperature (˚C)	Cycle
300	95	1
60	95	
60	52	40
60	72	
420	72	1

The aim of the present study was cloning of *tb10.4* gene in pcDNA3.1+ plasmid and evaluation of its expression in eukaryotic cells.

Materials and Methods

This study was performed at Mashhad University of Medical Sciences (Mashhad, Iran) from April 2012 to March 2013.

DNA extraction

Cloning of *tb10.4* fragment in pcDNA3.1+ vector

Fifty micro liters of the PCR product was used for electrophoresis in 1.5% agarose gel. Purification of gel was performed using Invitek DNA extraction kit (California, USA). For *tb10.4* enzymatic digestion, a mixture containing 5 μl *Xba*I (50U/μl), 4 μl *Eco*RI (10U/μl) and 5 μl 10X Buffer H (Fermentas, Germany) was mixed in one micro tube and 20 μl *tb10.4* was added to it (total volume was increased to 50 µl using DNase free water) and then were incubated at 37 ˚C for 16 h. PcDNA3.1+ plasmid was extracted with alkaline method. In this method, plasmid DNA was sedimented with different solutions. It was then extracted by washing with isopropanol. The extracted plasmid DNA was purified with Invitek DNA extraction kit (California, USA). For pcDNA3.1+ enzymatic digestion, a mixture containing 5 µl *Xba*I (50U/µl), 4 µl *Eco*RI (10U/µl) and 5 µl 10X Buffer H (Fermentas, Germany) was mixed in one micro tube. Following that, 10µl pcDNA3.1+ plasmid was added in it (total volume was increased to 50 µl using DNase free water) and was then incubated at 37 ˚C for 16 h. In the next step, digested and purified *tb10.4* fragments, were ligated to purified pcDNA3.1+ plasmid using *T4 DNA ligase* restriction enzyme (Fermentas, Germany). Ligation mixture contained 2 µl PEG, 2 µl T4 DNA ligase (5U/µl), 2.5 µl *T4 DNA ligase* 10X buffer (Fermentas, Germany), 12 µl *tb10.4* DNA (25 ng/µl), 6 µl pcDNA3.1+ plasmid (100ng/µl) and 0.5 µl DNase free water. It was incubated at 22 ˚C for 16 h.

Competent *E. coli* bacteria strain *JM109*, was prepared using $CaCl_2$ 0.5 M and pcDNA3.1+/*tb10.4* plasmid was transferred into competent bacteria using heat shock method (12).

Confirmed *tb10.4* cloning in pcDNA3.1+ vector

Transformed bacteria were inoculated on LB agar medium containing 100 µg/µl ampicillin and were incubated for 16h at 37 °C. Cloning *Tb10.4* gene in pcDNA3.1+ vector was confirmed by colony-PCR method (using *tb10.4* specific primers) and enzymatic digestion with *Eco*RI restriction enzyme.

HeLa cell culture

Eukaryotic HeLa cell was cultured in DMEM medium, which contained 10% fetal bovine serum (FBS), and 1% antibiotics and then incubated in 37 °C until the cells begin to grow and proliferation.

Transfection in eukaryotic cells

pcDNA3.1+/*tb10.4* recombinant plasmid was purified with alkaline method and transfected in eukaryotic HeLa cell with cationic liposome method using lipofectamine.

To confirm *tb10.4* gene expression, 48 h after transfection, the medium of cells were collected and used for RNA extraction.

RNA extraction and cDNA synthesis

RNA extraction was performed with RNX-PLUS kit according to the recommendations (CinnaGen, Iran). To remove the transfected vector, extracted RNA was digested by enzymatic digestion with *DNaseI*. Then, cDNA synthesis was performed with oligo dT primers kit (Invitrogen, San Di- ego, California).

Confirming the expression of *tb10.4* fragment

To confirm the expression of *tb10.4* fragment, PCR method was used using *tb10.4* specific primers as described at first in amplification of *tb10.4* fragment section.

Results

The extracted DNA was further used for PCR with specific primers. PCR products were subjected for electrophoresis on a 1.5% agarose gel, and 290 bp fragment of *tb10.4* gene was observed (Figure 1).

After purification of *tb10.4* products, they were digested with restriction enzymes. This fragment was further ligated to a pcDNA3.1+ plasmid and was transformed into a competent *E. coli JM109* strain.

After 16 h of transformation of competence bacteria and incubation at 37 °C, some colonies were grown on LB agar medium containing am-

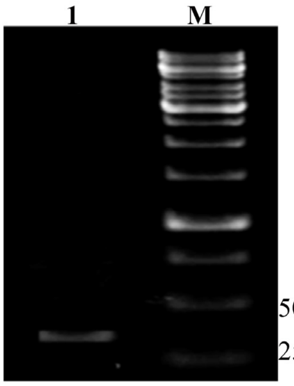

Fig. 1
PCR result for *tb10.4*. Lane 1: PCR products of a 290bp amplified fragment of *tb10.4* gene M: 1kb DNA size marker (SM0313, Fermentas, Germany).

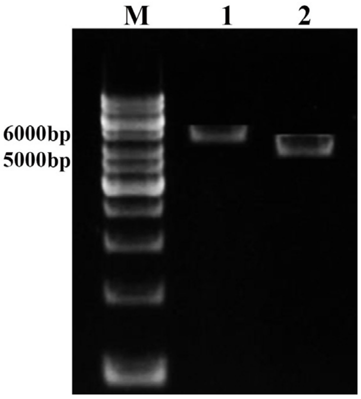

Fig. 2
RT-PCR results. Lane M: 1kb DNA size marker (SM0313, Fermentas, Germany). Lane 1: 290 bp fragment of *tb10.4* gene in RT-PCR.

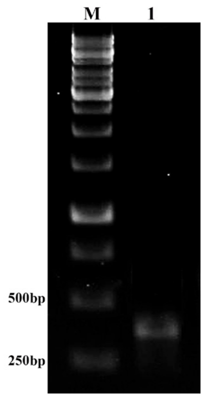

Fig. 3
Enzymatic digestion of pcDNA3.1+/*tb10.4* plasmid with *Eco*RI and *Xba*I restriction enzymes. Lane M: 1kb DNA size marker (SM0313, Fermentas, Germany). Lane 1: 5700 bp band of pcDNA3.1+/*tb10.4* recombinant plasmid digested with *Eco*RI. Lane 2: 5400 bp band of pcDNA3.1+ plasmid digested with *Eco*RI and *Xba*I.

picillin. The pcDNA3.1+/*tb10.4* recombinant vector was confirmed by colony- PCR using specific primers of *tb10.4*. Colonies with the specific plasmid were positive and showed the corresponding 290 bp size marker.

Results of the enzymatic digestion showed *tb10.4* fragment ligated in pcDNA3.1+ plasmid (Figure 2). Recombinant pcDNA3.1+/*tb10.4* was transfected into HeLa eukaryotic cell (grown on a DMEM medium culture) and cells incubated for 48 h at 37 °C. Finally, to confirm the expression of this gene in eukaryotic cells, RNA extraction, cDNA synthesis and RT-PCR were performed with a 290 bp fragment of *tb10.4* gene (Figure 3).

Discussion

One of the World Health Organization Millennium Development Goal is to reduce tuberculosis incidence by 2015. By designing and development of more effective drugs and vaccines compared to the conventional BCG (as currently being the only available vaccine), WHO target of decreasing the incidence of tuberculosis can be reached (9).

Since the completion of whole-genome sequencing of the causative agent *M. tuberculosis,* more than 100 DNA vaccines have been studied in animal TB models but still protective antigen for tuberculosis is not clear and this has created a major impediment to the development of tuberculosis vaccines (13).

In the past, many secreted proteins of *M. tuberculosis* were considered as candidate vaccines against tuberculosis. However, not all vaccines encoding antigens of *M. tuberculosis* were effective. Some plasmid DNAs encoding genes, such as 19 kDa lipoprotein, AhpC or crystalline alpha, rather than stimulating T-cell responses against the protein, were only able to stimulate non-protective antibody responses (13).

Another plasmid DNAs encoding 22 kDa protein, Pst-1 or HBHA of *M. tuberculosis*, provides no protection. Nevertheless, they could stimulate the antigen-specific antibody response and Th1-type immune response. So far, only a few DNA vaccine, encoding *ag85a*, *ag85b*, *esat-6*, *pst-3* and *hsp65* have shown promising degree of protection in mouse models. Therefore, they are possible candidate proteins for developing tuberculosis vaccines (13).TB10.4 is a recently identified protein encoded by the Rv0288 gene located in the *esx* cluster 3. TB10.4 appears to be essential for the virulence of *M. tuberculosis* (14, 15). The expression of Rv0288 is significantly downregulated in the attenuated *H37Ra* strain in comparison to the virulent *H37Ra* strain. Moreover, newly extensive identification of critical genes in *M. tuberculosis* includes the *esx* cluster 3 in the list of 600 genes essential for in vitro growth (16). Tb10.4 protein has conserved sequences in clinical isolates of *M. tuberculosis* (17).

In the present study, in attempts to produce a vaccine against *M. tuberculosis* strain H37Rv, *tb10.4* antigen was used. Tb10.4 stimulates immune responses and in TB patients *tb10.4* was even more strongly recognized than ESAT-6 (1), suggesting that it may be an ideal candidate to replace ESAT-6 (18).

The lack of diversity in TB10.4 sequence originated from 13 clinical isolates of *M. tuberculosis* (from different geographical locations) suggests that it has an important biological function (1). Ag-specific CD8+ T cells from infected mice produce several different cytokines following stimulation with the TB10.4 (4). Recently a zinc-binding site has been recognized in the TB10.4 protein involved in zinc ion acquisition (19, 20).

Desta Kassa et al. verified the immune response against several mycobacterial antigens, including five classical and 64 nonclassical antigens in active-pulmonary-tuberculosis (TB) patients. Most of the study participants (84.8%) responded to the TB10.4 as classical *M. tuberculosis* antigen (21).

The present study was victorious in cloning and expression of *tb10.4* secretory protein from *M. tuberculosis H37Rv* strain. *EcoRI* and *XbaI* restriction enzymes were used for cloning. For transformation, *E. coli* strain JM109 was used. PcDNA3.1+ Vector was used to import into eukaryotic cells. To confirm the expression in eukaryotic cells, RNA extraction and RT-PCR and cDNA synthesis was performed.

A phagosome is a vesicle produced around a particle absorbed by phagocytosis in which pathogenic microorganisms can be destroyed and digested. Many mycobacteria, including *M. tuberculosis* (22, 23), manipulate the host macrophage to hamper nitrous acid- comprising lysosomes from fusing with phagosomes and creating mature phagolysosomes. Such immature phagosome maintains an environment desirable to the pathogens inside it (24).Since there is no obvious homology to known protein from other organisms, this protein (TB10.4) has possible important mycobacterium-specific functions, which may be related to the intracellular region of the macrophage phagosome. In this regard, expression of this molecule may be extremely up regulated during intracellular growth (25).

Protective immune response against TB is mainly mediated through cellular immunity. In addition, it is dependent on activation of macrophages and granuloma formation. *M. tuberculosis* in macrophage is resistant to microbicide substances. However, these microbiocide substances effectively destroy other phagocytic bacteria. This is one reason that enables *M. tuberculosis* to stop the activation of macrophage by IFN-γ and IL-12 cytokines. Furthermore, deficiency in IFN-γ, IL-12 or their receptors, increased sensitivity to mycobacterium infection (26). In future, vaccination has a major role in the final goal of global eradication of tuberculosis (27, 28). Several TB vaccine candidates have shown sufficient promise in pre-clinical testing in different animal models to certification for initial phase I [safety] testing in human subjects (28).

This is a very important step for any new TB vaccine, and initially needs a brief study in healthy, PPD (Purified Protein Derivate) negative persons (usually adults). Additional Phase I trials may be performed on PPD+ individuals, children, infants, or other groups (28).

Often these trials are related to the phase IIA trial in which clinical samples are gathered for measurement of immunological response to the vaccine. Safety and immunogenicity are preconditions for any new TB vaccine to be accepted further in phase III [efficacy] trials (28). The critical concerns in the clinical trials of these vaccines change a little depending on the vaccine type. For example, for the living vaccines (e.g. recombinant BCG strains), the main concern is safety. For the subunit vaccines (e.g., proteins or peptides), the main experiment is the development of a safe and efficient adjuvant. For the DNA vaccines, the concern is effective transfer strategies, which ensure long-lasting protection (28, 29).

Construction of antigen expressing vectors that affecting the immunogenicity of *M. tuberculosis,* not only makes it possible to build a library of different antigens of this bacteria; it is possible to determine the effect of these antigens to stimulate the immune system and pathogenicity of them. In addition, these antigens can be used in the preparation of vaccines and designing of diagnostic kits.

Conclusion

The choice of *M. tuberculosis* antigens for expression by genetic TB vaccines is a critical determinant of vaccine efficacy. TB10.4 is a recently identified low molecular weight secreted *M. tuberculosis* protein, a member of the ESAT-6 family. TB10.4 antigen can be recognized by T cells activated by BCG immunization or *M. tuberculosis* infection. Successful cloning provides a basis for development of new DNA vaccines against tuberculosis. In this study, we prepared a plasmid encoding *tb10.4* fragment. The desired expression vector can be used as a vaccine in future studies. In addition, it can be administered with other TB vaccines in animal models.

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Conflict of interest

The authors declare that there is no conflict of interests.

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