Production of recombinant Human T Lymphotropic Virus type 1 Tax protein in Rosetta (DE3) bacterial host

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ABSTRACT

HTLV1 is the first detected retrovirus causing disease in human. The physiopathology of HTLV1 related diseases was mainly linked with its Tax protein characteristics. Use of mutant Tax proteins accompanied by immune regulator drugs could help treating HTLV1 associated myelopathy patients as a modulator of potent immune response against this viral protein. Since Tax protein is not commercially available, production of recombinant Tax protein was targeted for this study. Coding sequence of Tax protein (containing R222K mutation) in the pcDNA3.1(+) was digested with *Bam*HI and *Xho*I restriction enzymes, and then removed and inserted into the expression vector pET32a(+) within the same cutting sites and cloned into *E.coli* DH5α. Recombinant vector was confirmed with enzymatic digestion, colony PCR, and sequencing of cloned gene. *E.coli* Rosetta (DE3) was transformed with the recombinant plasmid and the expression was induced. The expression of protein was assayed with SDS-PAGE and western blot using monoclonal antibodies against Tax and 5His epitope. Finally, antigenic characteristic of the recombinant protein was evaluated by western blotting against patient sera. Presence of Tax protein band in the SDS-PAGE and western blot was confirmed. Western blotting of the recombinant protein with patient sera showed the band related to Tax protein. The recombinant protein is well produced and could be detected by patients' sera, making it eligible to be used as a recombinant viral antigen for future purposes.

Keywords: HTLV1; Tax; Cloning; SDS-PAGE; Western Blot

INTRODUCTION

HTLV1 (Human T Lymphotropic Virus Type 1) is the first human retrovirus discovered in 1980 [1]. Morphologically, HTLV1 is type C of delta virus category which is similar to lentiviruses (Human Immunodeficiency Viruses), with ability to long-term infection of human cells. HTLV1 is able to transform primary human T cells and creates immortal T cell's in vitro [2]. At first, pathological outcome of this virus, *i.e*. leukemia (lymphoid cancer) of T cells in adult (Adult T Cell Leukemia (ATL)) was detected three years before the discovery of the virus [3]. Accordingly, second disease raised by this virus was diagnosed which is a chronic nervous disease of lower limbs (TSP) (Tropical Spastic Paraparesis) [4], that was later related to HTLV1 (HTLV1 Associated Myelopathy (HAM)) as myelopathy [5]. HTLV1 as a disease is not prevalent and this virus is not the same in every part of the world. Just some regions are considered as endemic centers [6]. This virus is more prevalent in the Caribbean countries, south of Japan, Southern America and recently it is reported in northeastern Iran and Mashhad, so that the infection of this virus is reported in some regions of Khorassan Province up to 3% [7, 8]. As one of the most common transmission routes is via breast feeding, safety measures like avoiding breast feeding of newborns by infected mothers can prevent up to 97% of such cases [9]. Transmission by sex can also be highly prevented by using physical prophylaxis. Systematic screening of the donated bloods in blood transfusion services is another preventive measure which has been applied in several countries since 1986 and in high prevalent province of Khorassan in Iran for the past few years [8]. By applying these methods, the spread of the

disease could be very well controlled, without need for developing any vaccine. However, neither preventive immunization approaches, nor any of the above mentioned methods can help 15-20 million people who are already infected and 5-10% of whom that may develop serious outcomes in the future. So, in the last few years some attempts have been made to develop drug therapies or therapeutic vaccines for HTLV1 [10-13]. The physiopathology of HTLV1 related diseases is tightly mixed with extra ordinary properties of its Tax protein that has an important role in virus production and pathogenesis of HTLV1 related diseases [14]. In fact, Tax is the primary agent for oncogenic role of HTLV1, which leads to ATL, and strong immune response to this protein is the prime suspect for HAM-TSP pathogenesis. In other words, this protein is important in the life cycle and biology of the virus. It also is required for replication and to change the structure of host cell to meet the needs of the virus [10, 15, 16]. On the other hand, Tax is also one of the most conserved parts of this virus. This characteristic is an advantage for a vaccine candidate, because a conserved antigen could work for all subtypes of the pathogen for which the vaccine is designed against. For this purpose, any attempt to use it in a vaccination design should be after inactivation of this protein using changes happening in its coding sequence. For this project, in order to express protein, Tax cDNA with reported R222K mutation was used. This mutation is used to decrease Tax intervention in cell signaling pathway. In this mutant protein, lysine amino acid has replaced arginine [17]. It is reported that ubiquitination of Tax plays an important controlling role in the activation of NFKB pathway [18,19]. Therefore, lysin instead of arginine in this region may cause a ubiquitination site for Tax, and dramatically decrease activation of NFKB [17].

Use of different types of mutant Tax proteins in association with immune-modulator substances could act as strong immune-modulator for designing the vaccine. These harmful immune responses cause HAM-TSP disease and modulating their responses is a promising way for healing people affected by this disease [20, 21]. For this reason, the present study has sought the production of new Tax protein composition with the mentioned characteristics.

MATERIALS AND METHODS

In order to clone recombinant *Tax* gene, its coding sequence, including 1062 base pairs and containing R222K mutation, was cut out of pcDNA3.1(+)-Tax Eukaryotic vector using *Bam*HI and *Xho*I restriction enzymes, and was then inserted into the prokaryotic expression vector pET32a(+) within the same cutting sites and cloned into *E.coli* DH5α. Recombinant vector was confirmed with enzymatic digestion, colony PCR and sequencing of the cloned gene (Takapou zist Co.). For colony PCR the following primer pair was used:

* forward Primer: T7 promoter

5´ - TAA TAC GAC TCA CTA TAG GG-3´

*Reverse primer

5´ - TGT CTC GAG GAC TTC TGT TTC AC-3´

Sequence of *Tax* in vector pET32a(+) was confirmed and Plasmid pcDNA3.1-Tax was used as positive control and plasmid pET32a(+) as negative control in colony PCR. Recombinant vector after confirmation for accuracy of cloning was transferred to the expressing host *E.coli* Rosetta (DE3) and expression was induced. After optimizing the effective conditions, the recombinant protein was expressed in optimal condition of IPTG 1mM, 30 ˚C temperature, 2xYT+1%Glc culture and 6 hours incubation after induction in form of fusion with His-tag. The expression of the recombinant protein was assayed with SDS-PAGE and Western blot analysis.

In order to confirm Tax protein and its expression with His-tag fusion, Western blot is performed with mouse anti Tax monoclonal Ab produced (Abcam) with a dilution of 1/200 and penta anti-His monoclonal Ab, (Sigma) in a dilution of 1/2000. The antigenisity of Tax was evaluated by western blot using sera of HTLV1 infected people and was compared with uninfected people. For this study, serum samples for patients as well as for healthy people were prepared with dilution of 1/500. The sera were absorbed with bacterial lysate to remove non-specific antibodies against E.coli. Fifteen different sera samples were used for this analysis, including 10 patients (infected or HAM-TSP) and 5 control samples. . In order to achieve best result in the process of western blot, method optimization was performed through changing in antibody concentration

and other effective factors.

RESULTS

The result related to colony PCR and vector pET32a (+)-Tax recombinant vector is demonstrated in Figure 1. Development of band 1600 base pair in columns 1 to 14 relates to the cloned Tax gene in vector pET32a(+).The SDS PAGE analysis shows development of 50 kDa protein band after induction comparing with sample before induction of which suggests successful expression of Tax protein (Figure 2). A protein band was observed in the western blot with mouse anti Tax monoclonal Ab and penta anti-His monoclonal Ab, at almost 55 kDa (Figure 3). A similar band was observed after western blotting using patients' sera (Figure 4). The result of western blot with serum of one of people in the control group is also shown in Figure 4 for comparison.

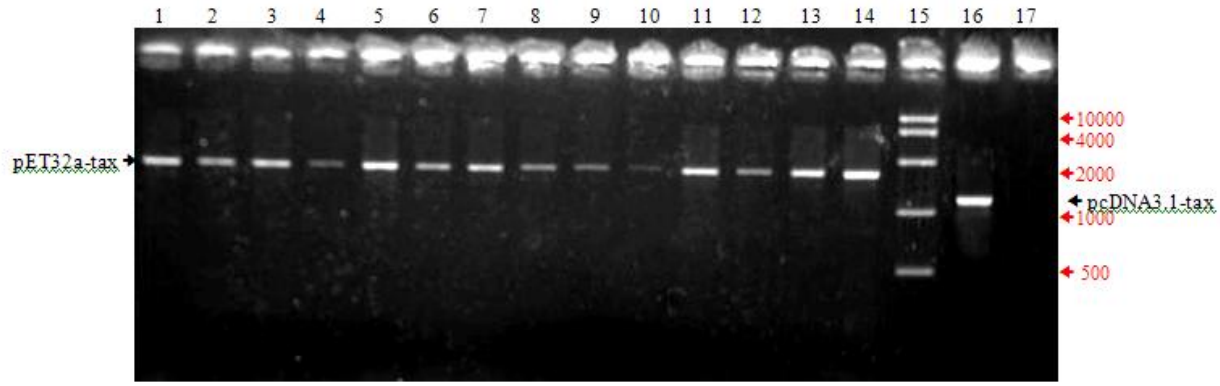

Figure 1. Colony PCR test confirms *tax* insertion into pET32a(+). Columns1-14: PCR product using transformed colonies. Column15: marker (Fermentas SM1123). Column16: PCR product using pcDNA3.1-tax as positive control. Column17: PCR product using pET32a(+) as negative control

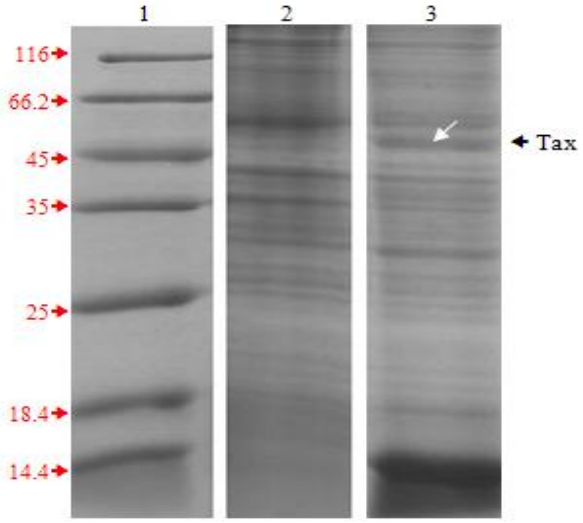

Figure 2. SDS-PAGE analysis for expression of recombinant protein in Rosetta (DE3) transformed with pET32a-tax. Column1: marker (Fermentas SM0431). Column2: before induction sample with IPTG. Column3: after induction sample

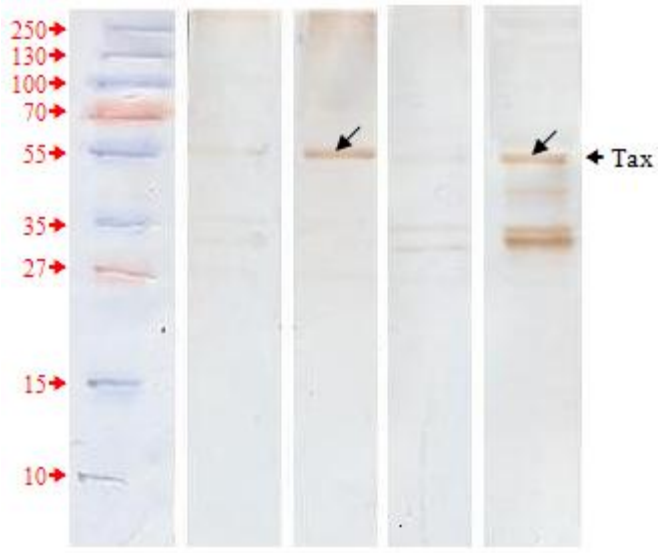

Figure 3. Analysis of recombinant Tax protein expression with anti-Tax monoclonal Ab and Penta anti-His monoclonal Ab. Column1: marker (Fermentas SM1811). Column2: before induction sample blotted with anti-Tax monoclonal Ab. Column3: after induction sample blotted with anti-Tax monoclonal Ab. Column4: before induction sample blotted with Penta anti-His monoclonal Ab. Column5: after induction sample blotted with Penta anti-His monoclonal Ab

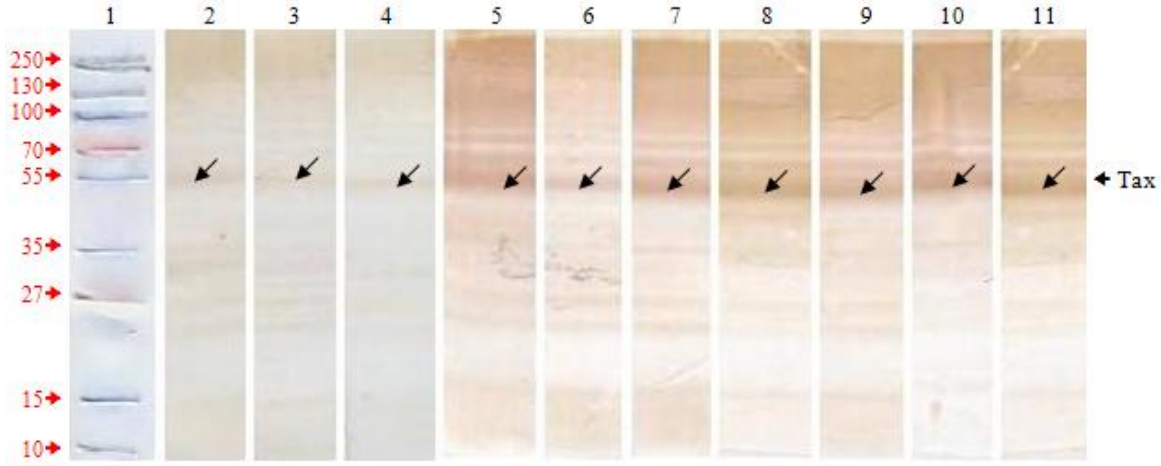

Figure 4. Evaluating of antigenic characteristic in recombinant Tax protein by western blotting against patient serum. Column1: marker (Fermentas SM1811). Columns2-11: after induction sample blotted with HAM-TSP patient

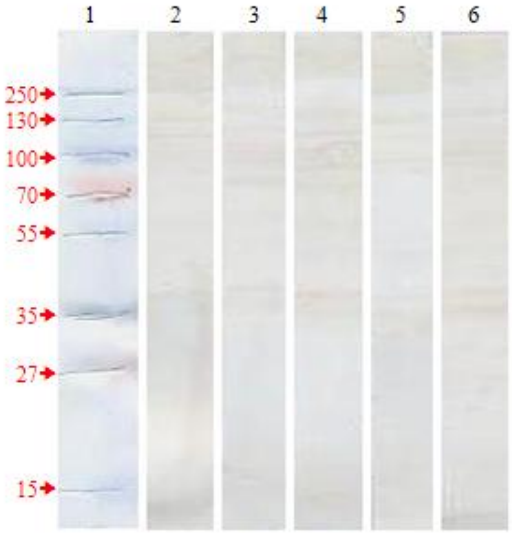

Figure 5. Evaluating of antigenic characteristic in recombinant Tax protein by western blotting against healthy people as control. Column1: marker (Fermentas SM1811). Columns2-6: after induction sample blotted with control group

DISCUSSION

HTLV1 virus is a real health problem in some parts of Iran and up to 3% of general population is infected by this virus in some regions of Khorasan Province. All these infected people fear the disappointing possible outcomes of their infection and are looking for a helpful therapy. On the other hand, preventive measures for blocking the transmission of this virus don't help those who are infected with this virus and between 5 to 10 percent of these people will show progressive diseases in their life. Therapeutic vaccine might be one of the effective methods for treating such patients. Many efforts have been performed during recent years for treatment of HTLV1 using medicine and therapeutic vaccine [14]. Tax is the immune-dominant protein for CTL response in HTLV1, while it is one of the most conserved parts of this virus. These characteristics are considered as important advantage for producing a candidate vaccine [14, 22]. However, most worrisome concern about Tax is its intracellular adverse effects; for this reason, any effort to use it for producing vaccine shall be done after inactivation of harmful effects of this protein using change in its genome. Therefore, production of recombinant Tax protein with mutation of R222K was performed [23-26]. Since cloning process is implemented through sub cloning but no PCR, thus the gene was not prone to adverse effects of PCR process (such as appearance of point mutation), accordingly, mistake in cloning seems absolutely impossible. However, for confirmation of cloning accuracy, colony PCR confirms *Tax* in vector pET32a(+). Whereas Forward primer (T7) is selected from sequence of vector, cloned *Tax* gene must be longer (approximately 500 bp) than *Tax* gene in positive control, therefore, according to Figure 1, after electrophoresis of PCR product in all selected colonies, band 1600 of base pair was observed in agarose gel comparing to the approximately band of 1100 base pair in sample of positive control; although no PCR product was achieved from pET32a(+) PCR. In this manner, after ensuring correct cloning and expression vector; effective condition of expression process such as inducer concentration, incubation time after induction, expression temperature, bacterial media culture and expression host have been considered. It is expected that Tax protein be expressed with 353 amino acid and molecular weight of 40 kDa. The leader polypeptide with 170 amino acid adds about 15 to 18 kDa to molecular weight of recombinant protein. So, in case of successful expression, a 55 kDa band will be observed. However, recombinant Tax protein is observed with approximate weight of 50 kDa in SDS-PAGE gel (Figure 2). Such observation may be due to error in measuring with the used marker in SDS-PAGE comparing with used marker in western blot (regarding usage of different markers in each technique).

Bioinformatics studies suggest that *Tax* gene has 44 rare codones, including 5 codones related to Arginine, 5 codones coding isoleucine, 10 codones of leucine and 24 codones coding proline, which are very rarely used in conventional *E.coli* expression system (http://nihserver.mbi.uda.edu/PACC). It may reduce expression level of Tax in *E.coli*. Rosetta (DE3) carries plasmid which encodes tRNA related to rare codones in eukaryotes. Consequently, expression of Tax protein is increased in this host [27-29].

Through study on effective factors of protein expression, it was realized that decreasing temperature from 37 to 30˚C after induction with IPTG may cause increase in expression of recombinant Tax protein. Decreasing temperature may provide suitable folding condition for the protein, so stability of soluble protein and finally protein yield in soluble form could be increased [27]. Since maximum activity of cellular protoases is happens in 37˚C, therefore, if Tax protein expression is optimal within this temperature, effect of proteases may cause protein decomposition and prevents determination of expression. Therefore, decrease in temperature could be effective in successful protein expression through prevention of cellular proteases activation.

For optimization of Tax protein expression, 1% glucose in 2xYT medium, significantly increased expression comparing with the medium without it. The better expression of Tax protein with glucose could be attributed to suppressing effect of glucose for leak expression of T7 RNA polymerase before induction with IPTG [30-35]. Western blot of Tax protein with exclusive monoclonal antibody against Tax epitopes demonstrated a 55kDa band which is in concordance with the expected molecular weight (Figure 3). In order to confirm expression of Tax protein with histidine tag, western blot of expressed recombinant protein was performed with

specific antibody against this tag. Achieved results of blotting have shown one 55kDa band corresponding to the expected Tax protein as well as a 35kDa band (Figure 3). It seems that this 35 kDa band is related to a protein of *E.coli* that was detected non-especially by Penta anti-His monoclonal due to its histidine content.

Since final goal of this study had been to use this protein in vaccine design, presence of antibody against Tax in serum of patients comparing with healthy people (control group) was evaluated by western blot to confirm antigenic characteristic of Tax protein. It is well documented that antibodies against Tax protein exist in most patients (who have antibody against antigens of HTLV1). Existence of 55 kDa band in sample after induction according to Figure 4, suggests the antibodies of the patients can react with our expressed Tax protein. Whereas blotting with patient's serum has had always background due to non special antibodies against *E.coli*, the results of before and after induction must be compared for presence or absence of the desired band. This band is not observed in before induced samples as indicated in Figure 4.

After optimizing expression condition, it was observed that the expression host is the most effective in expression of Tax recombinant protein. Actually, Rosetta (DE3) having plasmid which codes tRNAs of some rare codones in *E.coli*, considerably influenced on expression of recombinant Tax.

Study of western blot for recombinant protein using serum of patients suggests the expressed protein is well reactive to patients' sera. Measuring the amount of these antibodies has prognostic value for such patients. However, low level expression of Tax protein in this study is considered as weak point, limiting it for using it as vaccine. Therefore, further optimizing the expression of this protein is suggested for next steps.

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