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Research Article

Truncated MTA-1: A Pitfall in ELISA-Based Immunoassay of HTLV-1 Infection

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HTLV-1 causes adult T-cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). Recombinant envelope glycoprotein is used in production of diagnostic enzyme-linked immunosorbent assay (ELISA) kit. There are some reports that a significant percentage of Iranian HTLV-1 infected patients showed no seroreactivity with MTA-1 peptide, while HTLV-1 had been confirmed by PCR detection methods or ELISA kits containing a cocktail of HTLV-1 specific peptides. This report describes experiments designed to determine whether some discrepancies between ELISA and PCR results could be due to truncation of immunodominant epitopes using immunoassay method. We have cloned the MTA-1 epitope of env gene from HTLV-1 in NotI/NdeI sites of pET22b(+) expression vector. Sequencing analysis of recombinant plasmids revealed an insertion of a cytosine in position 271 causing a stop codon in the MTA-1 protein translation. SDS-PAGE analysis also failed to reveal the presence of the desired protein. Subjects with a mutant HTLV-1 env gene were shown to be seronegative using ELISA, but positive with PCR.

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1. INTRODUCTION

Infection with human T-cell lymphotropic virus type-1 (HTLV-1) is a growing medical problem, with over 20 million estimated infections worldwide [1]. HTLV-1 has been identified as the etiologic agent of two distinct human diseases: adult T-cell leukemia (ATL) and a chronic, progressive demyelinating disorder known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2]. The virus has also been associated with a number of autoimmune diseases, including Sjögren's syndrome, uveitis, and an inflammatory arthropathy [3]. The regions of HTLV-1 endemicity, with proportionately higher rates of infection, are clustered in southern Japan, the Caribbean, South America, the southern United States, equatorial Africa, and northeastern Iran [4, 5]. Three major routes of HTLV-1 transmission are mother to child via breast milk, sexual intercourse, and blood transfusion [6, 7]. Otherwise, HTLV-1 is not easily transmissible, since cell-to-cell contact is presumably required [6, 8]. Retroviruses can employ a variety of different cell surface proteins as receptors for binding and entry into host cells. Entry involves an interaction between the surface protein (SU) of the virus envelope glycoprotein (env) and the cellular receptor with subsequent fusion of the cellular and viral membranes mediated by the transmembrane (TM) region of env [9, 10]. According to current diagnostic criteria, immunoreactivity with p24 and one of the envelope proteins (usually gp46) is considered diagnostic of HTLV-I or HTLV-II infection; however, this immunoreactivity pattern still results in a significant proportion of false negative. MTA-1 peptide from the HTLV-1 gp46 region demonstrated the highest percentage of reactivity with HTLV-I positive sera, particularly among subjects of Japanese ancestry [11, 12]. There are some reports that

a higher percentage of Iranian HTLV-1 infected patients showed no seroreactivity with MTA-1 peptide, while HTLV-1 infection of patients had been confirmed by PCR detection methods or ELISA kits containing a cocktail of HTLV-1 specific peptides [4].

Some of these discrepancies could be explained by variation in immune response at different population. The other cases may be as a result of truncation of gp46 region in HTLV subtybes. Previously, it has been demonstrated that point mutations or deletion of glycosylation sites can result in a nonfunctional env protein [13].

This report describes experiments designed to determine whether some discrepancies between ELISA and PCR results could be due to truncation of immunodominant epitopes using immunoassay method.

2. MATERIALS AND METHODS

To prepare MTA-1 insert DNA, primers were chosen based on their locations within the prototype HTLV-1 genomic sequence available in the Gen-Bank database with accession number AF.33817. In order to put sticky end into blunt-ended amplified molecule, the sequences of restriction enzymes *Nde I* and *Not I* were incorporated into the 5′ ends of forward and reverse primers, respectively, with regard to cloning site of pET-22b(+) vector (Novagen, Madison, WI, USA), and restriction sites flanked by 3 spacer nucleotides at the 5′ end to allow for efficient digestion. The PCR amplification of DNA fragment of interest was performed for 35 cycles at the following temperatures: 10 cycles; 1 minute at 94°C, 45 seconds at 55°C, 2 minutes at 72°C, and 25 cycles; 45 seconds at 94°C, 1 minute at 65°C and 2 minutes at 72°C with a final extension of 20 minutes at 72°C in a DNA thermal cycler (Perkin-Elmer 480). Final composition of the reaction was 100 ng of pool of four DNA samples, 20 pmole appropriate primers, 1.5 mM dNTP mixture, and one unit (u) of *Pfu* DNA polymerase in the buffer containing $MgSO_4$ in 25 μl reaction volume. Sequences of the primers were as follows.

Forward primer: 5′-GCGCATATGGCTCCAGGATAT-GACCCCATC-3′.

Reverse primer: 5′-ATAGCGGCCGCGGAGCGGGAT-CCTAGGGTG-3′.

Expected product size of 455 bp fragment was amplified, gel purified using CoreOne gel extraction kit. Amplified DNA insert was sequentially digested and followed by a second gel purification.

Double digestion of plasmid DNA (5493 bp) was performed using *Not I* and *Nde I* restriction enzymes at 37°C, overnight. After purification, cut plasmid DNA was dephosphorylated with calf intestine phosphatase enzyme. The modified and digested pET-22b(+) plasmid was gel purified prior to ligation with the insert.

The appropriate amount of insert used in the ligation assay was calculated using the following equation: [(amount of vector, ng) × (size of insert, kb)/(size of vector, kb) × (molar ratio of insert/vector)] = ng of insert DNA. pET-22b(+)/MTA-1 constructed by ligating MTA-1 sequence into the pET-22b(+) expression vector via *Not I–Nde I* sites, and the reactions were carried out by T4 DNA ligase enzyme in 10 μl volume. T4 DNA ligase was then inactivated by heat treatment for 20 minutes at 65°C to increase the efficiency of transformation. Plasmid carrying the gene was used to transform bacterial cells (*E. coli* DH5a) using Inoue transformation method.

The cells were grown overnight at 37°C on SOB plates containing 50 μg/mL ampicillin. Plasmid DNA was extracted using Bioneer plasmid extraction kit (Bioneer, Korea, USA); screening was performed using PCR colony with insert-specific PCR primers, and restriction analysis and recombinant plasmid sequenced.

For protein analysis, a recombinant pET-22b(+)/MTA-1 plasmid was transformed into expression host *E. coli* BL21(DE3) strain containing a chromosomal copy of the gene for T7 RNA polymerase. Preparation of recombinant cells was followed by induction and optimization of expression of target protein using a range of IPTG concentration from 0.1 mM to 1 mM added to a growing culture. Cells were collected from the growth medium by centrifugation, 14000-rpm, 1 minute, at room temperature. The pelleted cells were resuspended in extraction buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 10% Glycerol, 100 mM KCl, and 0.5% Triton X-100) and subjected to centrifugation at 14,000 rpm for 20 minutes. Supernatants were removed; 250 μL 2X SDS/PAGE sample buffer was added to cells and equal volumes of supernatant mixed with 2X sample buffer, incubated at 95°C for 5 minutes and subjected to reducing sodium dodecyl sulfate SDS-polyacrylamide gel electrophoresis (PAGE).

3. RESULTS AND DISCUSSION

pET-22b(+) vectors were chosen as a convenient and powerful system for cloning and expression of recombinant protein in *E. coli* [14]. Extraction of plasmid, digestion, isolation, ligation, transformation, identification, and PCR were performed as described previously [15]. The conditions of the ligation reactions were first subjected to a number of optimizations for various parameters [16]. The temperature of 16°C resulted in desirable result. In the experimental sets focusing on the amount of T4 DNA ligase, both 10 and 20 units of enzyme gave high efficiency. The time of the ligase reaction was influential as 4-hour ligation reaction resulted in a high number of recombinant plasmids than the overnight ligation.

One of the strategies for high-efficiency cloning of sticky-end DNA molecules involves dephosphorylation of vectors with calf intestinal phosphatase (CIP), which catalyses the hydrolysis of 5′-phosphate groups from DNA. Treatment with CIP also increased the ligation efficiency of the PCR-amplified inserts.

Using these strategies as well as the optimized ligation conditions, three different molar ratios of insert to vector DNAs were tested. The ratios of 1 : 1 gave significantly low transformation efficiency, while the insert to vector ratio of 3 : 1 resulted in the highest number of transformants.

As shown in Figure 1, Lanes 2 and 4 showed digested plasmid from transformed clones with no insert using *Alw*

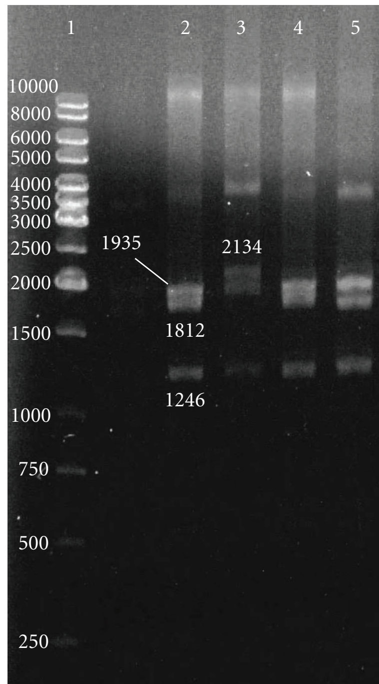

Figure 1: *Alw 441 restriction analysis of plasmids, extracted from transformed clones.* Plasmids of transformed clones are visualized on ethidium bromide-stained 1% agarose gel after purification and digestion with *Alw 441*. Lane 1 : 1 kb DNA Ladder; Lane 2, 4: digested plasmid from transformed clones with no insert using *Alw 441* restriction enzyme; Lane 3, a transformed clone containing pET-22b(+) with a 455 bp MTA-1 insert; Lane 5 indicates *Alw 441* restriction map of nonrecombinant pET-22b(+) (negative control).

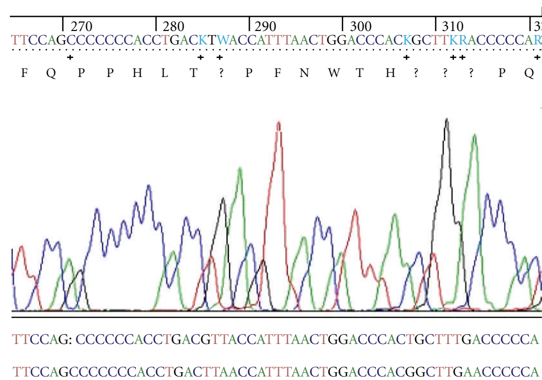

Figure 2: *Sequencing analysis*. An insertion of a cytosine in position 271 causing a stop codon in the MTA-1 protein translation.

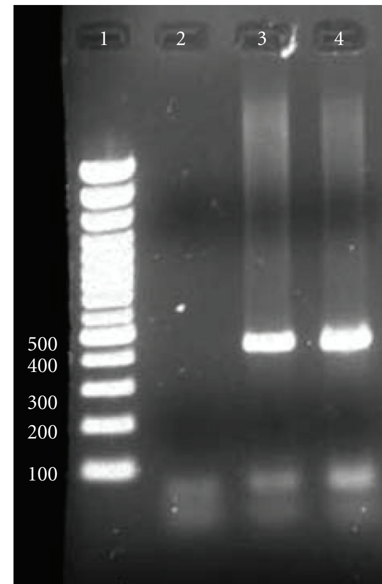

Figure 3: *Amplification of HTLV-1 MTA1-coding region*. A 455 bp fragment was amplified from the MTA-1 region of HTLV-1 genome. PCR products were electrophoresed in a 1.5% agarose gel, stained in ethidium bromide, and photographed. Lane 1, 100 bp ladder; lane 2, negative control; lane 3, PCR product from an ELISA positive patient; Lane 4, PCR product from an ELISA negative patient.

441 restriction enzyme. One of three transformed clones (Lane 3) contained recombinant pET-22b(+) carrying a 455 bp fragment. The presence of this fragment changed *Alw 441* restriction map of recombinant pET-22b(+) (Figure 1, Lane 3) in comparison with nonrecombinant pET-22b(+) (Figure 1, Lane 5).

Sequencing analysis showed an insertion of a cytosine in position 271 causing a frame shift and stop codon in protein translation (Gen-Bank accession no. DQ911752) (Figure 2). All four DNA samples used in this study send for sequencing and showed the same results. For further investigation, SDS-PAGE analysis was performed to detect potential MTA-1 protein. However, this analysis failed to reveal the presence of the desired protein band 37 KDa. It is likely that the MTA-1 truncated protein resulted from 271insC mutation is an unstable and degradable protein giving no band in SDS-PAGE.

Screening for HTLV-1 has become routine in blood banks in the most developed nations. The screening process is initiated with an HTLV-1-specific enzyme-linked immunosorbent assay (ELISA). Samples which are found to be repeatedly positive by ELISA are confirmed through a PCR assay or, more commonly, a commercially available Western blot assay. The standardized ELISA assay incorporates viral proteins obtained from a number of recombinant HTLV-1 glycoproteins, which are included to increase the sensitivity and specificity of the assay. In a significant number of cases, which can be demonstrated throughout the world, the HTLV-1/2 ELISA is negative, but HTLV-1 PCR is positive. The causative agent(s) and medical significance of the HTLV-1 seroindeterminate status are unclear. A number of potential explanations have been provided for this serological finding, including (i) infection with a truncated or deleted form of HTLV-1, (ii) infection with a novel retrovirus bearing significant homology to HTLV-1, and (iii) infection with prototype HTLV-1 at viral loads which are below the range of current methods of detection. Previous studies have had limited success in addressing these possibilities.

The presence of truncated form of HTLV-1 gp46 protein could have been implicated as potential agents of disease in

a number of cases. Such truncated form of HTLV-1 could theoretically account for an incomplete banding pattern on an HTLV-1-Western blotting due to absence or alteration of crucial immunodominant viral epitope.

The 271insC mutation in MTA-1 envelope genomic sequences causing a truncated protein can explain the seronegative (ELISA negative) HTLV-1 infected individuals who are HTLV-1 PCR positive. Although samples found to be repeatedly negative by HTLV-1-ELISA, HTLV-1 genomic sequences were readily detectable by PCR in the peripheral blood lymphocytes of these infected individuals (Figure 3). The positive results were also confirmed by sequencing.

Therefore, in addition to variation in immune response at different population, some of the discrepancies between PCR detection methods and ELISA kits containing MTA-1 peptide from the HTLV-1 gp46 region could be explained by truncation of gp46 region in HTLV subtybes. In consistent with this notion, it has been demonstrated that point mutations or deletion of glycosylation sites can result in a nonfunctional env protein [13].

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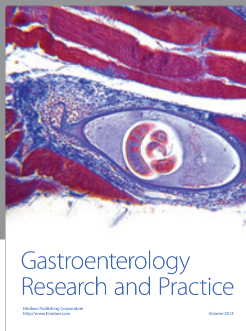

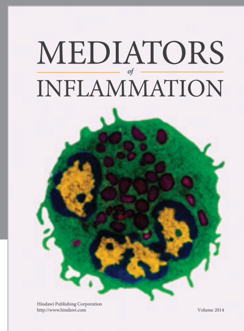

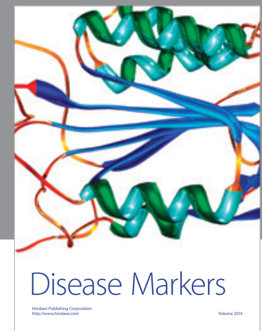

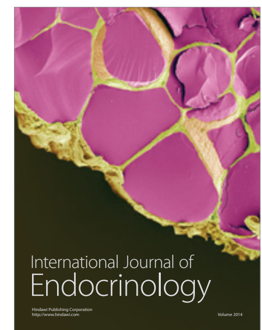

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