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Original Article

Investigation of the Relationship between HTLV-1 Infection and MMP-3 Gene Expression in HTLV-1 Positive Cardiac Patients

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Abstract

Background and Aims: Human T-lymphotropic virus type 1 (HTLV-1) is a member of retroviridae family that causes ATL and HAM/TSP. Many inflammatory diseases are associated with this virus, such as Sjögren's syndrome, Hashimoto's thyroiditis, Uveitis and also Atherosclerosis. HTLV-1 performs in long latency period and can activate the immune responses in coronary vessels. Activated immune system produces inflammatory factors such as TNF-α cytokine that can increase Matrix Metalloproteinases as a main factor in patients with coronary artery diseases (CAD). We aimed to determine the relation between this virus infection and MMP-3 expression in Atherosclerosis patients.

Materials and Methods: 44 patients (13 individuals CAD+ HTLV-1+, 8 individuals CAD- HTLV-1+, 13 individuals CAD+ HTLV-1- ——and 10 individuals CAD- HTLV-1-) were selected and MMP-3 gene expression was measured by Real-time polymerase chain reaction.

Results: There was no significant correlation between MMP-3 gene expression and HTLV-1 expression level in HTLV-1-positive cardiac and Atherosclerosis patients.

Conclusions: Although the main risk factor in Atherosclerosis patients is MMP-3, despite our expectation, no significant correlation was observed in our statistical analysis within four examined groups ($p \geq 0.05$). This evaluation showed higher MMP-3gene expression but not significant in CAD+ HTLV-1+ group compared to CAD+ HTLV-1- group. The larger sample sizes may help to improve the outcome to reach a significant level.

Keywords: HTLV-1, MMP-3, TNF-α, Atherosclerosis, Real-time PCR

Introduction

Human T-lymphotropic virus type 1 (HTLV-1) a deltaretrovirus is a genus of the retroviridae family. Primary chronic inflammation due to HTLV-1 pathology characteristics, can cause some diseases, including tropical spastic paraparesis/HTLV-1-associated myelopathy (HAM/TSP) (1, 2) and ATLL (Adult T-cell Leukemia/Lymphoma) (3).

In the last decades, the determinants of the atherosclerosis plaque forming was assumed to be smooth muscle cells proliferation, interaction between injured endothelial and platelet aggregation and releasing platelet-derived growth factors (4, 5). Today, these views about atherosclerosis have been changed. It is described as a chronic inflammation in immunological mechanisms

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(4, 6, 7). Hypercholesterolemia and oxidized LDL can activate macrophages which either increase CD40 molecules and leukocyte adhesion (ICAM-1, VCAM-1) or activate platelets and release proinflamatmory factors (8-11). Released proinflammatory factors from macrophages such as tumor necrosis factors (TNF) and Interleukin-1 (IL-1), enhance the expression of tissue factors including Matrix Metalloproteinases (MMPs), endothelin-1, platelet activating factors, plasminogen activator inhibitor-1, E-selectin, P-selectin, Interlukin-8 and monocyte chemotactic protein-1 (MCP-1). In the second step after these changes, the intra-plque processing occur which mediate CD40/CD40L interactions between antigen presenting cells and CD4+ T-Lymphocytes (11-16). Matrix metalloproteinases (MMPs) are defined as proteolytic enzymes which degrade component of extracellular matrix (ECM) and basal matrix (BM). Proteolytic disruption of ECM and BM causes leukocyte infiltration in inflammation site. Matrix Metalloproteinases (MMPs) including gelatinase B (MMP-9) and steromelysin (MMP-3) play the main roll during all phases of developing human atherosclerosis (16-18). MMPs are released by activated T-cells and macrophages (16, 19, 20). Obviously, the most important factor involved in the pathological mechanism of acute coronary syndrome (ACS) is matrix metalloproteinase activation in the extracellular matrix.

Physiologically, MMPs have a double function in embryogenesis and tissue restructuring that causes either activation or cell proliferation or migration of vascular smooth muscle cells to the tunica intima and leads to construct atherosclerotic plaques. On the other hand, despite this function, they degrade the fibrous cap of the plaque and cause its disruption then the plaque becomes instable or crumbly that ends to luminal thrombosis and thromboembolic stroke. Thus, the plaque disruption is a main cause for acute myocardial infarction (16, 21, 22).

Collection of ECM components and new capillaries construction during plaque formation, engage major changes in the vascular wall structure then atherosclerotic plaques appear along with the atresia, stenosis, rupture and thrombosis (23-28). Despite all the mentioned points, there is a correlation between MMP rising and Cytomegalovirus (CMV) and also other pathogens (23-28). Other studies have also demonstrated that in the patients with HAM/TSP disease, the expression of MMP-9 and MMP-2 increase (29).

HTLV-1 mediates malignant and inflammatory diseases which has been recognized with either distinct pathological conditions or common features of increasing the number of HTLV-1-infected cells that have migrated/infiltrated into the lesions (30). Although some studies on T-cells infected with HTLV-1 have demonstrated MMP-9 over-expression, other studies have revealed deregulation of MMP expression in ATL patients infected with HTLV-1 (25).

In this study attempt was made to determine the relation between MMP-3 expression and HTLV-1 infection as a noticeable factor in developmental process to make a plaque by infecting macrophage or T-cell which induce chronic inflammation in patients with coronary artery diseases.

Methods

Subjects and inclusion criteria for the study. The patients were chosen based on the information of their cardiovascular disease symptoms and angiography experience in Razavi and Imam Reza Hospital in Mashhad. HTLV-1 infection was checked by ELISA and was confirmed with quantitative PCR analysis (31, 32). Forty four patients (13 individuals CAD+ HTLV-1+, 8 individuals CAD- HTLV-1+, 13 individuals CAD+ HTLV-1- and 10 individuals CAD- HTLV-1-) were selected. The criteria for inclusion of the patients were satisfactory and the coronary artery disease was confirmed by angiography, with no history of insulin-dependent diabetes, and the use of corticosteroid medication. The CRP and RF tests were also negative with no history of autoimmune disease. The informed consent was obtained from participants. Ethic permission was obtained from Mashhad

Table 1. Primers and probe Beta 2µ, GAPDH and MMP-3

Genes	Primers	Probe
Beta 2µ	Forward: 5'-TTGTCTTTCAGCAAGGACTGG-3 Reverse: 5'-CCACTTAACTATCTTGGGCTGTG-3'	Fam- TCACATGGTTCACACGGCAGGCAT-BHQ1
MMP-3	Forward: 5′- CCCACTCTATCACTCACTCACAG -3′ Reverse: 5′- CAAAGGACAAAGCAGGATCACAG -3′	Fam- CCTGACTCGGTTCCGCCTGTCTCA -BHQ1
GAPDH	Forward: 5′- TCGGATTTCAGCAAGCTGTGG -3′ Reverse: 5′- GGATTAATGCTGCTTGGCCCGTG -3′	

University of Medical Sciences to conduct the research.

Pro-viral load measurement. PBMCs were separated using Ficoll density-gradient centrifugation 1077 (Sigma, Dorset, UK). Real-time PCR was performed using a commercial absolute quantification kit (Novin Gene, Iran) to measure the pro-viral load of HTLV-1 and specific primers and a fluorogenic probe (Rotorgen Q Real-Time PCR machine, Qiagen, Germany). The HTLV-1 copy number was reported as an actual amount of cellular DNA by means of quantification of the albumin gene as the reference gene. HTLV-1 and albumin DNA concentrations were calculated from 5-point standard curves. The normalized value of the HTLV- I pro-viral load was calculated as the ratio of (HTLV-1 DNA copy numbers/albumin DNA copy numbers/2) ×104 and expressed as the number of HTLV-1 pro-viruses per 104 PBMCs.

RNA isolation and cDNA synthesis. Total RNA was isolated using TriPure isolation reagent (Roche, Mannhein, Germany) according to the manufacturer's instruction. The mRNA was reverse transcribed using First Strand cDNA Synthesis kit (Fermentas, St.Leon-Roth, Germany). Reverse transcription was performed at 42°C for 60 minutes, followed by inactivation at 70°C for additional five minutes. The cDNA was stored at −20°C until use. In order to verify the integrity of synthetic cDNA, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) primers (Table 1) were prepared and the presence of cDNA was confirmed by observing 500 bp bands on an agarose gel (data not shown).

Real-time polymerase chain reaction analysis. A semi-quantitative Real-Time Polymerase Chain Reaction (RT-PCR) was performed on the cDNA samples using the TaqMan® Master Mix (Takara, Otsu, Japan) in the Rotor Gene System (Q-6000 Machine; Qiagen, GmbH, Germany) according to the manufacturer's instructions. Primers and probes for beta-2 microglobulin (b2M) (the reference gene) and MMP3 were designed using Beacon Designer 7 software (PREMIER Biosoft International, Palo Alto, CA, USA). The primer and probe sequences are shown in Table 1.

Standard curves for target and reference genes were prepared using serial dilutions of cDNAs. The relative quantity of b2M, a low-molecular-weight protein presented in all nucleated cells, was applied as a housekeeping gene to normalize the relative quantity of each gene and control for errors between the samples.

Statistical analysis. Statistical analysis was performed using GraphPad Prism Software, version 7 (GraphPad Software, Inc., San Diego, CA). Statistical procedures were performed using the Kruskal– Wallis and Mann-Whitney U tests. The significant level was considered less than 0.05, with a confidence interval of 95%.

Results

Demographic data and clinical impact. This study was conducted based on the information of the symptomatic heart patients that had been checked with angiography method in two hospitals in Mashhad; Razavi Hospital and Imam Reza Hospital. HTLV-1 infection was checked by ELISA and PCR analysis. Thereby , 44 patients (M=24, F=22) were selected and divided in four groups (CAD- HTLV1-, CAD+ HTLV1-, CAD- HTLV1+, CAD+, HTLV1+). The mean age of the participants was 60.43 ± 1.33. Statistical analysis of test samples using

KrusKal-wallis Test, indicated no significant difference in age among the groups (p=0.11) (Figure 1).

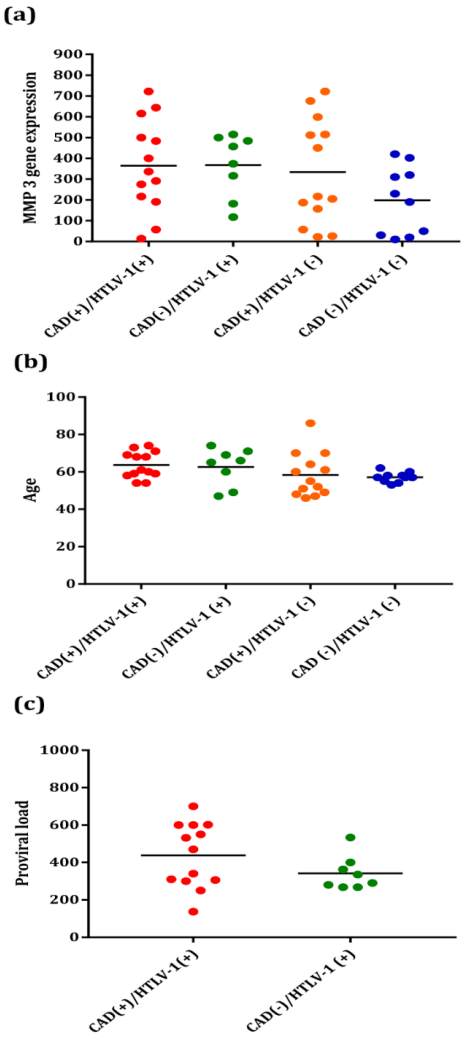

Fig. 1. (a) MMP-3 gene expression by Real-time polymerase chain reaction analysis in four examined groups. (b) The mean age of the participants in four examined groupsusing KrusKal-wallis Test. (c) The mean HTLV-1 pro-viral load in CAD+ HTLV+ and CAD- HTLV+ groups.

HTLV – 1 Pro-viral load. The mean HTLV-1 pro-viral load in HTLV-1 infected individuals was obtained 438.2 ± 47.96 in CAD+ HTLV-1+ group and 342.3 ± 32.24 in CAD- HTLV-1+ groups. No significant difference was observed between two groups by Mann-Whitney U tests analysis (p=0.17). Figure 1(c) shows the mean HTLV-1 pro-viral load.

MMP-3 mRNA expression. The mean of MMP3 mRNA expression in the CAD+ HTLV-1+ group was 364.9±61.3, in CAD-e HTLV-1+ group was obtained 368.1±53.58, in CAD+ HTLV-1- showed 334.2±70.17 and in CAD- HTLV-1- was198.2±51.27. There was no significant difference among the groups by Kruskal– Wallis test analysis (p=0.23) (Figure 1(a)).

Based on the Spearman test, no significant difference was observed in MMP-3 gene expression in the studied groups (p=0.2) and no significant correlation was obtained among gene expression of MMP-3, pro-viral load, age, artery stenosis and gender in either group (p=0.2).

Discussion

In this study we examined the relationship between MMP-3 gene expression as a risk factor in coronary artery diseases and HTLV-1 pro-viral load. The relationship among these factors and demographic parameters such as age, sex, artery stenosis grade were evaluated and no association was observed among the groups under study. Although, the statistical analysis on four examined groups, in (α = 95%) showed no significant result , this evaluation in (α=90%) showed that MMP-3 expression did not increase significantly in CAD+ HTLV1+ groups compared to CAD+ HTLV-1- group. Therefore, in the case of increasing the sample numbers, this examination may or may not indicate the significant differences between CAD+ HTLV1+ group and CAD+ HTLV-1- group and also the role of virus can be suspected in increasing MMP-3 as an important risk factor in atherosclerosis. In all the studied groups no significant relationship was found between the expression of MMP3 and HTLV-1 pro-viral load whereas pro-viral load in the HTLV-1+ CAD+ group showed higher level than HTLV-1+ CAD- . It has been suggested that a high pro-viral load in peripheral blood mononuclear cells is considered as a risk factor for the development of ATLL (3). In this study, HTLV-1 pro-viral load was analyzed as a main and viable risk marker for the prognosis and development of coronary artery diseases. In earlier studies, matrix metalloproteinases

(MMPs) were introduced as proteolytic enzymes which degrade the components of the ECM and BM and play main roll during all phases of human atherosclerosis by recruitment and migration of immune effector cells to the inflammation site in vessels (13, 18-19). Hereby, in this study no significant statistical difference was observed between the expression of MMP-3 among those groups which might be due to the small sample size, whereas a previous study reported the prevalence of virus in population of coronary artery disease was higher than general population (3). Accordingly, this conclusion can't discover a relation between the virus and MMP-3 as a main factor in the cardiovascular diseases. Other studies reported that in HAM/TSP diseases, HTLV-1 increased MMP-3 gene expression (16, 20). Although it has been guessed through this study that increased MMP-3 gene expression by HTLV-1 virus may affect the patients with coronary artery disease, our result was against this outcome and MMP-3 gene expression did not change in either group. Previous studies demonstrated that HTLV-1 infection increased proinflammatory cytokines, including TNF-α and Matrix Metalloproteinases (33, 34) in atherosclerosis patients, whereas this statistical analysis showed different results. In this research, HTLV-1 has not interfered with atherosclerosis patients by elevated MMP-3 and this conclusion is the same as a report published by Glauco In 2015 (35). In other study, HTLV-1 was introduced as an independent risk factor to increase atherosclerosis which was opposite to our results.

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