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Research paper

Analysis of CTLA-4 + 49A/G gene polymorphism in cases with leprosy of Azerbaijan, Northwest Iran

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ABSTRACT

Leprosy, which is developed by the obligate intracellular *Mycobacterium leprae* (ML); has different manifestations, associated with the host immune responses. The protective immune response against ML includes T-cell-mediated immunity. The CTLA-4 has a great impact as a negative regulator of the immune response and maintenance of peripheral tolerance.

This study analyzed the relationship between CTLA-4 + 49A/G gene polymorphism and clinical manifestation of leprosy disease and susceptibility among the Azeri population living Northwest Iran.

One hundred and ninety-two leprosy patients and 185 healthy controls participated in the study. CTLA-4 + 49A/G genotyping was conducted via tetra-primer amplification refractory mutation system–polymerase chain reaction (T-ARMS–PCR) analysis.

The allelic and genotypic frequencies of + 49A/G gene polymorphism were similar in controls and patients. However, older ages, older age of onset and over-representation in male were observed in lepromatous leprosy patient carriers of GG genotype. The current study demonstrates that although CTLA-4 + 49A/G polymorphism was not correlated with a higher genetic risk for leprosy, the presence of a GG genotype was associated with older ages, older age of onset and over-representation in male in Iranian Azeri population.

1. Introduction

Leprosy is an infectious disease (it is caused by the obligate intracellular *Mycobacterium leprae* (ML)), that influences the skin and peripheral nerves (Modlin, 2010; Montoya and Modlin, 2010). Leprosy is a critical health problem worldwide, with the highest incidences in Africa, Asia and Latin America (Vannberg et al., 2011).

Exposure to the bacillus is necessary, but this alone does not mean an individual will develop clinical symptoms of the disease (Quintana-Murci et al., 2007). Human genetic factors affect the clinical course of the disease and the acquisition of leprosy (Alcais et al., 2005; Alter et al., 2011; Shields et al., 1987). Development of damage in leprosy patients is often caused by the host immune responses which are driven to mycobacterial elimination (Fitness et al., 2002). Leprosy involves widespread clinical and histopathological manifestations. This variety puzzled and frustrated clinicians and researcher; hence, this diversity was based on the capability ability of the host to create a cellular immune response to M. leprae (Scollard et al., 2006). Currently, most research workers use Ridley Jopling criteria, which has a basis on lymphocyte and macrophage populations in the lesions of skin and defines a five-point leprosy spectrum with borderline tuberculoid (BT) leprosy and polar tuberculoid leprosy (TT), polar lepromatous leprosy (LL), and borderline lepromatous leprosy (BL) at the end and borderline-borderline (BB) in the middle (Ridley and Jopling, 1966). Tuberculoid leprosy patients have a fairly successful *M. leprae* specific cell-mediated immune response. Their lesions are specified characterized by

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epithelioid cell granulomas, participation of lymphocytes (mainly of Th1 type), and few if any bacilli were detectable. Contradictorily, the specific cellular immunity against *M. leprae* is almost absent in the lepromatous form (Faber et al., 1978); with diffuse dermal infiltrates which are characterized by weak young macrophages with a heavy load of bacilli and a few T-cells which are mainly of the Th2 type (Yamamura et al., 1991). So, a protective immune response in leprosy is relies on the cellular arm of the immune system, particularly on helper T-cells generation which activates cells from the monocytic lineage to destruct the bacilli they harbor, along with effector T-cells, making them capable of killing these infected cells. Thus, T-cell activation is a major phase in leprosy immunity (Murray et al., 2007). The co-stimulatory and co-inhibitory receptors interaction (e.g., CD28 and CTLA-4) expressed on T cells with the ligands (e.g., B7-1 and B7-2) on APCs affects the duration and magnitude of responses of antigen specific T cell (Dilmec et al., 2008). CTLA-4 is exclusively expressed on activated CD4 + and CD8 + T cells and connects the same ligands, B7-1 and B7-2, as CD28 but with a 20- to 100-fold greater affinity (Chistiakov and Turakulov, 2003; Linsley et al., 1994). Nevertheless, whereas CD28 renders an important costimulatory signal necessary for the commencement and development of T cell immunity (Greenwald et al., 2002); CTLA-4 actually function to down-regulate T cell function (Krummel and Allison, 1995; Walunas et al., 1994) and is important for preventing T cell activation and peripheral tolerance and immunological anergy and susceptibility to T cell-mediated infectious diseases (Liu et al., 2010; Walunas et al., 1994).

The human CTLA4 gene was mapped to chromosome 2q33 and includes four exons (Kristiansen et al., 2000). Polymorphisms are detected in the CTLA4 gene and are associated with various susceptibilities to a wide range of infectious diseases and T cell-mediated autoimmune diseases (Almasi et al., 2015; Liu et al., 2013) (Chen et al., 2010; Danilovic et al., 2012; Hajilooi et al., 2014). CTLA4 + 49A/G polymorphism in exon-1, leads to a threonine-to-alanine amino acid variation in the leader peptide which is considered as a sequence variant that affects the gene expression and disturbs the immune regulation (Chistiakov and Turakulov, 2003; Ueda et al., 2003).

Because of the critical role of T cell activation in immunity against leprosy; and the inhibitory effects of CTLA4 on the T cell activation, it was explored, for the first time, whether the polymorphism in the exon1 A49G of CTLA4 gene was correlated with clinical manifestation of disease and susceptibility among the Azeri population of Northwest Iran.

2. Material and methods

2.1. Patients and controls

One hundred and ninety-two inactive treated leprosy patients, which consist of 73 females and 119 males who were residing in Bababaghi Hospice in Tabriz, enrolled into this study.

Diagnosis of leprosy was established according to World Health Organization criteria. With classification according to the Ridley-Jopling system, there were 108 LL, 71 TT, 9 BB and 4 BT leprosy patients in this study. Their performance status was quantified by Karnofsky Performance Status (KPS) scale (Mor et al., 1984). Performance was graded as mild (KPS 80–100), moderate (KPS 50–70) and severe (KPS 0–40). Furthermore, we determined other clinical variables such as age, sex, age at presentation of the disease and clinical manifestation of disease. This study has been performed from May 2013 to October 2017.

The control group consisted of 185 healthy volunteers (88 females and 97 males) matched for ethnicity and gender. Control subjects were screened by a physician using a questionnaire to ensure the absence of any clinical evidence or family history of autoimmune diseases, cancers, asthma and chronic infectious diseases such as leprosy.

Toward minimize genetic heterogeneity; we included unrelated individuals with a history of at least three generations of Azeri origin from Northwest Iran. The project was approved by the Ethics Committee of Tabriz University of Medical Sciences, and informed consent was obtained from all patients and healthy subjects.

2.2. Tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR)

EDTA-added whole blood was collected from leprosy patients and controls. Genomic DNA was extracted from peripheral blood leukocytes by the proteinase K method. CTLA4 + 49A/G genotyping was performed by T-ARMS–PCR technique, as previously described by Balbi et al. (2007). In T-ARMS–PCR, outer (common) primers generate a non-allele specific control amplicon, along with two inner (allele-specific) primers (in opposite orientation) and generate allele-specific amplicons. The allele-specific amplicons have differing lengths and can be simply isolated through standard gel electrophoresis, since the mutation is asymmetrical to the outer (common) primers.

Three bands consist of a 229 bp band for two outer primers (control), a 162 bp for A allele and a 120 bp for G allele primers for a specified heterozygous case. The T-ARMS-PCR assay diagram is depicted in Fig. 1.

The concentration of each primer, primer sequences, and the sizes of amplicon are listed in Table 1.

The reaction of PCR was performed in 20 μL reaction containing 150 ng of genomic DNA, 150 μM dNTP, 2.5 μL of complete buffer (having Mg-Cl_2), and 1 unit from Taq polymerase (BIORON, Germany) and the appropriate concentration of every outer and inner primer (see Table 1). The cycling program consisted of initial denaturation at 95 °C for 10 min, followed by 35 denaturation cycles (94 °C for 50 s), annealing temperature (62 °C for 30 s) and extension (72 °C for 30 s) and a final extension cycle 72 °C for 7 min. Products of PCR were isolated through standard electrophoresis over 2·5% agarose gel with DNA Safe Stain (CinnaGen, Tehran, Iran). A representative T-ARMS-PCR analysis is displayed in Fig. 2.

2.3. Statistical analysis

Statistical calculations were carried out using Epi Info 2002 (Centers for Disease Control and Prevention, Atlanta, GA USA), SPSS (version 16.0, SPSS Inc., Chicago, USA). Qualitative data are presented as the number of (%) and were analyzed using Fisher's exact tests and Pearson Chi-square test, with Yates' correction where appropriate. Data were analyzed with one-way ANOVA and Kruskal-Wallis tests for multiple comparisons. Statistical significance was defined as $P < 0.05$. Hardy-Weinberg proportions were determined by applying the equation $(p^2 + 2pq + q^2)$.

3. Results

3.1. CTLA4 + 49A/G polymorphism

CTLA4 + 49A/G polymorphism was not significantly deviated from the Hardy–Weinberg's equilibrium either in leprosy patients or in healthy controls. The allele and genotype frequencies in case and control groups are shown in Table 2.

As indicated in this table, no significant differences were revealed for CTLA-4 + 49A/G genotype and allele frequencies between leprosy patients and the control group ($P = 0.779$, and $P = 0.694$; respectively), and odd's ratio (OR) = 0.925, and 95% confidence interval (CI) = 0.670–1.275 for + 49G allele.

3.2. Clinical characteristics of patients with CTLA-4 + 49A/G genotypes

The clinical characteristics of the patient population are shown in Table 3.