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Research paper

A novel approach to investigation of the pathogenesis of pterygium based on assessment of promoter hyper-methylation and expression profile of *CTLA4* gene
A credible report of *CTLA4* gene expression in human eye tissue

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ABSTRACT

Background: Pterygium is the human eye lesion whose prevalence in the general population is estimated about 2%. The disease, in extreme phase, can lead to visual disturbance and eventually causes complete loss of vision due to the lesion growth over the papillary axis. Pterygium invasive tissue is a tumor-like tissue that is initially identified and then is attacked by cytotoxic T cells. Cytotoxic T lymphocyte associated antigen 4 (CTLA4), as a modulator molecule of the adaptive immune system, plays a critical role in maintaining peripheral T cell tolerance by diminishing its responsiveness and increasing its activation threshold. The aim of this study is to investigate the association between some epigenetic changes of the *CTLA4* gene, such as promoter methylation and gene expression, and pathogenesis of pterygia.
Materials and methods: Genomic DNA was extracted from 75 formalin-fixed, paraffin-embedded tissues of pterygia and 70 specimens of normal conjunctiva from eyes without pterygium as the control group, collected from Sistan and Baluchestan population. *CTLA4* gene promoter methylation was carried out by methylation-specific PCR technique. The gene expression analysis was done on extracted total RNA from 20 healthy and 23 pterygium tissue samples using Real-Time PCR technique.
Results: Promoter methylation changes of *CTLA4* gene were not statistically different in patients with pterygium in comparison with healthy controls (OR = 1.614; 95% CI = 0.57–4.75; *P* value = 0.37). However, gene expression level of CTLA4 was remarkably different in patients and healthy controls (Mean ± SD: 1.343 ± 0.133 and 2.027 ± 0.219, respectively; *P* value = 0.009).
Conclusion: This is a credible evidence of *CTLA4* gene expression in human eye tissue. This first hand attempt of investigating the association of epigenetic changes of the *CTLA4* gene and pathogenesis of pterygia, indicated a significant intensification of the gene expression of *CTLA4* in patients with pterygia. We suggest that increasing *CTLA4* gene expression can be a trigger which promotes pterygium enlargement. However, further studies on more populations with larger sample sizes need to be done to verify this hypothesis in the future.

1. Introduction

Human pterygium which abides as one of the most controversial ocular diseases, is a chronic condition characterized by the invasion of a wing-shaped fibro-vascular portion of the bulbar conjunctiva into the cornea (Cimpean et al., 2013; Perra et al., 2002; Anguria et al., 2014). The disease in its extreme phase can lead to visual disturbance and eventually causes complete loss of vision due to the lesion growth over the papillary axis (Narsani et al., 2008; Kralj et al., 2008).

The lesion prevalence is estimated about 2% in the general population (Detorakis and Spandidos, 2009). This condition is also more frequent in environments with dusty, windy, hot and dry weather (Narsani et al., 2008).

Existent reports signify that several risk factors contribute to pterygium pathogenesis. Some researches indicate that sunlight exposure appears to be the main factor in pterygia incidence by inducing chronic inflammation, growth factor production or DNA damage; while others propose that genetic factors are crucial for pterygium occurrence and that sunlight or chronic inflammation are only triggers which promote its enlargement. However, there is no report merging these factors in the pathogenesis of this complex disease (Anguria et al., 2014; Vergas, 2012).

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Pterygia imitate a tumor-like tissue by having several features including hyper-proliferation, hyper-vascularization, changing the extracellular matrix, anti-apoptotic behavior, invasion and high recurrence rate (Bradley et al., 2010).

Adaptive immune cytotoxic T cells initially recognize the invasive agents such as tumor cells and then attack them. T cell responses are regulated by both stimulatory and inhibitory signals. The balance between these two against signals is significant for efficient immune responses. The excessive transmission of inhibitory signals can lead to insufficiency of T cell responses. Reciprocally, the excess stimulatory signaling can cause the autoimmune diseases (Schaefer, 2004).

The *CTLA4* gene is a protein-coding gene, belonging to the immunoglobulin superfamily. This gene is sited on the chromosome 2q33.2 and encodes a protein which transmits an inhibitory signal to T cells (http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTLA4&search=ctla4, n.d.).

CTLA4 is a glycoprotein expressed on both $CD4^+$ and $CD8^+$ T cells. This immune-modulatory molecule is a fundamental negative regulator for T cell immune responses and has a crucial role in the maintenance of peripheral T cell tolerance by reducing T cell responsiveness and increasing the T cell activation threshold. The molecular mechanism of CTLA4 inhibition is still under debate. Nevertheless, some theories are available in this area (Walker and Sansom, 2011; Gattinoni et al., 2006).

Various studies signify that the binding of CD28 to ligand brings about co-stimulation of T cell functions, such as its proliferation and cytokine production. This ligation decreases T cell activation threshold as well. These stimulatory roles of CD28 are against the inhibitory roles of CTLA4. These two immune-regulatory molecules are different in their ability to bind to ligands. CTLA4 affinity and attraction to interact with its ligands is remarkably higher than CD28. Accordingly, it has been suggested that CTLA4 can act as antagonist of CD28–ligand interactions by contesting for binding to ligands (Walker and Sansom, 2011).

NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA. NFκB is involved in cellular responses to stimuli such as stress, cytokines, free radicals and ultraviolet irradiation. This nuclear factor plays a key role in regulating the immune response to infection. Incorrect regulation of NFκB has been linked to cancer, inflammation, autoimmune diseases and improper immune development (Prajapati et al., 2010). The nuclear transcription factor κB is activated through some cytoplasmic signal transduction cascades. According to current evidences, these signaling pathways seem to be a crucial target of CTLA4 inhibition (Schaefer, 2004). Activation of NFκB is critical for T cell response to antigen and is required for its successful entrance to S-phase of cell cycle. Moreover, entering into S-phase and succeeding proliferation are required for T cell efficient response (Rossman et al., 2006; Schaefer et al., 2004).

Due to lack of information in this area, the aim of the present study was to investigate the association between some epigenetic changes of the *CTLA4* gene, such as promoter methylation and gene expression, and the pathogenesis of pterygia.

2. Materials and methods

2.1. Study subjects

This case–control study comprises 75 formalin-fixed, paraffin-embedded tissues of pterygia and 70 specimens of normal conjunctiva from eyes without pterygium as a control, that were collected in Alzahra specialized ophthalmology hospital, Zahedan, Iran, during 2011 to 2013. The pterygium group consists of 31 male and 44 female specimens (mean age ± SD: 53.24 ± 2.308), as well as, the control group consists of 30 male and 40 female specimens (mean age ± SD: 51.12 ± 2.886).

The demographic information of studied statistical population is given in Table 1.

Table 1
Demographic table of studied statistical population.

		Case (N = 75)	Control (N = 70)	*P* value
Mean age ± SD		51.12 ± 2.887	53.24 ± 2.308	0.56
Median age		57	55	
Age range		12–86	2–90	
Gender	Male	31	30	0.85
	Female	44	40	

SD: Standard Deviation, N: Number.

2.2. DNA preparation and bisulfate modification

Genomic DNA was extracted from pterygia and normal tissue specimens using the QIAamp DNA Mini Kit (QIAGEN), according to the manufacturer's instructions, and then its quality was estimated by a spectrophotometer.

Bisulfite treatment of DNA samples was carried out as previously described, with slight modifications (Patterson et al., 2011). Briefly, genomic DNA (1 μg) in a volume of 45 μl was denatured by 1 M NaOH for 10 min at temperature of 37 °C, 30 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite was supplemented and mixed. Samples were incubated under mineral oil at temperature of 50 °C for 16 h. Modified DNA was purified using the Wizard DNA Clean-Up System (Promega), according to the manufacturer's procedure, and eluted within 50 μl of sterile distilled deionized water. Modification was perfected by supplementing 20 μl of 1 M NaOH, and the samples were incubated for 5 min at room temperature, then carried on precipitation with 3 μl of 2.5 M ammonium acetate and 210 μl of 100% ethanol. Purified and modified DNA was preserved in 20 μl sterile distilled deionized water and used in the next step.

2.3. Methylation-specific polymerase chain reaction (MS-PCR)

Methylated and unmethylated primers were designed, using online software MatPrime, from the preferred sequence of *CTLA4* gene promoter as shown in Table 2. Methylation-specific PCR (MSP) analysis of promoter region of *CTLA4* gene was performed in 25 μl PCR reactions containing 16.0 μl of sterile distilled deionized water, 2 μl of bisulfite-treated genomic DNA (80 ng), 2.5 μl of 10 × PCR buffer, 2 μl of $Mgcl_2$ (25 mM), 1 μl of dNTPs mix (10 mM), 0.5 μl of each primer (10 mM), and 0.5 μl of HotStar Taq (5 U/μl).

MSP amplification for *CTLA4* gene began with an initial denaturation step: 5 min at 95 °C, followed by 40 cycles consisting of [30 s at 95 °C (denaturation step), 30 s at 52 °C for methylated primer (M) and at 50 °C for unmethylated primer (U) (annealing step), and 30 s at 72 °C (extension step)], which was accomplished with a final extension step at 72 °C for 10 min.

Parallel with each set of MSP reactions, negative control (no sample) and positive control were run. 10 μl of each MSP reaction products was electrophoresed on 2% agarose gel and visualized under ultraviolet illumination after staining with ethidium bromide, as shown in Fig. 1.

Table 2
Primer sequences for methylation and real-time reactions.

Gene	Primer sequences (5′ → 3′)	Annealing temperature (°C)	Product size (bp)
CTLA4 M	F: GAGATTAGTTTGGTTAATATGGCGA R: CCAAATTAAAATACAATAACGCGAT	52	182
CTLA4 U	F: GAGATTAGTTTGGTTAATATGGTGA R: CCCAAATTAAAATACAATAACACAAT	50	183
CTLA4 (real-time PCR)	F: CACAAGGCTCAGCTGAACCT R: AGGTGCCCGTGCAGATGGAA	62	295

M: methylated, U: un-methylated, F: forward, R: reverse, bp: base pair, °C: centigrade degree.