·Basic Research·

Quantitative detection of the expression level of transforming growth factor-β and its receptors in pterygium with RT- PCR

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Abstract

• AIM: To quantitatively investigate transforming growth factor-β (TGF-β) and its receptors in normal bulbar conjunctival tissues and pterygium tissues.

• METHODS: Thirty cases of pterygium patients were randomly selected to undergo surgical resection of pterygium lesion, and the normal margin of bulbar conjunctival tissues were collected as control. Gene expression was detected quantitatively by the method of quantitative real-time PCR (QRT-PCR) analysis.

• RESULTS: The expression level of TGF-β 1 and TGF-β 2 was $4.26\times10^{-7}\pm1.45\times10^{-7}$ and $1.08\times10^{-10}\pm0.68\times10^{-10}$ in normal bulbar conjunctival tissues, while $10.67\times10^{-7}\pm7.47\times10^{-7}$ and $8.23\times10^{-11}\pm6.63\times10^{-11}$ in pterygium tissues. The expression level of TGF-β RI and TGF-β RII was 0.003015 ± 0.0036 and $5.33\times10^{-5}\pm5.05\times10^{-5}$ in normal bulbar conjunctival tissues, while 0.000379 ± 0.000281 and $1.002\times10^{-5}\pm9.04\times10^{-6}$ in pterygium tissues. The expression level of TGF-β 1 and TGF-β 2 in pterygium was elevated ($P<0.01$). TGF-β 1 expression level in pterygium increase 2.9±2.8 times than in normal conjunctiva. TGF-β 2 expression level in pterygium increase 7.5 ±1.4 times than in normal conjunctiva. The expression level of TGF-β RI in pterygium was significantly lower ($P<0.05$). The expression level of TGF-β RII in pterygium was significantly lower ($P<0.01$).

• CONCLUSION: QRT-PCR is an effective method to quantitatively detect gene expression in eye. The upregulation of TGF-β 1 and TGF-β 2 and down-regulation of their receptors expression may play an important role in the pathogenesis of pterygium, which is noteworthy further investigation in diagnosis and treatment of pterygium.

• KEYWORDS: transforming growth factor-β ; receptor; pterygium; quantitative real-time PCR; gene expression

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INTRODUCTION

The pterygium is one common disease frequently-occurring in ophthalmology department, which until now still had no consensus to its pathogenesis. Clinic also lacks ideal methods for permanent therapy. Therefore, it is urgently needed to study its pathological change essence and seek the effective measures for prevention. The pathology research indicated that the principal constituents of pterygium become the fibroblasts by the massive proliferations, causing the corresponding pathological. In recent years, it is indicated that the growth factors play an important role in inflammation and the proliferation of fibroblasts[1]. Transforming growth factor-β (TGF-β) is such kind of multi-peptides that are able to regulate the cell growth and differentiation, which is reported to participate in pterygium pathology process [2]. But its concrete mechanism is still not discovered. We used quantitative real-time PCR (QRT-PCR) to determine the expression of TGF-β1 and TGF-β2 as well as their receptors in pterygium and normal bulbar conjunctiva tissues, exploring their roles in the process of pterygium lesions.

MATERIALS AND METHODS

Materials Thirty cases of pterygium patients were randomly selected with surgical resection of pterygium tissues, collecting 2mm×5mm from the top of the normal margin of bulbar conjunctival tissues as control. All the patients have no other lesions of cornea and conjunctiva.

Methods Specimen Preparation: under aseptic conditions, normal bulbar conjunctival tissues and pterygium tissues were obtained through surgery, drawing rapidly into liquid nitrogen immediately, using RNA Extraction Kit Trizol Reagent (Invitrogen) to extract the total RNA, UV spectrophotometry to measure its purity and content, 4g/L formaldehyde denaturing gel electrophoresis test its integrity. Primer design: In accordance with QRT-PCR primer design principles, TaqMan probe fluorescence was chosen as a fluorescent FAM group report, TAMRA as quenching groups. PCR primers and TaqMan probe were designed according to TGF-β1 and TGF-β2 and its type I and II receptors and refer to 18S cDNA sequence. Primer sequences were as follows:

TGF-β1-F: 5'>AAC TAC TGC TTC AGC TCC AC<3'
TGF-β1-R: 5'>TGT GTC CAG GCT CCA AAT GTA<3'
TGF-β1-TM: FAM 5'>CAG AAG TTG GCA TGG TAG CCC TTG GG<3'TAMRA
TGF-β2-F: 5'>ATG TGC AGG ATA ATT GCT GCC<3'
TGF-β2-R: 5'>TGG TGT TGT ACA GGC TGA GG<3'
TGF-β2-TM: FAM 5'>TGT TGT GTG TCT GAA CTC CAC AGA T<3'TAMRA
TGF-βRI-F: 5'>ACC TTC TGA TCC ATC CGT T <3'
TGF-βRI-R: 5'>CGC AAA GCT GTC AGC CTA G <3'
TGF-βRI-TM: FAM 5'>CAG AGC TGT GAG GCC TTG AGA GTG <3'TAMRA
TGF-βRII-F: 5'>CCC TAC TCT GTC TGT GGA TGA<3'
TGF-βRII-R: 5'>GAC GTC ATT TCC CAG AGT AC<3'
TGF-βRII-TM: FAM 5'>CAG GTG GGA ACA GCG AGA TAC ATG G <3'TAMRA
18S-F: 5'>GTA ACC CGT TGA ACC CCA TT <3'
18S-R: 5'>CCA TCC AAT CGG TAG TAG CG <3'
18S-F-TMP: FAM 5'>ATG GGG ATC GGG GAT TGC AAT <3'TAMRA

Quantitative RT-PCR Reactions Take out 5μg RNA, using Superscript II retroviral to reverse transcriptase cDNA. The reaction system and the operation were carried out according to specification. Using pterygium and normal bulbar conjunctival tissues cDNA as a template, TGF-β1, TGF-β2 primer, TGF-βI and II receptor primer were respectively added, corresponding quantitative PCR reaction with add in the TaqMan probe, three-hole repeated. PCR reactions happen in 96-well plates. Reaction System consist of the 50μL: 10 ×PCR buffer 5μL, $MgCl_2$ 1.5mmol/L, upstream and down-stream primers 0.1μmol/L, 0.1μmol/L TaqMan probe, 5 U Taq enzyme. Set over in each cycle of degeneration, and the process automatically record the final 10 percent the average fluorescence value of cycle time in the last cycle at the end of the PCR. Fluorescence types choose FAM-490, in accordance with the procedures set excitation and emission spectra selection filters which were 490nm and 530nm.

Quantitative PCR Analysis

All specimens Point curve were recorded, the default analysis mode was the background of the clawback (Background Substrated), choose baseline PCR deduction (PCR Base Line Substrated) mode for data analysis and correction. In the adjusted baseline cycle (Baseline Cycles) and the computational domain values (Threshold Value), drawn Ct values (Threshold Cycle). The ratio of TGF-β and 18S gene of the initial template was $2^{Ct18s\text{-}Ct\ TGF\text{-}\beta}$, to represent expression of TGF-β gene in the normal conjunctival tissues and pterygium relative to level of 18S. Ct β1 was on behalf of TGF-β1 Ct value, Ct β2 was on behalf of TGF-β2 Ct value. TGF-β receptor gene and 18S ratio of the initial template for the $2^{Ct18S\text{-}\ CtTGF\text{-}\beta\ R}$ were on behalf of pterygium and normal conjunctival of TGF-β receptor gene relative expression level of 18S. CtTGF-βR1 represent TGF-β I receptor Ct value, Ct TGF-βR2 represent TGF-β II receptor Ct value. All Ct value was got from the median.

Statistical Analysis Three holes of each specimen were determined, and their average value mean ±SD was calculated. Each group was compared using t test.

RESULTS

Extraction of RNA in Pterygium and Normal Conjunctival Tissue Using 4g/L formaldehyde denaturing gel electrophoresis to identify of 18S and 28S strip which was clear. There was no significant degradation of RNA (Figure 1). UV spectrophotometry measured OD260/280, the ratio was 1.8-2.0. RNA extraction was high purified and can be used for the experimental study.

Quantitative RT-PCR Analysis the Expression of TGF-β1 and TGF-β2 in the Normal Conjunctival Tissue and Pterygium The results showed that the average expression levels of TGF-β1 in the normal bulbar conjunctival tissues and pterygium were $4.26\times10^{-7}\pm1.45\times10^{-7}$and $10.67\times10^{-7}\pm7.47\times10^{-7}$. TGF-β 2 were $1.08\times10^{-10}\pm0.68\times10^{-10}$ and $8.23\times10^{-11}\pm6.63\times10^{-11}$. TGF-β1 expression level in pterygium increase 2.94±2.81 times than in normal

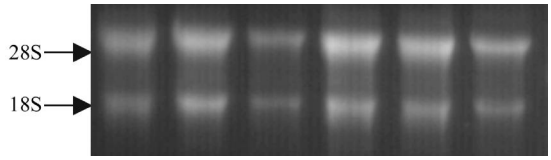

Figure 1 Gel electropherogram of RNA extraction

conjunctiva. There was significant difference (P<0.01). TGF-β2 expression level in pterygium increase 7.5 ±1.4 times than in normal conjunctiva. A statistically significant difference can also be found (P<0.01). It can be presumed that the average expression level of TGF-β1 and TGF-β2 in pterygium was far higher than in normal bulbar conjunctival tissues. TGF-β1 expression in normal conjunctiva tissue was higher than TGF-β2, but the change extent of TGF-β2 was larger than TGF-β1. TGF-β may play an important role in the development of pterygium lesions.

Quantitative RT -PCR Analysis with the Normal Conjunctival Pterygium of TGF -βRI, TGF -βRII Expression The results showed that the average expression of TGF-βRI in normal bulbar conjunctiva tissue and pterygium was 0.003015±0.0036 and 0.000379±0.000281, TGF-βRII was $5.33\times10^{-5}\pm5.05\times10^{-5}$ and $1.002\times10^{-5}\pm9.04\times10^{-6}$. The difference of TGF-βRI was significant (P<0.05). Expression of TGF-βRII level in pterygium was significantly lower than that in normal conjunctiva (P<0.01). It can be supposed that expressions of TGF-βRI and TGF-βRII were decreased in pterygium. Besides, there was more TGF-βRI than TGF-βRII both in pterygium and in normal bulbar conjunctival tissues, suggesting that the decreased TGF-βRI and TGF-βRII may lead to the increase of TGF-β secretion, resulting in the development of pterygium lesions. TGF-βR was closely related with pterygium.

DISCUSSION

Pterygium is a common disease in the ophthalmology department. It is generally believed that such excessive stimulation of sandstorms, a long-term soot, dust, sunlight (ultraviolet radiation) and the pollen are factors of pterygium. Various complex factors are involved in the pathologic process with which chronic inflammation of the conjunctiva may also be correlated. Pterygium is a proliferation disease the clinical performance of which is the new tissue kept on hyperplasia and corneal infiltration. Pathological studies show that the major components of pterygium is a large number of proliferate fibroblast, led by corresponding lesions. Recent studies show that cytokines play an important role in the inflammatory response and the fibroblast proliferation [3-4]. TGF-β is one of the important factors. Research has indicated that TGF-β1 was strong expressed in the primary pterygium tissue. Exogenous TGF-β1 has mild suppression on cultured normal conjunctival fibroblasts, and promoting effect on the cultured fibroblasts of pterygium in a dose-dependent manner [5]. TGF-β may play an important role in the abnormal proliferation of pterygium, but the exact mechanism of positive and negative control and signal transduction pathway research needs to be further confirmed.

TGF-β is a kind of polypeptide that can regulate cell growth and differentiation, can regulate the expression of a variety of target genes in cell differentiation, proliferation, migration and play an important role in the regulation of apoptosis. It can demonstrate promotion or inhabitation of cell proliferation and differentiation based on different target cells. For example it could facilitate fibroblasts, osteoblasts, and other mesenchymal cell to proliferate, but has strong inhibition for many epithelial cells, endothelial cells and lymphoidand[6]. Study of TGF-β expression in the pterygium will help on the understanding of their sources and mechanisms. Because TGF-β must bind to specific receptor and through Smads protein that is an intermediary molecule can transduct extra cellular signal to nuclear biological effects. Therefore, studying the role of TGF-β in the course of the disease at the same time, should also observe the corresponding receptor gene expression levels. TGF-βR is a cell surface receptor, including TGF-βRI, TGF-βRII and TGF-βRIII. TGF-βRI and TGF-βRII are imperative in TGF-β signaling transduction system, TGF-βRIII is the foundation of the action. TGF-βRI and TGF-βRII are receptors of serine/ threonine kinase receptors and they have common commencement action of TGF-β in the cytoplasm signal. Then the Smads protein is activated and the signal is transducted to the nuclei and the transcription of objective gene is regulated [7,8].

Our research shows that expression of TGF-β1 and TGF-β2 in pterygium was higher than that in normal conjunctival tissue. TGF-β1 in pterygium and normal bulbar conjunctiva was higher than the expression of TGF-β2 in the corresponding tissues, but the increasing level of TGF-β2 in pterygium was higher than TGF-β1, suggesting that TGF-β may play an important role in the development of pterygium

lesions, and that TGF-β2 may play regulatory role in the development of pterygium. In the pterygium lesions, our research results also suggest that TGF-βRI and TGF-βRII expression were significantly lower when compared with normal tissue. May ultraviolet affect the TGF-β/Smads signal transduction process, making the secretion of TGF-β to increase and change its regulation of target cells, to spur the occurrence and development of the pterygium diseases. Advanced study the role of TGF-β/Smads signal transduction process is needed.

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