

# Eotaxin-1 expression in tears of thyroid-associated ophthalmopathy

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## Research Article

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# Abstract

**Purpose** To explore changes of Eotaxin-1 concentration in tears of thyroid associated ophthalmopathy (TAO) patients

**Methods** This prospective observational study. 16 patients with TAO (32 eyes) and 18 healthy volunteers (36 eyes) were recruited. TAO patients were divided into active TAO (16 eyes) and inactive TAO derived from the active TAO patients after corticosteroid treatment (16 eyes). The tear concentration of Eotaxin-1 was measured per ELISA assay. Eotaxin-1 protein expression was evaluated per Western Blot.

**Results** The highest Eotaxin-1 concentration in tears was in inactive TAO patients, followed by patients with active TAO and the controls per ELISA. Western Blot analysis indicated that Eotaxin-1 expression in inactive TAO was also higher than in active TAO and the controls.

**Conclusion** The changes of Eotaxin-1 expression in tears of patients with active and inactive TAO suggested that orbital inflammation may be involved in the ocular surface damage of TAO.

## Introduction

TAO is a inflammatory autoimmune disorder of the orbit, characterized by inflammation and proliferation of the orbital tissue caused by CD4 + T cells and orbital fibroblasts [1]. Many Patients often have symptoms of ocular surface discomfort, which are caused by the changes of tear fluid proteins, which increase the tear fluid's osmolarity, and result in ocular surface damage [2]. Our previous study [3] has been shown increased IL-7 expression in tears and Orbital tissues of inactive TAO patients, which indicates that orbital inflammation may be involved in the pathogenesis of ocular surface damage in TAO. The process of TAO is an active phase initially, followed by an inactive (fibrotic) phase [4]. Interventions are generally effective in active TAO patients, but are ineffective in those of inactive TAO. Therefore, the early assessment of TAO disease activity is essential for optimal outcome of medical interventions [5].

Immune response plays a central role in the pathogenesis of TAO. The notion that T cell-mediated immunity contributes to TAO development has been widely accepted [6], but some studies have suggested that other potential signal molecules, such as chemokines, can be involved in the pathogenesis of TAO [7]. Chemokines exert their biological effects by interacting with Chemokines receptors in many homeostatic and pathological processes. Thus, an alteration of chemokine and/or chemokine receptor expression may lead to the persistence of an inflammatory reaction [8].

Eotaxin-1, encoded by the CCL11 gene, is a chemokine within the CC subfamily that is produced by a variety of cell types [9–11]. Binding of Eotaxin-1 to the chemokine receptors CCR2, CCR3, and CCR5 [12–13], Eotaxin-1 stimulates the migration of cells [8]. Effects of Eotaxin-1 have been involved in

various immune-mediated diseases [14]. Previous studies have shown that the level of Eotaxin-1 is increased in several chronic inflammatory diseases, including allergic rhinitis, rheumatoid arthritis, and ankylosing spondylitis [15–17]. Recent study reported that the reduction of the level of Eotaxin-1 significantly attenuates airway inflammation in experimental mice asthma [18], which indicating the potential use of eotaxin-1 as a biomarker for the diagnosis and assessment of inflammation severity and control.

In our study, we investigated the level of Eotaxin-1 in tears of patients with active TAO, inactive TAO and the controls per ELISA. Furthermore, the expression of Eotaxin-1 protein was determined per western blot analysis. This results showed that Eotaxin-1 may be related to the pathogenesis of the lacrimal gland and tear inflammation in ocular surface damage among TAO patients.

## Materials And Methods

### Subjects

A total of 16 patients who were diagnosed with TAO based on Bartley's criteria [19], were enrolled in the present study. We used the Bartalena Standard classification [20] for moderate-to-severe selection of TAO. Standard ophthalmic examinations such as visual acuity, slit-lamp examination, fundus examination, tear film break-up time, fluorescein staining and Schimer tests were performed for both TAO and control patients.

Exclusion criteria included patients suffering from ocular surface disorders, such as dry-eye syndrome, allergic ocular surface diseases or pterygium prior to development of TAO; a history of medication which may affect ocular surface parameters [21], such as rheumatoid arthritis drugs, topical anti-glaucoma drugs; wearing of contact lenses; patients with a history of steroid treatment or radiation therapy; and patients with other diseases that are related with TAO such as diabetes. Ophthalmopathy was evaluated based on the clinical activity score (CAS) following complete examination of the eyes. Patients with  $CAS \geq 3/7$  were defined as having active phase TAO, whereas,  $CAS \leq 2/7$  were defined as having inactive phase TAO [22]. Based on these criteria, 16 patients were classified as active phase TAO, while, 16 patients with inactive TAO were from the same patients with active TAO after corticosteroid treatment. In addition, 18 age-matched and sex-matched healthy volunteers were enrolled as the control group. There was no significant difference between the ocular parameters and disease course in our experiments.

The study was approved by the Ethics Committee of Inhuman Hospital affiliated with Shanghai Jiaotong University School of Medicine, Shanghai, China. All experiments were performed in strict accordance to the Declaration of Helsinki. Informed consent was obtained from each participating patient prior to taking part in the study.

### Tear collection and protein extraction

Unstimulated tear samples were collected from 32 eyes of the 16 TAO patients and 36 eyes of 18 healthy volunteers using disposable 10  $\mu$ l microcapillary pipettes (Microcap 10  $\mu$ l; Drummond

Scientific, Broomall, PA, USA) via the inferior meniscus. Tear samples were placed into 0.5 ml Eppendorf tubes and stored at -80°C for subsequent investigation.

Eotaxin-1 concentration was measured using Human Eotaxin-1 ELISA Kit (Abcam, UK) according to the manufacturer's instructions. The samples were divided into three groups: 16 patients with active TAO, 16 patients with inactive TAO-derived from the active TAO patients after corticosteroid treatment, and 18 healthy volunteers. For the experiment, tear samples were diluted 10 times with assay diluents. A total volume of 100µl from each sample was used for the assay.

#### Detection of protein expression in tears via western blot assay

Western blot was performed according to standard procedures. Briefly, total protein was quantified using BCA protein assay kit (Tiangen Biotech Co., Ltd, China) and equal amounts of protein was loaded into each well for separation via SDS-PAGE. Proteins were then transferred onto a PVDF membrane (Millipore, USA), blocked for 1 h at room temperature, then incubated with relevant antibodies at 4°C overnight. The membranes were then incubated with the HRP-conjugated secondary antibody for 1 h at room temperature and immunoreactive bands were reacted with ECL substrate and visualized under chemiluminescence imaging analyzer (GE, USA). GAPDH was used as the internal control to normalize protein loading. Eotaxin-1 primary antibodies was purchased from Santa Cruz Biotechnology and used at 1:2000 dilution. Western blot images were analyzed using Quantity One software (Bio-Rad, USA).

#### Statistical analysis

Eotaxin-1 concentration was expressed as mean  $\pm$  standard deviation. Statistical analysis of the data was performed per multivariate ANOVA and Bonferroni's adjusted test for multiple comparisons, automatically applied by computer program (System). The multiplication factor was calculated based on the number of comparisons applied. Statistical software (IBM SPSS Statistics 25.0; SPSS inc Chicago, IL) was used for analysis, and  $P \leq 0.05$  was considered as statistically significant.

## Results

#### Validation of protein expression

In order to verify the identified Eotaxin-1 expression, we performed western blot analysis, which indicated that the expression of Eotaxin-1 protein was significantly higher in inactive TAO and active TAO than in the control ( $p < 0.01$ , respectively). Furthermore, the protein expression in inactive TAO was significantly higher than in active TAO ( $p < 0.01$ ). These results indicate that the occurrence of TAO was associated with dynamic changes in immune regulatory proteins (Fig. 1).

#### Eotaxin-1 expression in the tear sample of TAO patients

We focused on Eotaxin-1 and further analyzed its expression level in tear samples by ELISA assay, which showed that the level of Eotaxin-1 in inactive TAO and active TAO was significantly

higher than in the control ( $p < 0.05$ , respectively). Furthermore, Eotaxin-1 concentration in inactive TAO was also higher than in active TAO ( $p < 0.05$ ). In healthy volunteers, the level of Eotaxin-1 in tears was approximately 739pg/ml. However, following the occurrence of TAO, the level of Eotaxin-1 increased rapidly, reaching an average of 1496 mg/ml in active TAO group, and as high as 1882 mg/ml in inactive TAO, indicating a 2.5-fold increase compared with the controls. These results suggested that Eotaxin may be implicated in the development and progression of the ocular surface damage of TAO (Fig. 2).

## Discussion

TAO has been shown to be associated with the typical signs and symptoms characteristic of dry eye. Our study found that Eotaxin-1 concentration in tears is significantly higher in inactive TAO than in active TAO and the controls, which, may be useful as a potential biomarker for inactive TAO.

The mechanism of changes on Eotaxin-1 expression in tears of TAO was not characterized. However, accumulating data suggested that the lacrimal gland and tear fluid may be directly involved in the ocular surface damage in TAO [23]. Danping Huang [24] reported that the lacrimal gland is significantly enlarged in active and inactive TAO than in the control, suggesting that inflammation of the lacrimal gland might be associated with the ocular surface damage. In addition, lacrimal gland acinar cells had a common target for autoimmunity with thyroidal and orbital tissues, which thus contributed to lacrimal gland impairment and ocular surface damage [23]. Lacrimal gland inflammation could increase the expressions of tear inflammatory cytokines in TAO [24]. Our previous study [3] showed that IL-7 was increased in tears of TAO patients, suggesting that orbital inflammation may be involved in the ocular surface damage of TAO.

Eotaxin-1, is a secreted protein produced by a variety of cell types, such as fibroblasts [10]. As immune modulators, chemokines exert in a wide variety of inflammatory and immune responses via the chemoattractive effects of immune cells, which, participate in the pathogenesis or progression in various disease [25]. Our study showed that elevated eotaxin-1 concentration is significantly detected in tears of the inactive TAO compared with the controls and active TAO. The reason for increased eotaxin-1 concentration was not established. Cho [26] found higher expression of Eotaxin-1 in that fibroblasts are increasingly proliferating in chronic stage, resulting in fibrosis. Furthermore, the ocular surface tissues, including lacrimal glands, cornea and conjunctiva, might be primarily responsible for the overexpression of Eotaxin-1 [27, 28]. Other reasons including aging, obesity, cigarette smoking can also affect Eotaxin-1 concentration [29-31].

In conclusion, our result suggested that Eotaxin-1 concentration in tears of inactive TAO is significantly more higher than in inactive TAO and the control, indicating a significant role of orbital inflammation in interpreting the ocular surface damage in TAO. Eotaxin-1 may be an ideal biomarker for TAO diagnosis and prevention using a clinical diagnostic test of non-invasive patient tear samples.

# Declarations

## Compliance with Ethical Standards

**Funding:** This study was funded by Medical-engineering cross fund of Shanghai Jiao Tong University (Grant No. YG2014MS72).

**Conflict of Interest:** HaiBo Tan declares that he has no conflict of interest. Kebo Cai declares that he has no conflict of interest.

**Ethical Approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Xinhua Hospital affiliated with Shanghai Jiaotong University School of Medicine, Shanghai, China and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent:** Informed consent was obtained from each participating patient prior to taking part in the study

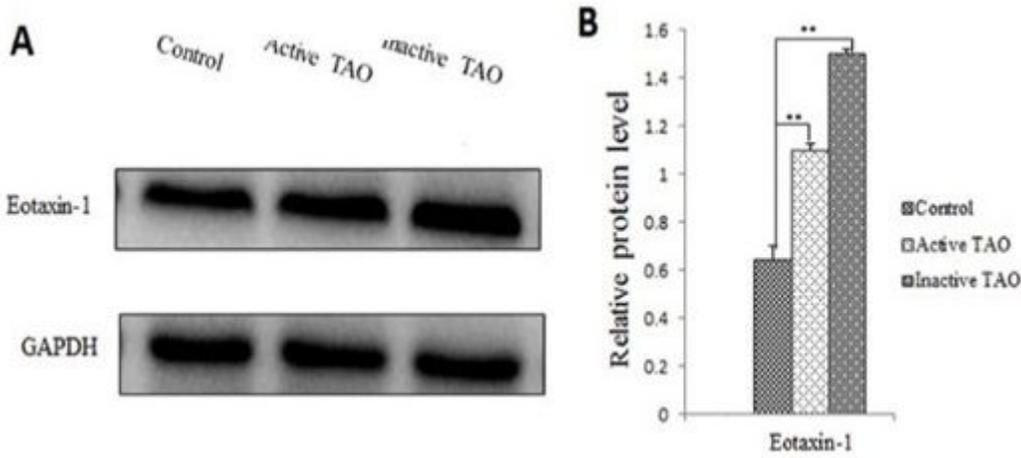
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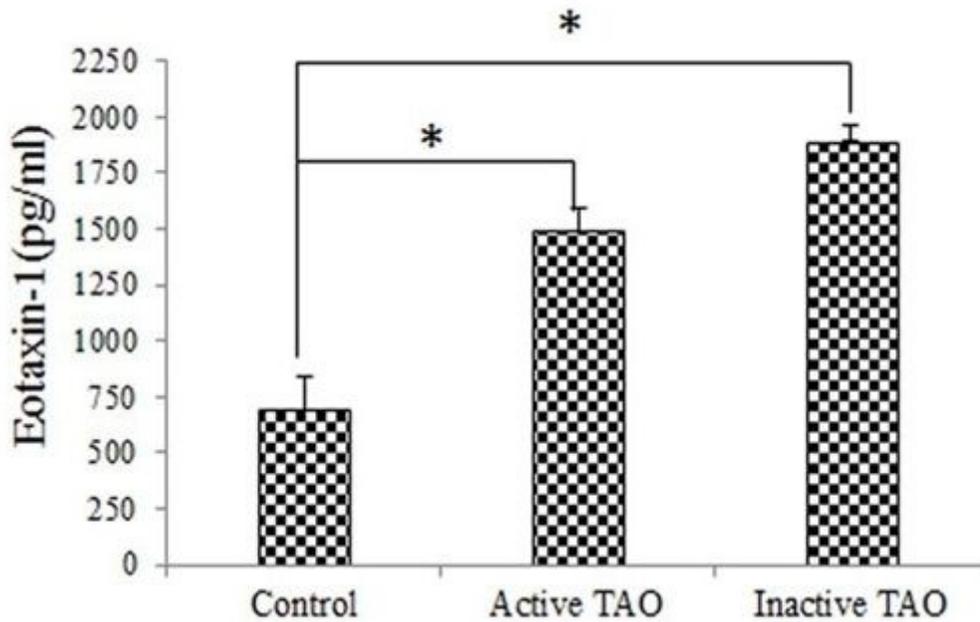
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## Figures



**Figure 1**

(A) The protein level of Eotaxin-1 was analyzed by Western blotting in healthy control and TAO groups. (B) By densitometric analysis, the histogram showed the relative protein level. GAPDH was used as internal reference protein. Data are expressed as the means  $\pm$  SD in the corresponding bar graph and statistical significance was determined by the Student's t-test. \* $P < 0.05$ , \*\* $P < 0.01$  indicated the comparison with control.



**Figure 2**

Eotaxin-1 concentration in tears of different groups was determined with ELISA. Experiment was done in duplicate. \* $P < 0.05$  compared with healthy control.