

Transcription factors T-bet/GATA-3/Foxp3 in peripheral blood of patients with chronic otitis media with effusion

Lin Yang^{1,2,3}, Shou-Qin Zhao^{1,2}, Ying Li^{1,2}, Ran Ren^{1,2}, Chen-Yi Wei^{1,2}, Jin-Song Yang^{1,2}, Bin-Bin Yang⁴

¹Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing 100010, China;

²Key Laboratory of Otolaryngology Head and Neck Surgery, Capital Medical University, Beijing 100010, China;

³Department of Otolaryngology Head and Neck Surgery, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China;

⁴Department of Medical Research Center, Beijing Tongren Hospital, Capital Medical University, Beijing 100010, China.

To the Editor: Otitis media with effusion (OME), also known as secretory otitis, is a disease in which secreted fluid accumulates in the middle ear cavity and is a major cause of hearing loss, both in children and adults.^[1] Causes of OME include recurrent infections, inflammatory conditions, malformations, and eustachian tube dysfunctions.^[2] Although most patients could recover spontaneously, by medication, or by surgery, some patients show frequent recurrence of otitis media for more than 3 months, called chronic otitis media with effusion (COME).^[1] The pathogenesis of COME is not completely understood. Increasing evidence suggests a central role of immunologic cells and their associated cytokines in the chronic inflammation that characterizes COME.

T lymphocyte is the predominant cell type in the immune response. Among these, helper T cells (Th cells), especially CD4⁺ T cells, account for the majority. Differentiation of naïve T cells into Th1 or Th2 subtypes is mediated by their respective cytokines and transcriptional factors. Th1 cells secrete interferon (IFN)- γ , and its differentiation is regulated by a specific transcriptional factor, T-bet. The differentiation into Th2 cell, which secretes cytokines interleukin (IL)-13, IL-5, and IL-4, is regulated by a specific transcriptional factor, GATA3.^[3] Regulatory T cells (Treg), also referred to as suppressor T cells, exist in several forms. The most well-known type expresses CD4, CD25, and Foxp3 (CD4⁺CD25⁺Foxp3⁺ regulatory T cell), and is regulated by a specific transcriptional factor, Foxp3.^[4] T-bet, GATA-3, and Foxp3 have been investigated in COME by different ways,^[5-7] but the results are still confusing. In our study, we explored the role of Th1/Th2/Treg-cell-specific transcription factors, T-bet, GATA-3, and Foxp3, respectively, in the pathogenesis of COME.

This study was conducted at the Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital. Twenty-seven patients (18 males, nine females; mean age: 26.7 \pm 16.6 years) and 20 healthy controls (11 males, nine females; mean age: 28.5 \pm 18.2 years) were recruited. Subjects more than 14 years old were considered as adults ($n = 19$ and $n = 15$ in COME and control groups, respectively), and those younger than 14 years were considered as children ($n = 8$ and $n = 5$ in COME and control groups, respectively).^[8]

Patients with COME mainly reported stuffiness in the ear accompanied by hearing impairment and secondary tinnitus. A small number of patients reported earache. Otorrhea or elevated body temperature was not observed. Otolaryngoscopic examination revealed retraction or bulging of tympanic membrane, with partially visible bubbles or fluid levels. Pure tone audiometry showed mild to moderate conductive hearing loss with or without partially mixed hearing loss. Tympanometry indicated type "B" or "C" tympanic pressure map with the absence of acoustic reflex. All patients with COME had a duration of disease for more than 3 months.^[1] Fourteen patients underwent tympanocentesis or tympanostomy tube insertion for the first time, while 13 patients for two or more episodes. Patients with chronic rhinitis, sinusitis, nasal polyps, nasopharyngeal carcinoma, and adenoid hypertrophy were excluded from the study. Patients with diabetes, tuberculosis, other serious systemic diseases, cancer, and blood disorders, and those with a history of steroid therapy in the immediately preceding 2 weeks, were also excluded. Healthy controls with no history of allergic disease, or adenoid hypertrophy, were included in the study.

Blood samples (2 mL) were collected in ethylenediaminetetraacetic acid (EDTA) tubes from all subjects, and

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Correspondence to: Prof. Shou-Qin Zhao, Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital; Key Laboratory of Otolaryngology Head and Neck Surgery, Capital Medical University, Beijing 100010, China
E-Mail: shouqinzhao@163.com

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500 μL blood was transferred into RNase-free Eppendorf tube, mixed with 500 μL TRIzol reagent (Thermo Fisher Scientific Inc., USA), vigorously vortexed for 30 s, and then kept in -80°C until further experiments. The above mixture (200 μL) was transferred into RNase-free Eppendorf tube, mixed with 1 mL TRIzol Reagent and vigorously vortexed for 30 s. Subsequently, 200 μL of chloroform was added, vortexed vigorously for 15 s, incubated for 2 to 3 min at room temperature, and centrifuged at $12,000\times g$ for 15 min at 4°C . The aqueous phase was removed into a new microcentrifuge tube, and 500 μL isopropanol was added; the tubes were then incubated for 10 min at room temperature, and centrifuged at $12,000\times g$ for 10 min at 4°C . The supernatant was removed. The pellet was washed with 1 mL of 75% ethanol, briefly vortexed and centrifuged at $7500\times g$ for 5 min at 4°C . The pellet was air dried at room temperature, and reconstituted with RNase-free water. Agarose gel electrophoresis was performed to check the quality of RNA extracted.

Reverse transcription was performed using a reverse transcription kit (Promega, USA) according to the manufacturer's instructions. Briefly, a mixture of 25 μL total volume containing 3 μL RNA, 0.5 μL Oligo (dT) 12 to 18 Primer, 5 μL $5\times$ Moloney murine leukemia virus (M-MLV) Buffer, 1.25 μL deoxy-ribonucleoside triphosphate (dNTP) mixture (10 mmol/L each), 25 IU RNase inhibitor (40 IU/ μL), 200 IU of M-MLV (200 IU/ μL), and RNase free dH_2O , was incubated at 42°C for 2 h, and then at 95°C for 15 min followed by cooling on ice.

Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using SYBR Premix Dimer Eraser (Takara, Japan). The reaction mixture: 5 μL $2\times$ SYBR Premix Dimer Eraser, 0.4 μL PCR forward and 0.4 μL PCR reverse primers (10 $\mu\text{mol/L}$), 0.2 μL $50\times$ Rox Reference Dye II, 3.5 μL dH_2O , and 0.5 μL cDNA template, was prepared and mixed on ice. The primers were designed, synthesized, and purified by SinoGenoMax Research Centre Co., Ltd., China. *Foxp3*: forward: 5'-CCAAACCAAGGCTCCATC-3'; reverse: 5'-CTCCGCACAGCAAACAGG-3'; *GATA3*, forward: 5'-TACCACAAGATGAACGGACAGA-3'; reverse: 5'-GGGTCTCCATTGGCATT-3'; *T-bet*: forward: 5'-GCTGCACATTGTGCGAAGTCA-3'; reverse: 5'-GCATTCTGGTAGGCGGTCA-3'; *GAPDH*: forward: 5'-ACAGCAACAGGGTGGTGGAC-3'; reverse: 5'-ACTCCGACCTTCACCTTC-3'. *GAPDH* was used as an internal control. The reaction was performed in ABI PRISM[®] Real-Time PCR System (ABI, USA) under the following conditions: 95°C 60 s, 1 cycle; 95°C 10 s, 60°C 50 s, 40 cycles for amplification; followed by 95°C 15 s (temperature gradually increased from 60°C to 95°C at 0.5°C/s) to obtain a melting curve. Melting temperature (T_m) of *T-bet*, *GATA-3*, *Foxp3*, and *GAPDH* was 77.1°C , 80.0°C , 78.2°C , and 83.5°C , respectively. Only one peak was observed in the melting curves. The relative expression of target genes was calculated by $2^{-\Delta\Delta C_t}$ method. Unlike the relative expression of individual genes calculated by $2^{-\Delta\Delta C_t}$ method, the expression ratio of *GATA3/T-bet* could be expressed directly by *GATA3 Ct/T-bet Ct*.

Data are expressed as mean \pm standard deviation. Independent samples *t* test was performed to compare data from two groups with equal or unequal variances. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., USA).

The expression of *T-bet* in COME patients was higher than that in controls (relative expression: 0.44 ± 0.64 vs. 0.16 ± 0.20 , $t = -2.17$, $P = 0.037$). At the same time, the mRNA expression of *GATA-3* and *Foxp3* in the COME group was also higher than that in the control group (relative expression: 0.89 ± 0.99 vs. 0.36 ± 0.36 , $t = -2.57$, $P = 0.015$, and 1.08 ± 0.79 vs. 0.45 ± 0.48 , $t = -3.35$, $P = 0.002$, respectively). This indicates that the Th1/Th2/Treg cells were increased in patients with COME, significantly on Th2 and Treg cells. *GATA-3/T-bet* expression ratio in peripheral blood of COME patients was significantly higher than that in normal controls ($t = 8.141$, $P < 0.01$). This suggests a higher expression of Th2 cells in peripheral blood during the progress of COME.

In summary, our study finds that the mRNA expression of *T-bet*, *GATA-3*, and *Foxp3* increases in the pathogenesis of COME, which means their associative cells, Th1, Th2, and Treg cells, may participate in the progress of COME, especially Th2 and Treg cells. Furthermore, the difference in *GATA-3/T-bet* ratio reminds the imbalance between Th1 and Th2 cells in COME. This study demonstrates the potential role of the transcription factors, T-bet, GATA-3, and Foxp3, in the pathogenesis of COME.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

None.

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