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Effect of vascular endothelial growth factor and its receptors in adult otitis media with effusion

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Abstract

Purpose Some studies have demonstrated that vascular endothelial growth factor (VEGF) plays a critical role in the pathogenesis of otitis media with effusion (OME) in animal models. However, the levels of VEGF and its receptors in adult OME have not been clarified. Our study was designed to detect the levels of VEGF and its receptors in adult OME and explore their relationship with effusion types, duration and prognosis of OME.

Methods 61 patients with secretory otitis media were enrolled including 21 males and 40 females, with an average age of 54.7 ± 17.5 years. The middle-ear effusions were collected by tympanocentesis or myringotomy. The protein concentrations were determined by enzyme-linked immunosorbent assay and messenger RNA by real-time quantitative PCR.

Results VEGF level was higher in AOME group, but not correlated with the recurrence of OME. VEGFR1 and VEGFR2 levels were lower in recurrent group compared with non-recurrent group. VEGFR2 level was higher in serous effusions than mucoid effusions. VEGF messenger RNA was positively correlated both with HIF-1α and MUC5B.

Conclusions VEGF and its receptors function to induce the production of middle-ear effusions (MEEs) at acute stage of OME rather than chronic or recurrent stage, which is mainly mediated by HIF-1α pathway. The formation of mucoid effusions is associated with MUC5B and VEGFR2, but not with duration and recurrence of OME.

Keywords Otitis media with effusion · Vascular endothelial growth factor · Receptor · Hypoxia-inducible factor-1α · Mucin 5B

Introduction

Otitis media with effusion (OME) is characterized as middle-ear effusion without acute ear infection [1]. It is a common cause of hearing loss in children. The incidence rate of OME is 20% in 2-year-old children but only 0.6% in adults in the UK [2]. The etiological factor of adult OME is different from paediatric OME and the effect of tympanotomy in adults is not as good as the latter [3].

The pathogenesis of OME is a complex assembly of anatomy, immunity, genetics, and environments [4].The main pathological change of OME is inflammatory reaction to bacteria or bacterial components including lipopolysaccharide, virus, and inflammatory factors such as TNF, IL-1b, IL-6, IL-8, and VEGF [5]. OME also induces mucosal angiogenesis, a physiological process in which new blood vessels extend from pre-existing vascular network. Angiogenesis is a dynamic balance via the mutual regulation of pro- and anti-angiogenic factors in the microenvironment and is influenced by hypoxia, inflammation, and tumors [6]. Among all the pro-angiogenic factors, VEGF is a mediator of hypoxia-induced angiogenesis and is essential for neovascularization in a variety of tissues under physiological and pathological conditions [7].

VEGF, also known as VEGFA, is mainly produced by vascular endothelial cells and binds to two tyrosine kinase receptors (RTKs), VEGFR-1 and VEGFR-2 [8]. VEGFR-1 or Flt-1, whose expression is regulated by HIF-1, is a negative regulator of VEGF activity [9]. VEGFR-2, also known as KDR or Flk-1, is a pivotal receptor mediating angiogenesis and vascular permeability [10]. Jung et al. [11] first detected the expression of VEGF protein and its mRNA in the middle-ear mucosa of rats with OME. Kohsuke et al. [12] examined the expression of VEGF in

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paediatric MEEs, demonstrating that VEGF production is a response to bacterial components in the middle-ear cavity.

Oxygen homeostasis is a key factor in the regulation of VEGF. Hypoxia can lead to pathological changes in the microenvironment and increase the expression of VEGF mRNA [13]. Cheeseman et al. [14] found that VEGF inhibitors and HIF-1 α inhibitors can reduce the severity of hearing loss and decrease the formation of middle-ear effusion, indicating that HIF-VEGF pathway plays an important role in the pathogenesis of OME.

Many studies suggest that VEGF plays an important role in the development of OME in children and animal models. However, no study has explored the expression of VEGF and its receptors in adult MEEs. The purpose of this study was to detect the expression levels of VEGF and its receptors VEGFR1 and VEGFR2 in adult MEEs and to analyse their correlation with HIF-1α and MUC5B. We also explore their relationship with effusion types, duration, and prognosis to provide clinical reference for the development of OME disease.

Materials and methods

Subjects

A total of 61 patients who were diagnosed with OME were enrolled in this study from the Department of Otorhinolaryngology Head and Neck Surgery, Beijing Anzhen Hospital from September 2016 to December 2017. There were 21 males and 40 females, with a mean age of 54.7 ± 17.5 years old (22–92 years).11 cases were secondary to upper respiratory tract infection, 3 cases underwent radiotherapy for NPC, 1 case was caused by air pressure injury after flight, and 1 case was after operation of malignant melanoma. Most patients complained about hearing loss and aural fullness and minority of them complained about tinnitus or mild earache. Patients were divided into an acute group and a chronic group according to the duration of OME. The former was defined as the duration of OME being less than 3 months [1] and consisted of 36 cases (59%), and the latter was more than 3 months and included 27 cases (41%). Patients were also divided into a recurrent group and a non-recurrent group by whether recurrence occurs at least three times in AOM within 6 months or at least four times in 12 months [18]. There were 6 cases (10%) in the recurrence group and 55 cases (90%) in the non-recurrence group. Informed consent was obtained from the patients for the experimental use of specimens. Approval for the study was granted by the Institutional Review Board of Beijing Anzhen Hospital.

Diagnostic and exclusion criteria

All the patients were examined by endoscopy, pure tone audiometry and an acoustic impedance test. The tympanic membrane of the OME patients presented a retracted, reddish or amber color, a shortened or disappeared light cone and liquid level or bubbles. Pure tone audiometry showed air-bone gap and the acoustic impedance was B or C type. Patients with symptoms of acute infection in the middle ear such as fever, severe earache, and purulent inflammation were excluded.

Collection and preparation of middle-ear effusions

Patients who were diagnosed with OME underwent tympanocentesis or myringotomy with grommet insertion. The middle-ear effusions were collected with sterile syringes and then were divided into serous or mucoid groups. Mucoid effusion is thick, turbid, rich in mucins with a high viscosity while serous effusion is thin and transparent. The samples were vortexed and spun in a centrifuge at 2000 rpm for 20 min. The supernatant was deposited in an EP tube and stored at − 80°. A total of 61 samples of middle-ear effusion were collected, of which 46 were for protein determination and 15 were for mRNA.

Protein expression of VEGF, VEGFR1, and VEGFR2 in MEEs

The concentrations of VEGF, VEGFR1, and VEGFR2 were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). The MEEs specimens were diluted five times according to the preliminary results. VEGF standards and diluted MEE samples were pipetted into the wells of a microplate precoated with a monoclonal antibody specific for VEGF and incubated for 120 min at room temperature. After washing three times, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells and incubated for 2 h at room temperature. The plate was washed again and luminol/peroxide substrate solution was added to the wells. After 25 min incubation at room temperature, stop solution was added and the color reactions were measured with a microplate luminometer. VEGFR1 and VEGFR2 levels in diluted MEEs were also determined by ELISA according to the manufacturer's instructions.

Real-time quantitative PCR (RT-qPCR) of VEGF, VEGFR1, VEGFR2,HIF-1α, and MUC5B in MEEs

Total RNA from 15 pooled samples of middle-ear effusions were isolated using Trizol Reagent (Invitrogen, CA,USA). RNA quantities were measured and the integrities were assessed by gel electrophoresis. Total RNA was used for first-strand cDNA synthesis with M-MLV reverse transcriptase (Takara, Otsu, Shiga, Japan). RT-qPCR was performed using TaqMan gene expression assays on the LightCycler 480II Real-Time PCR System (Roche, Rotkreuz, Switzerland). PCR conditions included initial denaturation at 95 °C for 5 min, 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s for 45 cycles. Gene expression levels were normalized to β-actin. All samples were run in duplicate. The primers for genes were used as in Table 1.

Statistical analysis

The concentrations of VEGF, VEGFR1, VEGFR2, HIF-1α, MUC5B protein, and mRNA were presented as mean ± standard deviation. Mann–Whitney test and Chi-square test were used for comparing data between different groups. Correlation between different cytokines was assessed by Spearman and Pearson analysis. The significance level of the *P* value was set to 0.05 and all calculations were performed using SPSS22 software (SPSS Inc., Chicago, IL, USA).

Results

ELISA results of VEGF, VEGFR1, and VEGFR2 in MEEs

35 of all the 46 (76%) MEEs were serous and 11 of them (24%) were mucoid. There were 23 (50%) MEEs in the AOME group and 23 (50%) in the COME group. 6 (13%) MEEs were recurrent and 40 (87%) were non-recurrent. No significant differences were found in age, gender, and laterals between these groups.

The protein expression of VEGF, VEGFR1, and VEGFR2 was detected in all the samples with the concentrations of 3465 ± 5494 pg/ml (139.7–24887 pg/ml), 8073 ± 4067 pg/ml (60.59–12839.19 pg/ml) and 4974 ± 2367 pg/ml (1819.94–16137.28 pg/ml), respectively. The incidences of serous and mucoid effusions in the AOME and COME groups are shown in Table 2. There was no statistical difference in the expression of VEGF in each group ($P > 0.05$). No correlation was found between the course of disease and the type of effusion by Chi-square test ($P > 0.05$).

The concentration of VEGF in AOME was higher than that in COME (5281 ± 7216 vs. 1648 ± 1662), (Fig. 1a, $P < 0.05$). However, the VEGFR1 and VEGFR2 levels in

Table 1 Oligonucleotide sequences of PCR primers and expected length of their PCR products

Primer	Oligonucleotide sequences	Base number	Length (bp)
β-Actin	F:TCCATCATGAAGTGTGACGT	20	154
	R:GAGCAATGATCTTGATCTTCAT	22	
VEGF	F:CCCAGGTCAGACGGACAGAAAGA	23	197
	R:GAAGCAGGTGAGAGTAAGCGAAGG	24	
VEGFR1	F:ACCTCACTGCCACTCTAATTGTCA	24	158
	R:GTGCCAGAACCACTTGATTGTAGG	24	
VEGFR2	F:TGTATGGAGGAGGAGGAAGTATGTG	25	175
	R:CCGTCTGGTTGTCATCTGGGATTA	24	
HIF-1α	F:GCTCCTATACAAGGCAGCAGAAAC	24	121
	R:GGTAATGAGCCACCAGTGTCCAA	23	
MUC5B	F:GAACGGCGTGCTTGTGTCTGT	21	133
	R:GGTGTTGTTGTGGAAGAGGCTGTA	24	

Table 2 VEGF levels in serous and mucoid MEEs of different groups

MEEs	Serous (pg/ml)	Ears	Mucoid (pg/ml)	Ears	*P* value
AOME	4684.10 ± 6497.72	18	7431.97 ± 9980.06	5	N.S.
COME	1741.76 ± 1741.76	17	1385.17 ± 1239.34	6	N.S.
rOME	2896.85 ± 2291.27	6	0	0	
nrOME	3329.10 ± 5407.85	29	4133.71 ± 7112.00	11	N.S.

MEEs middle-ear effusions, *OME* otitis media with effusion, *AOME* acute OME, *COME* chronic OME, *rOME* recurrent OME, *nrOME* non-recurrent OME, *N.S.* no significance

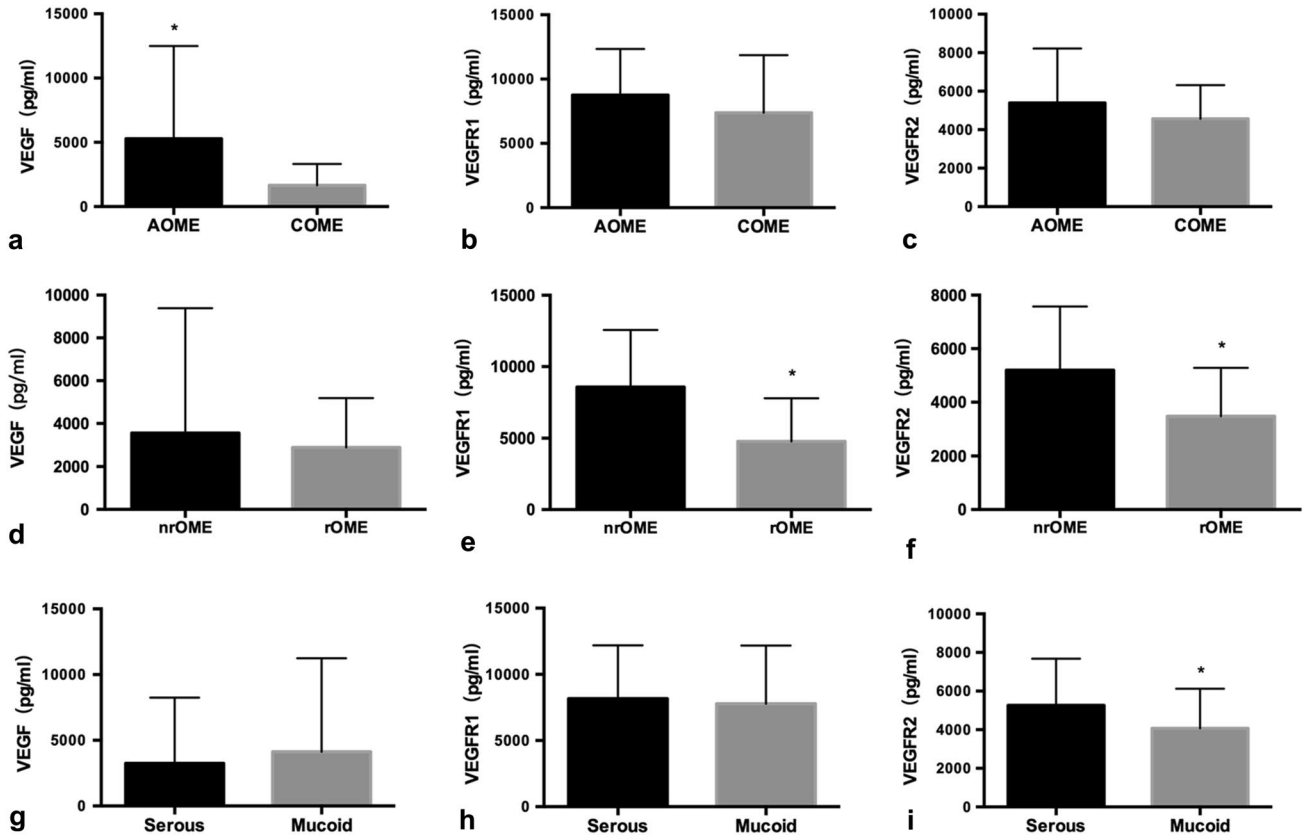

Fig. 1 Analysis of expression level of VEGF, VEGFR1, and VEGFR2 protein by ELISA in different groups. The expression of VEGF was significantly higher in AOME than COME (**a**). The levels of VEGFR1 and VEGFR2 protein were significantly higher in nrOME than rOME (**e, f**). Only KDR was significantly different in two types of MEEs (**i**). $^{*}P < 0.05$

the two groups had no significant difference (8763 ± 3575 vs. 8763 ± 3575, 5388 ± 2831 vs. 4561 ± 1756, respectively), (Fig. 1b, c). All the six recurrent MEEs were serous. There was no statistical significance in the VEGF level neither between the two effusion types in the nrOME group (Table 2) nor between the recurrent group and non-recurrent group (2896 ± 2291 vs. 3550 ± 5839, respectively), (Fig. 1d). Compared with the non-recurrent group, the expression of VEGFR1 and VEGFR2 was significantly decreased in the recurrent group (4778 ± 3008 vs. 5867 ± 4000, 3476 ± 1806 vs. 3476 ± 1806, respectively), (Fig. 1e, f). The VEGFR2 level in the serous group was significantly higher than that in the mucoid group (5258 ± 2415 vs. 4017 ± 2049 pg/ml), (Fig. 1i). No statistical significances were discovered in VEGF and VEGFR1 levels between two groups (Fig. 1g, h).

RT-qPCR results of VEGF, VEGFR1, VEGFR2, HIF-1α, and MUC5B in MEEs

The mRNA levels of VEGFR1 and HIF-1α in the AOME group were higher than that in the COME group and the MUC5B level was lower, but the differences were

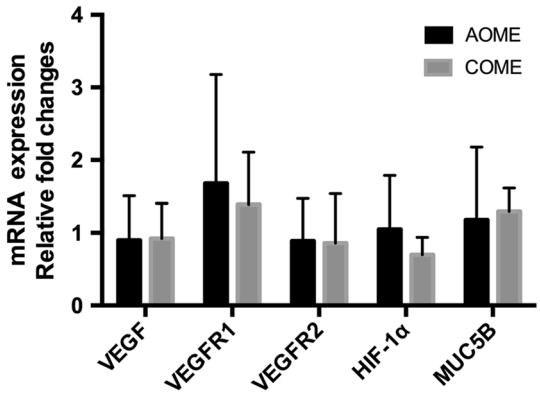

Fig. 2 RT-qPCR results of VEGF, VEGFR1, VEGFR2, HIF-1α, and MUC5B expression in MEEs of AOME group and COME group. The mRNA levels of VEGFR1 and HIF-1α were higher in the AOME group and the MUC5B level was lower, but the differences were not statistically significant. VEGF and VEGFR2 were expressed at the same levels in both groups. Data was mean of 11 MEEs in AOME group and 4 in COME group ± SD

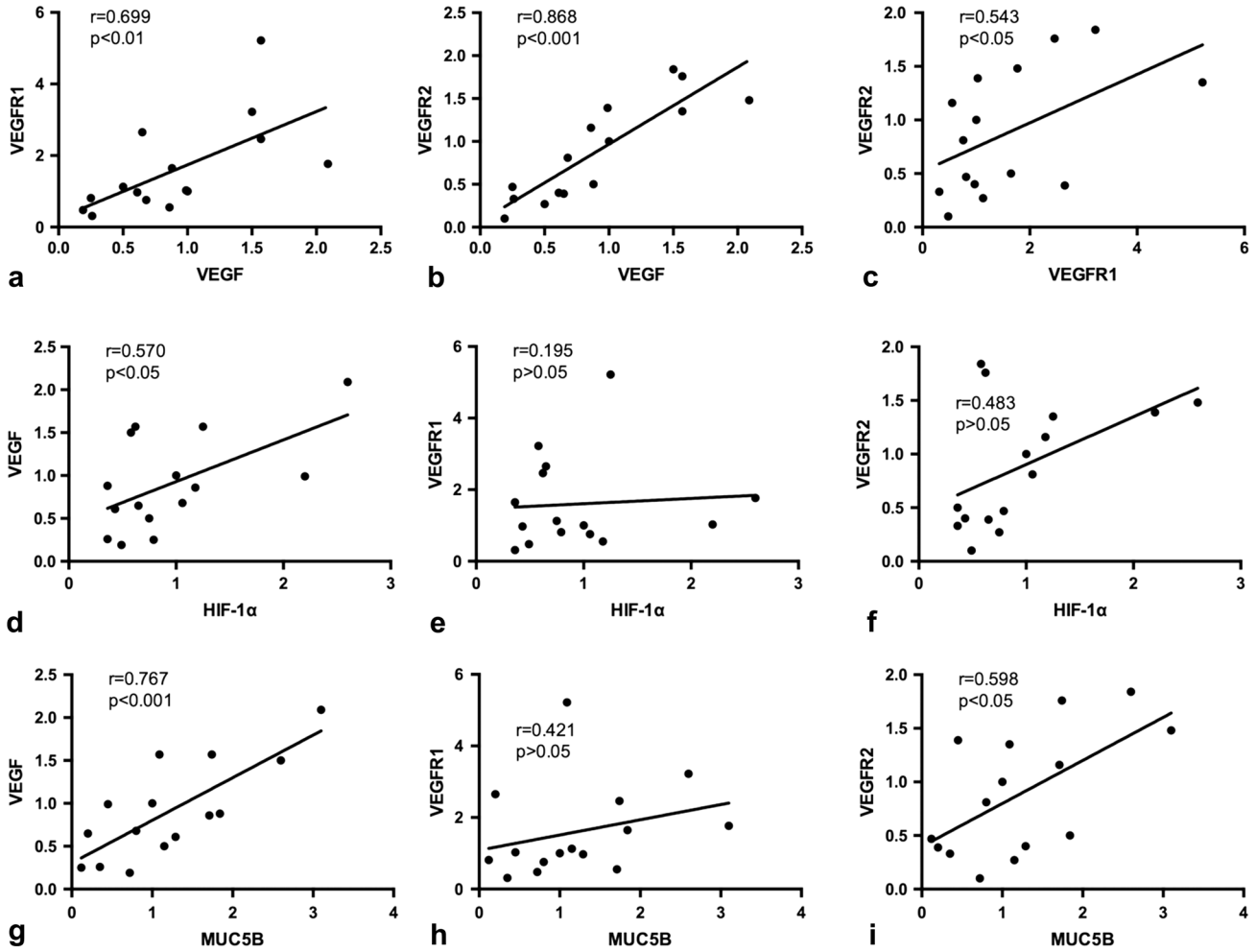

Fig. 3 Correlation results between VEGF, VEGFR1, VEGFR2, HIF-1α, and MUC5B mRNA levels by RT-qPCR. The level of VEGF mRNA was well correlated with those of VEGFR1 and VEGFR2, respectively (**a, b**).The level of VEGFR1 mRNA was significantly positively correlated with VEGFR2 mRNA (**c**).The level of VEGF mRNA was significantly correlated with both HIF-1α and MUC5B mRNA (**d, g**). There was not any significant correlation between VEGFR1 and HIF-1α or MUC5B mRNA levels (**e, h**). The level of VEGFR2 mRNA was correlated with MUC5B mRNA significantly (**i**), but not with HIF-1α mRNA levels (**f**). Data were mean of 15 MEEs ± SD

not statistically significant. VEGF and VEGFR2 were expressed at the same levels in both groups (Fig. 2). There were strong correlations between VEGF mRNA and its two receptors, VEGFR1 and VEGFR2 ($P < 0.01$, $P < 0.001$, respectively), (Fig. 3a, b). VEGFR1 and VEGFR2 mRNA levels were also correlated with each other ($P < 0.05$) (Fig. 3c). VEGF mRNA was significantly associated with HIF-1α and MUC5B mRNA, respectively ($P < 0.05$, $P < 0.001$), (Fig. 3d, g). However, the correlations between VEGFR1 with HIF-1α or MUC5B were not statistically significant (Fig. 3e, h). VEGFR2 mRNA was significantly correlated with MUC5B mRNA ($P < 0.05$), (Fig. 3i), but not with HIF-1α mRNA (Fig. 3f).

Discussion

OME also known as secretary otitis media and the middle-ear effusions can be divided into two types, serous and mucoid. In our study, the expression of VEGF and its receptors was detected in all adult MEEs, but no difference was found between serous and mucoid groups. Sekiyama Kohsuke et al. [12] measured the expression of VEGF in paediatric MEEs which was lower than our study (1678 ± 1795 vs. 3465 ± 5494 pg/ml) and was higher in mucoid MEEs than serous MEEs. This may be due to different etiological factors of OME in children and adults.

The occurrence of paediatric OME is mainly related to age, repeated upper respiratory tract infection and adenoid hypertrophy [15]. While OME in adults is usually caused by sinusitis, immunological diseases, Eustachian tube dysfunction [16], nasopharyngeal disease and gastroesophageal reflux [17]. The production mechanism of VEGF may be different between children and adults.

Our study indicated that VEGF expressed abundantly at the early stage of OME and the types of MEEs were independent of the OME duration. As a critical pathogenic component of bacteria, lipopolysaccharide can stimulate vascular smooth muscle cells and endothelial cells to release VEGF, which is the main source of VEGF [18, 19]. LPS can also promote the production and release of VEGF by activating inflammatory cells including fibroblasts, macrophages and cytokines such as IL-1, IL-2, IL-6, IL-8, TNF, and TGF [20]. The expressions of VEGFR1 and HIF-1α mRNA in AOME group were higher than those in COME group, and HIF-1α was positively correlated with VEGF mRNA expression. This indicates that obstructive Eustachian tube dysfunction leads to hypoxia in the middle-ear cavity at early stage of OME, which resulted in increasing expression of HIF-1α and then inducing overexpression of VEGF mRNA. It also demonstrates that HIF-1α-mediated VEGF pathway has a vital role in OME.

In our study, the levels of VEGF and its receptors were lower in COME group and rOME group. There are two main reasons causing OME to develop to chronic stage. One is that the Eustachian tube has a poor function thus fluid in the middle ear cannot be discharged in time. Another is that the function of Eustachian tube is normal, but the mucins are excessively accumulated in the middle-ear cavity [21]. The mucins in MEEs mainly consisted of secretory proteins, of which MUC5B is a dominant component secreted by the sub-endothelial glands [22]. Our study found that the VEGFR2 protein concentration was higher in serous MEEs and its mRNA was positively correlated with MUC5B. Also, the MUC5B mRNA in the COME group was higher than that in the AOME group. These findings indicate that VEGFR2 level increases at early stage of OME and function to dilate blood vessels, increase vascular permeability and promote the production of fluids in middle ear. At later stage, VEGFR2 play roles in accelerating angiogenesis and the growth of endothelial cells which further differentiate into mucous cells to promote mucus production by secreting MUC5B.

There are also some limitations to our study. First, we had a small sample size, especially of mucoid MEEs, and the effusions were difficult to collect. Second, the follow-up time was short thus only 6 cases of recurrent OME were enrolled in our study. All of these MEEs were serous thus the relationship between effusion type and OME prognosis could not be clarified precisely. Finally, since most of the effusions were collected during outpatient treatment, the amount of one fluid was not sufficient enough to be measured for protein levels and mRNA expression of all the cytokines and receptors. To improve our study, more inpatients should be enrolled and more muciod MEEs in different stages of OME should be collected. We also need to extend the follow-up time to estimate the occurrence of recurrent OME precisely.

Compliance with ethical standards

Conflict of interest Author Xiping Li received a study-related grant from Wu Jieping Foundation, China. Author Mengxiao Ye declares she has no conflict of interest.

Ethical approval All procedures in this study were in accordance with the ethical standards of the Institutional Review Board of Beijing Anzhen Hospital.

Informed consent Informed consent was obtained from the patients for the experimental use of specimens.

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