

TLR4 GENE EXPRESSION IN PATIENTS WITH CHRONIC SUPPURATIVE OTITIS MEDIA

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Chronic suppurative otitis media (CSOM) is an infectious disease of the middle ear that involves inflammation and accumulation of fluid behind the eardrum. The pathogenesis of CSOM involves reduced bacterial clearance due to impairment of Toll-Like Receptor (TLR) 4 pathway. TLR4 receptor serves as the molecular sensor for bacterial endotoxin (lipopolysaccharide) and activates inflammatory cell signaling for clearing the bacteria. Previous studies have shown that the expression of TLR4 gene is reduced in middle ear epithelia of CSOM patients. Whether the expression of TLR4 gene is reduced in leukocytes is not known. In our present study we aim to compare the expression of the TLR4 gene in the blood samples of CSOM patients and healthy controls. A case-control study was carried out by involving 16 participants in each group. The levels of the TLR4 gene expression were measured by using the qRT-PCR method. The median (interquartile range) ΔC_t values of the TLR4 gene expression was 4.85 (2.61- 8.55) in the patient group and 2.29 (-1.63-4.85) in healthy controls. Expression of the TLR4 gene in the leukocytes of CSOM patient group was reduced by ~5.9 fold compared to the control group and the difference was found to be significant ($P = 0.01$).

Key words: chronic suppurative otitis media, gene expression, toll-like receptor 4 (TLR4).

Otitis media (OM) is a group of complex infectious and inflammatory disease that affects the middle ear cavity. OM can be classified into two subtypes as acute and chronic. acute form of OM (AOM) can lead to chronic suppurative OM (CSOM). Chronic suppurative OM (CSOM) involves persistent infection and continuous otorrhea for more than 3 episodes in a month with a tympanic membrane perforation [1,2]. CSOM results in serious complications such as brain abscess, hearing loss, meningitis, and facial nerve paralysis [3]. The global estimate of prevalence rate ranges between 1-46% of CSOM [4]. *Pseudomonas aeruginosa* (37.21%) and *Staphylococcus aureus* (27.91%) are the most common bacteria observed in the middle ear fluids of CSOM patients [5].

CSOM is considered to be a multifactorial disease with a genetic component. Animal studies

have shown that the *TLR4* gene is involved in the development of CSOM. The *TLR4* gene codes for the type 1 transmembrane protein called Toll-Like Receptor 4 (TLR4) which functions as a pattern recognition receptor specifically for the lipopolysaccharide. The latter molecule is derived from the cell wall of gram-negative bacteria [6,7]. TLR4 is expressed in macrophages, monocytes, dendritic cells, and epithelial cells [8]. The binding of lipopolysaccharide to TLR4 receptor activates the intracellular cell signaling pathway which then results in the secretion of inflammatory cytokines. These cytokines then elicit a protective immune response against bacterial infection. Inactivation of the *TLR4* gene is assumed to compromise the development of a protective immune response and lead to persistent infection [9].

Consistent with the above view, down-regulation of the *TLR4* gene has been reported in the

middle ear epithelia of CSOM patients [10]. It is not known if this pattern is unique to middle ear epithelia or shared by other cell types that express the *TLR4* gene. This study was undertaken to answer this question. The aim of this study was to compare the expression of the *TLR4* gene in the blood samples of CSOM patients and healthy controls.

Materials and Methods

Study design. The case-control design was used to conduct the study. The case group comprised of CSOM patients ($n = 16$) and the control group comprised of age and gender-matched healthy controls ($n = 16$). The study was approved (SDUAHER/KLR/CEC/01/2017-18) by the Central Ethics Committee. Patients attending the Department of Otorhinolaryngology, R. L. Jalappa Hospital and Research Centre, Tamaka, Kolar, Karnataka, India, and the patients were enrolled between October 2018-August 2019.

Participant selection. CSOM was diagnosed based on otoscopic examination and the following criteria (i) perforation involving tympanic membrane (ii) chronic ear infection and (iii) hard of hearing. Inclusion criteria for the cases were (i) patients diagnosed with CSOM (ii) both genders in the age group of 18-60 years. Exclusion criteria for the CSOM patients were (i) acute otitis media without effusion, (ii) post-traumatic CSOM, (iii) cleft lip/palate, (iv) down's syndrome, and (v) otomycosis [11]. The patient information sheet and written informed consent form were obtained prior to the recruitment of study subjects. One ml of whole blood samples of both CSOM patients and healthy controls were collected in an EDTA vacutainer and used for quantification of *TLR4* gene expression.

Quantification of *TLR4* gene expression by qRT-PCR. The *TLR4* gene expression was performed by using quantitative reverse transcription polymerase chain reaction (qRT-PCR) CFX96 touch system (Bio-Rad, USA). Total RNA was isolated from 1 ml blood samples of both cases ($n = 16$) and controls ($n = 16$) using Trizol reagent (GeNei, Bangalore) based on protocol provided by the manufacturer's instruction [12]. The concentration and purity of the total RNA were measured using a spectrophotometer (PerkinElmer UV-VIS (Lambda 35), USA). RNA samples were stored at -20°C until further use. The RNA samples of good quality (260/280 ratio of >2) were used for conversion of cDNA. Total RNA samples were converted to cDNA using iScript cDNA conversion kit (Bio-Rad, USA). Real-time quantifi-

cation of *TLR4* and *GAPDH* genes were performed using SYBR green method (Bio-Rad, USA).

Following primers were used to quantify the expression of *TLR4* and *GAPDH* (Sigma, USA):

TLR4 gene:

Forward primer: 5' GAACCTGGACCTGAGCTT-TAAT 3'

Reverse primer: 5' GTCTGGATTTCACACCTGGA-TAA 3'

GAPDH gene:

Forward primer: 5' GATCATCAGCAATGCCTC-CT-3',

Reverse primer: 5' GACTGTGGTCATGAGTC-CTTC 3'

The composition of qRT-PCR reaction mixture is as follows: 2 μl of cDNA (1:10 dilution), 0.5 μl of forward and reverse primers, 5 μl of SYBR green, and 2 μl of nuclease-free water. The reaction conditions used for qRT-PCR were as follows: hold at 50°C for 2 min, 40 cycles of hold at 95°C for 10 min, denature at 95°C for 15 sec and anneal/extension at 54.3°C for 60 sec. At the end of the reaction, the Cycle Threshold (Ct) values were obtained from the analysis software. The expression of the *TLR4* gene was normalized using *GAPDH* as endogenous control and the fold change in *TLR4* gene expression was calculated using the $2^{-(\Delta\Delta\text{Ct})}$ method [13].

Statistical analysis. All the statistical analysis was carried out using SPSS Statistics V22.0 (IBM, USA); qualitative variables were represented by percentages. Differences between the two groups were compared by using non parametric test Mann –Whitney U test. P -value less than 0.05 were considered to be statistically significant.

Results

The demographic and clinical characteristics of the study participants were given in Table. The mean age of both CSOM patient and control groups was 31.75 ± 9.89 years. In both groups, 31.25% of the participants were male and 68.75% were female. Majority of the patients showed bilateral involvement of CSOM (68.75%). The common site of tympanic perforation was central (68.75%) followed by posterior (18.75%), and anterior locations (12.05%). Cholesteatoma formation was observed only in few patients (12.50%).

The *TLR4* gene expression was quantified by using qRT-PCR. The resulting ΔCt values did not show normal distribution. Therefore, median and interquartile range (IQR) was calculated for both

the groups. The median ΔCt of *TLR4* gene was 4.85 (2.61 to 8.55) in CSOM group and 2.29 (-1.63 to -4.85) in control group. The difference between the median ΔCt values of the CSOM and control groups was statistically significant ($P = 0.01$; Mann–Whitney U test). The results were represented graphically in Fig. 1. Comparative Ct method used to calculate the fold change in *TLR4* gene expression. The average *TLR4* gene expression in the CSOM group was found to be 0.17 compared to the control group. Thus, the gene expression in the CSOM group was about 5.9 times lower than in the control group.

Further, the *TLR4* gene expression data was analysed after subgrouping the CSOM patients based on the clinical profile. Significant difference was observed between unilateral and bilateral subgroups ($P = 0.03$; Mann–Whitney U test). The results are shown in Fig. 2. Downregulation of the *TLR4*

gene expression was severe in the bilateral subgroup than in the unilateral subgroup. There was no significant difference between patients with and without cholesteatoma ($P = 0.26$; Mann–Whitney U test).

Discussion

The purpose of this study was to compare the *TLR4* gene expression in CSOM patients and healthy controls. The results of this study show that the levels of *TLR4* gene expression are reduced in CSOM patients compared to healthy controls. This implies that the *TLR4* gene expression is down-regulated in CSOM. To the best of our knowledge, this is the first study to quantify the *TLR4* gene expression in the blood samples of CSOM patients.

The results of this study are in agreement with the pattern reported in the middle mucosa of CSOM patients. Si and co-workers reported that expression

Table. Demographic and clinical parameters of study participants

Parameter	CSOM patients ($n = 16$)	Controls ($n = 16$)
Age (Years) (mean \pm SD)	31.75 \pm 9.89	31.75 \pm 9.89
<i>Gender</i>		
Male	05 (31.25%)	05 (31.25%)
Female	11 (68.75%)	11 (68.75%)
<i>Ear affected</i>		
Unilateral	05 (31.25%)	NA
Bilateral	11 (68.75%)	NA
<i>Perforated TM</i>		
Anterior	02 (12.05%)	NA
Central	11 (68.75%)	NA
Posterior	03 (18.75%)	NA
<i>Disease duration</i>		
≤ 1 year	06 (37.50%)	NA
>1 year to ≤ 5 years	06 (37.50%)	NA
>5 year to ≤ 10 years	03 (18.75%)	NA
Since childhood	01 (06.25%)	NA
<i>CSOM</i>		
With Cholesteatoma	02 (12.50%)	NA
Without Cholesteatoma	14 (87.50%)	NA
<i>Presenting symptoms</i>		
Ear discharge	13 (81.25%)	NA
Hearing loss	09 (56.25%)	NA
Ear ache	16 (100.00%)	NA

CSOM – Chronic Suppurative Otitis Media; SD – Standard Deviation; NA – Not Applicable; TM – Tympanic membrane

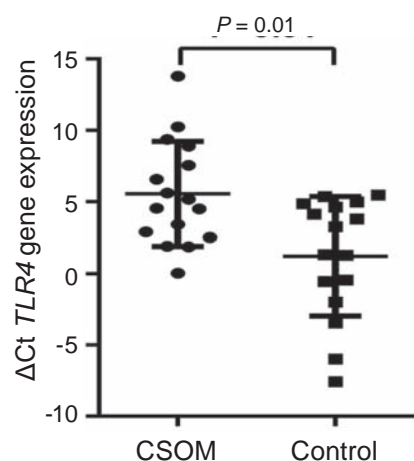


Fig. 1. Differential expression (ΔCt values) of *TLR4* gene in CSOM group vs. controls. Median $\Delta Ct_{CSOM} = 4.85$; relative gene expression = 0.17; Median $\Delta Ct_{Controls} = 2.29$; relative gene expression = 1.00

of the *TLR4* gene is lower in middle ear mucosal samples of CSOM patients compared to that of controls [14]. The *TLR4* gene expression levels were not altered in children with a history of OM compared to control in middle ear tissue samples [15]. Also, the *TLR4* gene expression is downregulated in middle ear tissue samples of CSOM patients in comparison to controls [16]. Together these report suggest that *TLR4* gene expression were downregulated in OM.

The role of the *TLR4* gene in the pathogenesis of otitis media has been established with animal studies. Two mice strains (C3H/HeJ and C57BL/6) with knock out of the *TLR4* gene have been developed. Carol and co-workers evaluated the visual changes in the tympanic membrane and auditory brainstem response in 7-8-month-old C3H/HeJ mice. The authors found that the C3H/HeJ mice developed OM spontaneously. Furthermore, increased hearing loss of mixed type and also inflammation in the middle ear were found [17]. Leichtle and co-workers evaluated the C57BL/6 mice and noticed an increase in the middle ear mucosal thickness, cel-

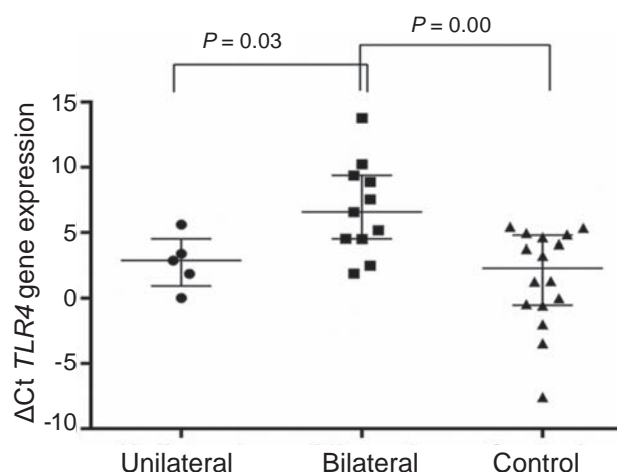


Fig. 2. Differential expression (ΔCt values) of *TLR4* gene in bilateral CSOM group, unilateral CSOM group and controls. Median $\Delta Ct_{Bilateral} = 6.57$; relative gene expression = 0.05, Median $\Delta Ct_{Unilateral} = 2.90$; relative gene expression = 0.65; Median $\Delta Ct_{Controls} = 2.29$; relative gene expression = 1.00

lular infiltration in the middle ear cavity. In addition, they reported that the bacterial clearance in the middle ear cavity was reduced [18]. These reports indicate that genetic variations are the likely cause for the impairment of Toll-Like Receptor 4 in OM. This view is supported by several genetic association studies [19-21].

Conclusion. The present study shows that the *TLR4* gene expression is downregulated in the leucocytes of CSOM patients.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЕКСПРЕСІЯ ГЕНА *TLR4* У ПАЦІЄНТІВ ІЗ ХРОНІЧНИМ ГНІЙНИМ СЕРЕДНІМ ОТИТОМ

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Хронічний гнійний середній отит (ХГСО) – інфекційне захворювання середнього вуха, яке супроводжується запаленням та скупченням рідини за барабанною перетинкою. Патогенез ХГСО характеризується зниженням бактеріального кліренсу через порушення сигнального шляху рецептора *TLR4*, який є молекулярним сенсором бактеріального ендотоксину (ліпополісахариду) і активатором протизапальної сигнальної системи клітин для знищення бактерій. Попередні дослідження показали, що експресія гена *TLR4* знижена в епітелії середнього вуха пацієнтів із ХГСО. Чи знижена експресія гена *TLR4* у лейкоцитах, невідомо. Метою дослідження було порівняти експресію генів *TLR4* у зразках крові пацієнтів із ХГСО та здорових осіб за допомогою кількісної ПЛІР. Проведено дослідження “випадок-контроль” за участю 16 осіб у кожній групі. Медіана (міжквартильний розмах) значення ΔC_t експресії гена *TLR4* складала 4,85 (2,61-8,55) у групі пацієнтів із ХГСО та 2,29 (-1,63-4,85) у групі здорових осіб. Експресія гена *TLR4* у лейкоцитах пацієнтів із ХГСО була знижена в ~5,9 раза у порівнянні з контрольною групою, і ця різниця виявилася статистично значущою ($P = 0,01$).

Ключові слова: хронічний гнійний середній отит, експресія генів, рецептор *TLR4*.

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