

## Expression of Toll-like Receptors 2 and 4 and Immunoglobulins in Children with Recurrent Otitis Media with Effusion

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**Background:** Toll-like receptors (TLRs) detect microbial infection and can directly induce innate host defense responses, which are thought to play critical roles in protecting the tubotympanum from infection. However, little is known about the relationship between TLRs, which are related to innate immunity, and immunoglobulins, which are related to adaptive immunity, in recurrent otitis media with effusion (OME). We therefore investigated the expression of TLR2 and TLR4 and immunoglobulin in children with OME.

**Methods:** The study population consisted of 72 children with OME, 31 with more than 4 episodes in 12 months or more than 3 episodes in 6 months (otitis-prone group), and 41 with fewer than 3 episodes in 12 months (non-otitis prone group). The expression in middle ear effusion of TLR2 and TLR4 mRNA, as determined by Real time-PCR, and the concentrations of IgG, IgA, and IgM, as determined by Enzyme-linked immunosorbent assay (ELISA), were compared between the two groups.

**Results:** Expression of TLR2 and TLR4 mRNA was lower in the otitis prone than in the non-otitis prone group, but the difference was not statistically significant ( $p > 0.05$ ). Between group differences in the concentrations of IgG, IgA and IgM in effusion fluid were not significant ( $p > 0.05$ ), and there were no correlations between immunoglobulin concentration and the expression of TLR2 and TLR4. **Conclusion:** Although there was a trend toward lower expression of TLR2 and TLR4 in the otitis-prone group, the differences, and those in immunoglobulin concentration, did not differ significantly between the otitis-prone and non-prone groups.

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### INTRODUCTION

Otitis media with effusion (OME) is a disease in which secreted fluid accumulates in the middle ear cavity and a major cause of hearing loss in children (1,2). Although most patients spontaneously recover, OME can frequently recur. Among the causes suggested for otitis media are Eustachian tube dysfunction, bacterial or viral infection, family history, and allergies (3), with inflammatory reactions induced by pathogen stimulation thought to be important (4). Persistent infection can lead to the accumulation of inflammatory mediators released due to inflammatory reactions, as well as to their secretion into effusion fluid. Thus the analysis of effusion fluid could be important in understanding the pathologic mechanism of OME.

The initial reaction against microorganisms in humans is triggered by the innate immune system. As part of the host defense mechanism, the innate immune system is important in the recognition of microorganisms, as well in the activation and control of adaptive immunity. Moreover, the innate immune system has been associated with sepsis, immunodeficiency, atherosclerosis, allergy, autoimmune diseases, and other diseases (5).

The first step in the activation of the human defense mechanism is the recognition of pathogens by macrophages. Macrophages recognize pathogen-associated molecular patterns (PAMPs), thus generating intracellular signals and the production of cytokines and chemokines, leading to the activation of the acquired immune system (6). One of the best known PAMPs is lipopolysaccharide (LPS), a component of

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the external wall of gram negative bacteria (7). Gram positive bacteria without LPS express other PAMPs, such as peptidoglycans, lipoteichoic acid, and lipoproteins.

The recognition and reaction of PAMPs is controlled by pattern-recognition receptors (PRPs). One well known PRP is CD14 (7), which acts together with Toll-like receptors (TLRs) in cellular activation. Of the 11 TLR subtypes currently known (8), the most actively studied are TLR2 and TLR4. Signal transduction by TLR consists of the activation of nuclear factor-kappaB (NF- $\kappa$ B) through the Toll/interleukin-1 (TIR) domain. Similar to interleukin-1 receptor (IL-1R) signaling, stimulation of the TLR receptor is activated through myeloid differentiation factor 88 (MyD88), IL-1R associated kinase (IRAK), NF- $\kappa$ B-inducing kinase (NIK), I $\kappa$ B kinase (IKK), and the NF- $\kappa$ B pathway (9). Activation of NF- $\kappa$ B leads to the expression of proinflammatory cytokines. In addition, activation of NF- $\kappa$ B, involved in acquired immunity by controlling the expression of cells expressing immune response receptors such as CD80 and CD86.

TLR2 has been shown to be a receptor primarily of gram positive bacterial cell wall constituents, such as peptidoglycans, lipoteichoic acid, and lipoproteins, as well being a receptor that reacts with endotoxic LPS of gram negative bacteria. TLR4 reacts with a major cell component of gram negative bacteria, as well as with harmful factors produced by gram positive bacteria (10-12).

Although the immunological etiology of recurrent otitis media has been studied many times, it is not clear when the innate immune system first reacts with pathogens invading the middle ear cavity. Using a mouse model in which the middle ears were infected with nontypeable *Haemophilus influenzae* (NTHi), TLR4 was shown to be important in the early accumulation of polymorphonuclear cells and the acceleration of function (13). Therefore, assessment of the expression of TLRs in the middle ear cavity in patients with recurrent OME is necessary to understand the process by which host immune reactions are induced and the process by which the innate immune system activates adaptive immunity.

In addition, several studies have shown that children with low serum immunoglobulin (Ig) A, IgG, and IgM concentrations during the initial stages of otitis media have a higher incidence of recurrent OME than those with lower serum Ig concentrations (14,15). Little is known, however, regarding the association between recurrence and the concentrations of immunoglobulin in the middle ear cavity. We therefore assayed the concentrations of immunoglobulins, which are as-

sociated with acquired immunity, the expression of TLRs, which are associated with innate immunity, and their relationship in patients with recurrent OME.

## MATERIALS AND METHODS

### Subjects

The study population consisted of 72 pediatric patients who visited the Department of Otorhinolaryngology at Kyung Hee University Hospital from March 2005 to August 2007 and underwent ventilation tube insertion for chronic OME. Of these, 31 children had been treated more than 4 times in the previous year or more than three times in the previous six months, without improvement (otitis-prone group) and 41 children had been treated 1~2 times in one year (non-otitis prone group). The use of samples and the purpose of experiment were explained to parents and guardians, and written informed consent was obtained. Children suspected of having head and neck anomalies, systemic disease, or congenital or acquired immunodeficiency were excluded.

### Enzyme-linked immunosorbent assay (ELISA)

All samples of middle ear effusion were stored at  $-80^{\circ}\text{C}$ , and their IgG, IgA, IgM concentrations were measured by ELISA. Briefly, 50  $\mu\text{l}$  of 1:100 goat anti-human IgG, anti-human IgA, and anti-human IgM (Bethyl Laboratories, Montgomery, Tex, USA) in coating buffer were placed in each well of a 96 well plate and incubated overnight at  $4^{\circ}\text{C}$ . The wells were washed 6 times, blocking antibody was added and 50  $\mu\text{l}$  of each sample was then added to each well, and the plates were incubated at room temperature for 3 hours. The wells were washed 6 times, and purified goat anti-human IgG, anti-human IgA, and anti-human IgM conjugated to horseradish peroxidase in PBS/Tween/BSA solution was added, and the plates were incubated at room temperature. The plates were washed 6 times, substrate solution (2,2'-AZINO-Bis (3-ethylbenzothiazoline-6-sulfonic acid)) was added, and the optical absorbance was measured at 450 nm (Bethyl, Montgomery, TX, USA).

### RNA extraction

Cultured cells were lysed by adding 1 ml RNA-Bee solution (Tel-test, Inc, Friendswood, TX, USA) to a 60 mm culture dish. Each was mixed with 0.2 times volumes of chloroform, kept on ice for 5 minutes, and centrifuged at  $12,000\times g$  for 15 minutes at  $4^{\circ}\text{C}$  (Eppendorf 5402, Hamburg, Germany). An

equal volume of isopropanol was added to each supernatant, and each was allowed to precipitate at room temperature for 10 minutes. Following centrifugation at 12,000×g for 5 minutes at 4°C, the supernatant was discarded, and each pellet was washed with 800 μl of 75% ethanol, centrifuged at 7,500×g for 5 minutes at 4°C, and dried in air for 10 minutes. Each pellet was resuspended in DEPC-water, and the RNA concentration of each sample was measured at 260 nm using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, England).

### Reverse transcription-polymerase chain reaction

To synthesize complementary deoxynucleotide acid (cDNA), 1 μg RNA was adjusted to 10 μl with DEPC-treated water, heated at 70°C for 5 minutes and kept on ice for 5 minutes. To each sample was added 2 μl 10x reaction buffer (100 mM Tris-HCl, pH9.0, 500 mM KCl, 1% Triton X-100), 4 μl 25 mM MgCl<sub>2</sub>, 2 μl 10 mM deoxynucleoside triphosphates (dNTPs), 0.5 μl (40 units/μl) ribonuclease (RNase) inhibitor, and 15 units AMV reverse transcriptase (Promega, Madison, WI, USA), after which the volume was adjusted to 20 μl and the samples were incubated at 42°C for 1 hour, 95°C for 5 minutes, and 4°C for 5 minutes.

The TLR2 and TLR4 primers are shown in Table I. Each PCR reaction consisted of 2 μl cDNA, 5 μl 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>), 4 μl 2.5 mM dNTPs, 20 pmol of sense and antisense primer, and 2 units Taq DNA polymerase (Takara, Shiga, Japan), adjusted to 50 μl with sterile distilled water. The amplification protocol consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles (25 for β-actin) of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by a final extension

at 72°C for 10 minutes (Biometra, Gottingen, Germany). A 10 μl aliquot of each PCR product was mixed with 2 μl 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% sucrose) and electrophoresed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide using 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 90 volts for 1 hour. Bands were assessed using the Gel Doc 1,000 gel documentation system (Bio-Rad, Hercules, CA, USA).

### Real-time PCR

To a 1.5 ml test tube was added 2.4 μl 25 mM MgCl<sub>2</sub>, DNA master SYBR green I containing 2 μl Lightcycler fast start enzyme, and 10 pmol sense and antisense primer, with the final volume adjusted to 18 μl with distilled water. To each was added 2 μl cDNA, and the samples were aliquoted into capillary tubes and centrifuged at 700×g for 5 seconds and placed in a Lightcycler amplifier (Roche Applied Science, Mannheim, Germany). The annealing temperature for TLR2, TLR4 and β-actin was 55°C, and depending on the size of PCR products, real-time PCR was performed at 25 bp/second. The crossing point of TLR2 or TLR4 with β-actin was applied to the formula,  $2^{-(TLR2/4-\beta\text{-actin})}$  and the relative amounts were quantitated.

### Statistic

Between group differences in immunoglobulin and TLR expression were compared using the Mann-Whitney U test, and the correlation between the expression level of TLR and immunoglobulin concentration was analyzed by Spearman correlation analysis. All statistical calculations were performed using SPSS 11.5 for Windows, and p values lower than 0.05 were considered statistically significant.

**Table I.** The real-time RT-Polymerase chain reaction primer used for detect TLR2 and TLR4 mRNA in otitis media effusion

gene	Sequence	Product size (bp)
TLR2	5'-GCCAAAGTCTTGATTGATTGG-3' 5'-TTGAAGTTCTCCAGCTCCTG-3'	347
TLR4	5'-TGGATACGTTTCCTTATAAG-3' 5'-GAAATGGAGGCCACCCCTTC-3'	507
β-actin	5'-CTTCTACAATGAGCTGCGTG-3' 5'-TCATGAGGTAGTCAGTCAGG-3'	305

TLR: Toll like receptor.

**Table II.** Concentrations of IgG, IgA and IgM in effusion fluid of the otitis prone and non-prone groups. All samples of middle ear effusion were stored at -80°C, and their IgG, IgA, IgM concentrations were measured by ELISA. Between group differences in immunoglobulin were compared using the Mann-Whitney U test

	Concentration of Ig in effusion fluid [Mean ± SD (ng/ml)]		p-value
	Otitis prone group	Non-prone group	
Ig G	3,160 ± 6,073	2,066 ± 2,168	0.300
Ig A	312 ± 211	377 ± 288	0.299
Ig M	301 ± 176	400 ± 554	0.347

## RESULTS

### Patients

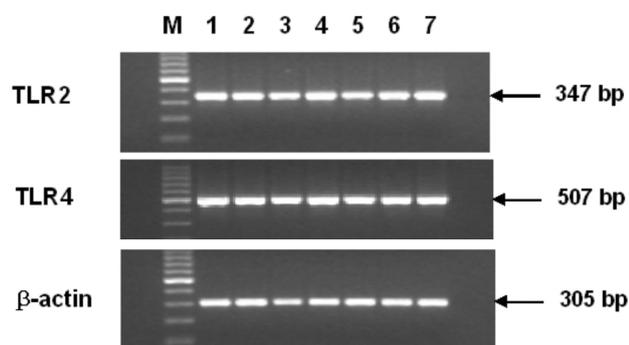
The otitis-prone group consisted of 31 children, 18 boys and 13 girls, of mean age 3.7 ( $\pm 2.2$ ) years. The non-otitis prone group consisted of 41 children, 24 boys and 17 girls, of mean age 4.8 ( $\pm 2.8$ ) years.

### Antibody in the effusion (Table II)

The concentrations of IgG, IgA, and IgM in the otitis prone group were 3,160 $\pm$ 6,073 ng/ml, 312 $\pm$ 211 ng/ml, and 301 $\pm$ 176 ng/ml, respectively. In the non-otitis prone group, the concentrations of IgG, IgA, and IgM were 2,066 $\pm$ 2,168 ng/ml, 377 $\pm$ 288 ng/ml, and 400 $\pm$ 554 ng/ml, respectively. None of these differences was statistically significant ( $p > 0.05$ ).

### Expression of TLR2 and TLR4 mRNA in the effusion (Fig. 1, Table III)

We observed expression of TLR2 and TLR4 mRNAs in the effusion fluid of both the otitis prone and non-otitis prone groups. The relative expression of TLR2 and TLR 4 in the otitis prone group were 0.130 $\pm$ 0.084 and 0.050 $\pm$ 0.037. In the non-otitis prone group, the relative expression of TLR2 and TLR4 were 0.179 $\pm$ 0.121 and 0.058 $\pm$ 0.033. In both groups, the level of expression of TLR2 was higher than that of TLR4. Although the level of expression of these two mRNAs differed in the two groups, the differences were not statistically significant ( $p > 0.05$ ).



**Figure 1.** Expression of TLR2, TLR4 mRNA in Otitis media effusion. mRNA expression was measured by RT-PCR and resolved on 2% agarose gel by electrophoresis. Lane 1, 100 bp DNA ladder; lane 2, normal PBL; lane 3-7, patients. TLR2, TLR4 and  $\beta$ -actin mRNA were expressed in all patients. TLR: Toll like receptor.

### Correlation of Ig concentrations and TLR mRNAs (Table IV)

We observed no correlations between the concentrations of IgG, IgM, and IgA and the expression levels of TLR2 and TLR4 mRNAs in either the otitis prone or the non-otitis prone group ( $p > 0.05$ ).

## DISCUSSION

OME is an inflammatory reaction caused by infection, in which effusion develops in the middle ear cavity. About 5% of children are otitis-prone, defined as the recurrence of otitis media more than 3 times in 6 months or more than 4 times per year (16). Analysis of the constituents of effusion fluid can provide important information, enhancing understanding of the immune response to infection in the middle ear and enabling differentiation of otitis-prone from non-otitis prone

**Table III.** Expression of TLR2 and TLR4 mRNA in effusion fluid of the otitis prone and non-prone groups. The expression in middle ear effusion of TLR2 and TLR4 mRNA, as determined by Real time-polymerase chain reaction (RT-PCR). The crossing point of TLR2 or TLR4 with  $\beta$ -actin was applied to the formula,  $2^{-CTLR2/4-\beta actin}$  and the relative amounts were quantitated. Between group differences in mRNA were compared using the Mann-Whitney U test

	Relative expression in effusion fluid (Mean $\pm$ SD)		p-value
	Otitis prone group	Non-prone group	
TLR 2	0.130 $\pm$ 0.084	0.179 $\pm$ 0.121	0.276
TLR 4	0.050 $\pm$ 0.037	0.058 $\pm$ 0.033	0.573

TLR: Toll like receptor.

**Table IV.** Correlation between expression of TLR 2, 4 and concentration of Ig G, A, M in effusion fluid. The correlation between the expression level of TLR and immunoglobulin concentration was analyzed by Spearman correlation analysis

		Prone group	Non-prone group
TLR 2	Ig G	$r = -0.038$ ( $p = 0.912$ )	$r = -0.098$ ( $p = 0.708$ )
	Ig A	$r = -0.088$ ( $p = 0.796$ )	$r = 0.073$ ( $p = 0.782$ )
	Ig M	$r = 0.043$ ( $p = 0.899$ )	$r = 0.010$ ( $p = 0.970$ )
TLR 4	Ig G	$r = 0.041$ ( $p = 0.906$ )	$r = -0.080$ ( $p = 0.760$ )
	Ig A	$r = -0.132$ ( $p = 0.669$ )	$r = -0.002$ ( $p = 0.992$ )
	Ig M	$r = 0.049$ ( $p = 0.887$ )	$r = -0.029$ ( $p = 0.911$ )

r: Spearman's correlation coefficient. TLR: Toll like receptor.

patients.

Human immune response to external antigens consists of innate and acquired immunity, which perform complementary actions. Innate immunity is not dependent on the clonal expansion of antigen (Ag) specific lymphocytes, and thus a long induction period is not required to trigger innate immune responses. In addition, the innate immune system recognizes only microbial non-self antigens and PAMPs that are commonly present in microorganisms. The pattern recognition receptor of innate immunity recognizes these PAMPs, following which it triggers the expression of cytokines and chemokines and initiates other *in vivo* defense mechanisms, including the production of reactive nitrogen and oxygen and anti-peptides (17). These characteristics differentiate innate from adaptive immune receptors, in that the binding of the latter (i.e. T cell receptors) depends on structural specificity, regardless of the origin of Ag. Moreover, after the innate immune receptors recognize PAMPs, CD80 and CD86 are induced on antigen presenting cells is induced, indicating that the innate immune system induces the response of the acquired immune system (18).

TLRs recognize the characteristic set of molecular patterns that have not been detected in normal vertebrate animals, thus distinguishing self and pathogens and having a major function in the initial host defense. To date, 11 types of TLR have been detected in humans. These receptors are expressed primarily on first line defense cells against infection, including dendritic cells, macrophages, neutrophils, and T and B cells (19). TLRs have extracellular domains rich in leucine, as well as Cys-rich area transmembrane domains (8). The cytoplasmic domains of TLRs have a structure similar to that of IL-1 receptor, referred to as the TIR area (18). One common feature of signal transduction mediated by TLR is the activation of NF- $\kappa$ B through the TIR domain. Upon TLR recognition of its ligand, it forms a complex with MyD88, which has the identical TIR domain (20). The C-terminal TIR domain of MyD88 binds to the TIR domain of TLR, and the N-terminal death domain of MyD88 binds to the Ser/Thr kinase domain of IRAK-1 or IRAK-2. IRAK-1, in turn, activates NIK by interacting with tumour necrosis factor receptor-associated factor 6 (TRAF6). NIK activation induces the phosphorylation of IKK, and IKK induces the phosphorylation of I $\kappa$ B, resulting in the activation of NF- $\kappa$ B. Activated NF- $\kappa$ B migrates to the nucleus, where it acts as a transcriptional factor (3,8) and induces the activation of cytokines or accessory molecules. Thus, NF- $\kappa$ B plays a role of a bridge be-

tween the innate and adaptive immune systems (9).

The area in the human body that contacts antigens or pathogens for the first time is the upper respiratory tract, which therefore plays an important role in immune function. Particularly, the epithelial area of the upper respiratory tract functions both as a barrier membrane as well as through its interaction with pathogens, thus activating both the innate and adaptive immune systems. The presence of TLR on the surfaces of the upper respiratory tract suggests that these receptors are involved in the specific recognition of pathogens (21,22). TLRs are also expressed on intestinal epithelial cells and epithelial cells in the oral cavity, thus demonstrating their role as sentinels in areas in contact with the external environment (22,23). In addition, TLR2 expression has been detected on epithelial cells in the middle ear cavity in patients with otitis media induced by *Haemophilus influenzae* (24).

The immune system of children may play a causative role in recurrent otitis media induced by *H. influenzae* (25). The immune systems of non-otitis prone children recognize antigens such as P6, and produce antibodies to all strains of *H. influenzae*, thus preventing disease recurrence. In contrast, the immune systems of otitis prone children recognize antigens of specific strains such as P2 but do not recognize P6. Thus, these children cannot produce antibodies against all strains and become susceptible to infection by pathogens of other strains. These findings suggest that immune reactions at the time of initial pathogen infection are important in the onset of recurrent otitis media. Particularly, although immune cells such as T and B cells are relatively scarce in the middle ear cavity, this cavity is pathogen free, suggesting that the middle ear cavity is protected by the innate rather than the adaptive immune system (26).

Recently, Emonts et al found that TLR4 299 A/A genotype were associated with otitis-prone condition. Finally, they suggested that variation in innate immunoresponse genes in the promoter sequences might result in altered cytokine production that leads to altered inflammatory responses and, hence, contributed to otitis-prone condition (27).

On the basis of these findings, we hypothesized that innate immunity induced after first pathogen invasion of the middle ear cavity and the process of acquired immunity may be important in the characterization of the onset of recurrent otitis media. Thus, expression of TLR may modulate not only the induction of innate immune reactions but future susceptibility to local infection. Furthermore, a deficiency in immune reactions caused by the reduction of TLR expression may influ-

ence the process occurring at the onset of recurrent otitis media.

We found, however, that although the expression levels of TLR2 and TLR4 mRNA tended to be lower in the otitis-prone group, they did not differ significantly from those in the non-otitis prone group. Therefore, the reduced innate and acquired immunity against pathogens was not due to the reduced expression of TLRs.

When we compared the concentrations of Igs in the effusion fluid of the otitis-prone and non-otitis prone groups, we found that IgG, IgM, and IgA were slightly higher in the otitis prone group, but the concentrations in the two groups did not differ significantly. Serum concentrations of Igs in patients with recurrent otitis media were lower than in a general control group or in patients with non-recurrent otitis media, with some of these differences being significant (14,28). We did not correct concentrations according to age and gender, but our results could not confirm findings showing that Ig concentrations in serum and exudates had clinical significance in patients with recurrent otitis media (29). In addition, since the normal Ig concentrations in exudates has not been established, we could not determine whether any of our patients had an immune deficiency. The higher IgG concentration in the otitis prone group may have been due to the stimulation of antibody production by repeated infection.

Recent studies have revealed the process by which external antigens stimulate TLRs, which activate the proliferation and differentiation of B cells through stimulation of the B cell receptor, T helper cells, and cytokines. The thymus-dependent antibody reaction is generally induced by two types of signals, the B cell receptor and T-helper cells. If these signals are absent or low, however, TLRs can stimulate B cells and control the immunoglobulin isotype switch (30). We therefore hypothesized that there may be a relationship between Ig concentrations and the expression of TLR2 and TLR4 in the middle ear cavity, both in the prone and the non-prone groups. However, in all cases, no significant correlation was observed.

In conclusion, despite our hypothesis, that the levels of expression of TLR2 and TLR4 mRNA and Ig concentrations would be lower in patients with recurrent than non-recurrent OME, we found that the differences were not significant. We also found no correlation between the level of expression of TLR mRNA and immunoglobulin concentration. For ethical reasons, we could not harvest the middle ear mucosa from these patients and we therefore performed experiments on

exudates. Although some mucosal epithelial cells or inflammatory cells may have been present in the retained fluid, the presence of TLR mRNAs and Igs was not due to these cells but rather the cells were present in the fluid that developed after inflammatory reactions.

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