

Title page

Title:

Expression of Toll-like Receptors in Chronic Otitis Media and Cholesteatoma

Authors:

Haruka Hirai, Shin Kariya, Mitsuhiro Okano, Kunihiro Fukushima, Yuko Kataoka, Yukihide Maeda, Kazunori Nishizaki

Authors' affiliations and addresses:

Department of Otolaryngology-Head and Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Corresponding author:

Shin Kariya, MD, PhD

2-5-1, Shikata-cho, Kita-ku, Okayama 700-8558, Japan

E-mail: [skariya@cc.okayama-u.ac.jp](mailto:skariya@cc.okayama-u.ac.jp)

TEL: +81-862357307

FAX: +81-862357308

**Abstract:**

**Objective:** Otitis media is one of the most common infectious diseases, especially in young children. Multiple factors affect the onset or development of otitis media. Human toll-like receptors recognize associated patterns and play a critical role in innate immune mechanisms. Toll-like receptors are considered to be important factors for clearance of infection and resolution of inflammation in otitis media. The purpose of this study was to evaluate the histological expression of toll-like receptor 2, which recognizes many kinds of pathogen-associated molecular patterns, and toll-like receptor 4, which recognizes lipopolysaccharide on Gram-negative bacteria, in tissue samples from patients with chronic otitis media and middle ear cholesteatoma.

**Methods:** Human middle ear tissue samples from 12 patients with chronic otitis media (n=7) and acquired middle ear cholesteatoma (n=5) were examined. Normal control middle ear samples without any inflammation were also included (n=7). The expressions of toll-like receptors 2 and 4 in middle ear tissues were examined immunohistochemically.

**Results:** Only one normal control middle ear sample showed weak expression of toll-like receptor 2, and toll-like receptor 4 was not observed in all control samples. On the other hand, both toll-like receptors 2 and 4 were markedly expressed in chronic otitis media and cholesteatoma. There was a significant difference between chronic otitis media and normal controls in the expressions of both toll-like receptors. Significant up-regulation of toll-like receptors 2 and 4 was observed in cholesteatoma as compared with control samples.

**Conclusions:** Toll-like receptors 2 and 4 were strongly expressed in chronic otitis media and middle ear cholesteatoma. These findings suggest that toll-like receptors may play a principal role in human chronic otitis media and cholesteatoma.

**Keywords:**

Otitis, ear, infection, innate immunity

## Introduction:

Toll-like receptors are membrane proteins that play a crucial role in the induction and activation of innate immunity in the course of infection. At least ten subtypes of toll-like receptors have already been identified in humans, and they are considered to be involved in the recognition of pathogen-associated molecule patterns in the innate immune system [1]. Toll-like receptor 4 (TLR-4) is one of the toll-like receptors recognizing toxic pneumolysin ligand produced by Gram-positive bacteria, as well as binding to lipopolysaccharide. Lipopolysaccharide is the major component of Gram-negative bacteria and is frequently detected in otitis media [2, 3]. Toll-like receptor 2 (TLR-2) recognizes many kinds of pathogen-associated molecular patterns.

Recent studies have shown that expression of toll-like receptors was observed in middle ear samples in acquired cholesteatoma and otitis media with effusions [1, 4]. To the best of our knowledge, the comparison of toll-like receptor expressions between chronic otitis media and normal controls in human middle ear tissue has not been reported. The purpose of this study was to show the presence and localization of TLR-2 and TLR-4 in middle ear samples in patients with chronic otitis media, patients with cholesteatoma, and normal controls.

## Materials and methods:

### Samples:

Middle ear tissue samples were obtained from 7 patients with chronic otitis media (mean age  $\pm$  standard deviation (SD),  $67.1 \pm 2.4$  years: range, 64-70 years) and from 5 patients with middle ear cholesteatoma (mean age  $\pm$  SD,  $36.0 \pm 27.0$  years: range, 6-63 years).

The control middle ear tissue samples were collected from 7 patients without any middle ear inflammation undergoing cochlear implant surgery (mean age  $\pm$  SD,  $38.0 \pm 22.7$  years: range, 5-61 years).

The expressions of TLR-2 and TLR-4 in middle ear tissue were examined immunohistochemically. This study was approved by the Institutional Review Board of Okayama University (IRB approval number, RINRI-1435) and was in compliance with the Declaration of Helsinki.

### Immunohistochemistry:

Paraffin-embedded samples were sectioned at a thickness of 4  $\mu$ m. Sections were deparaffinized, rehydrated, and pretreated using Liberate Antibody Binding Solution (COSMO BIO Co., Ltd., Tokyo, Japan) for antigen retrieval. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide ( $H_2O_2$ ), and nonspecific protein binding was blocked with skim milk. The tissue sections were then incubated with rabbit anti-toll-like receptor 2 or 4 polyclonal antibody (TLR-2, ab24192; TLR-4, ab13556; Abcam Inc., Cambridge, UK) overnight at 4°C. For visualization, the LSAB™2 kit, the

StreptABCComplex/HRP kit, and diaminobenzidine substrate (DAKO, Glostrup, Denmark) were used according to the manufacturer's instructions.

The reaction was assessed by blinded investigators under light microscopy according to the method of Szczepański [4]. Briefly, the rating score was classified as: (-), no positive reaction; (+), 1-10 positive cells; (++) , 11-100 positive cells; and (+++), over 100 positive cells per high power field (×400).

#### Statistical analysis:

For statistical analysis, a Chi-Square test was performed at a significance level of  $p < 0.05$  using SPSS (IBM, New York, NY, USA).

#### Results:

TLR-2 and TLR-4 showed similar expressions. The middle ear samples from control subjects showed no expression of TLR-4. Both TLR-2 and TLR-4 were expressed in the middle ear mucosa and granulation tissue in patients with chronic otitis media and cholesteatoma. [Fig. 1] Positive immunostaining for TLR-4 was observed in mucosal epithelial cells, infiltrating inflammatory cells, and macrophages. Positive immunostaining for TLR-2 was also observed in mucosal epithelial cells and infiltrating inflammatory cells. [Fig. 2]

The expression of TLR-2 in chronic otitis media was (-) in 1 case, (+) in 2 cases, (++) in 2 cases, and (+++) in 2 cases. The cholesteatoma samples showed (-) in 0 case, (+) in 2 cases, (++) in 2 cases, and (+++) in 1 case. The control samples showed (-) in 6 cases, (+) in 1 case, (++) in 0 cases, and (+++) in 0 cases. The immunohistochemical staining score of TLR-2 was significantly higher in chronic otitis media and cholesteatoma than in control samples (chronic otitis media,  $p = 0.048$ ; cholesteatoma,  $p = 0.026$ ).

The expression of TLR-4 in chronic otitis media was (-) in 0 case, (+) in 4 cases, (++) in 2 cases, and (+++) in 1 case. The cholesteatoma samples showed (-) in 0 cases, (+) in 0 cases, (++) in 3 cases, and (+++) in 2 cases. The control samples showed (-) in all 7 cases. Significant expression of TLR-4 was observed both in chronic otitis media and cholesteatoma as compared with normal control samples (chronic otitis media,  $p = 0.003$ ; cholesteatoma,  $p = 0.002$ ).

#### Discussion:

Toll-like receptors are members of the pattern-recognition receptor family that detects specific molecules associated with microbial pathogens. Toll-like receptors are key regulators of both innate and adaptive immune responses and comprise a family of germ line-encoded transmembrane receptors. The activations of toll-like receptors lead to the mobilization of other innate immune molecules, such as cytokines, chemokines, and interferons, as well as proteases, defensins, collectins, lysozyme, lactoferrin, and other antimicrobial intermediates [5]. These receptors recognize conserved microbial structures

called pathogen-associated molecular patterns, which are invariant within a given class of microorganism. Many pathogen-associated molecular patterns have now been recognized and their respective toll-like receptors identified; these include peptidoglycan (which binds to TLR2), synthetic double-stranded RNA (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), and CpG DNA motifs associated with bacterial DNA (TLR9) [6]. Although the participations of toll-like receptors are necessary to defend humans against microbial invasion, the abnormal responses of toll-like receptors also cause the development of many diseases [7, 8].

Lipopolysaccharide, a major component of the cell wall of Gram-negative bacteria, is a potent immune stimulator. An experimental animal study showed that injection of lipopolysaccharide into the middle ear can mimic the pathological changes of otitis media: mucosal inflammation, leukocytosis, edema, middle ear pressure abnormalities, and an infiltrate of macrophages into the subepithelial space [9 - 11].

Non-typeable *Haemophilus influenzae* is one of the most prominent bacterial pathogens of human otitis media and activates the TLR-4 signaling pathways [5]. Toll-like receptors are considered important factors in the pathogenesis of otitis media, but the role of toll-like receptors in chronic otitis media is controversial. The activation of toll-like receptors induces various transcription factors, including NF- $\kappa$ B, and subsequently results in high expression of proinflammatory cytokines such as IL-1 and TNF- $\alpha$  [4, 5, 12]. Toll-like receptor-deficient mice show reduced bacterial clearance after middle ear infection with bacterial pathogens [5]. In contrast, overexpression of toll-like receptors is observed in severe infection, and a TLR-4 antagonist (E-5564; Eisai Co., Ltd., Tokyo, Japan) is expected to be a novel remedy for sepsis [12 - 16].

In the present study, TLR-2 and TLR-4 were strongly expressed in the mucosal epithelium and infiltrating inflammatory cells both in chronic otitis media and middle ear cholesteatoma. These findings suggest that TLR-2 and TLR-4 may play principal roles in human chronic otitis media and middle ear cholesteatoma.

A recent study reported that TLR-2 and TLR-4 might play a different role in the pathophysiology of chronic otitis media and cholesteatoma [17]. The limitations of our preliminary study are small sample size and lack of age-matched normal controls. Further studies are needed to dissect the role of toll-like receptors in pathogenesis of middle ear diseases, especially in children.

Conflict of interest statement:

All authors disclose no financial and personal relationship with other people or organization that could inappropriately influence the work.

Acknowledgement:

This work was supported by Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan.

Reference:

- [1] Lee YC, Kim C, Shim JS, Byun JY, Park MS, Cha CI, et al. Toll-like receptors 2 and 4 and their mutations in patients with otitis media and middle ear effusion. *Clin Exp Otorhinolaryngol* 2008;1:189-195.
- [2] Kariya S, Okano M, Fukushima K, Nomiya S, Kataoka Y, Nomiya R, et al. Expression of inflammatory mediators in the otitis media induced by *Helicobacter pylori* antigen in mice. *Clin Exp Immunol* 2008;154:134-140. Erratum in *Clin Exp Immunol* 2008;154:432.
- [3] Kariya S, Okano M, Aoji K, Kosaka M, Chikumoto E, Hattori H, et al. Role of macrophage migration inhibitory factor in otitis media with effusion in adults. *Clin Diagn Lab Immunol* 2003;10:417-422.
- [4] Szczepański M, Szyfter W, Jenek R, Wróbel M, Lisewska IM, Zeromski J. Toll-like receptors 2, 3 and 4 (TLR-2, TLR-3 and TLR-4) are expressed in the microenvironment of human acquired cholesteatoma. *Eur Arch Otorhinolaryngol* 2006;263:603-607.
- [5] Leichtle A, Lai Y, Wollenberg B, Wasserman SI, Ryan AF. Innate signaling in otitis media: pathogenesis and recovery. *Curr Allergy Asthma Rep* 2011;11:78-84.
- [6] Kopp E, Medzhitov R. Recognition of microbial infection by Toll-like receptors. *Curr Opin Immunol* 2003;15:396-401.
- [7] Prince LR, Whyte MK, Sabroe I, Parker LC. The role of TLRs in neutrophil activation. *Curr Opin Pharmacol* 2011;11:397-403.
- [8] Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. *Int Immunopharmacol* 2007;7:1271-1285.
- [9] DeMaria TF, Apicella MA, Nichols WA, Leake ER. Evaluation of the virulence of nontypeable *Haemophilus influenzae* lipooligosaccharide htrB and rfaD mutants in the chinchilla model of otitis media. *Infect Immun* 1997;65:4431-4435.
- [10] Kariya S, Schachern PA, Cureoglu S, Tsuprun V, Okano M, Nishizaki K, et al. Up-regulation of macrophage migration inhibitory factor induced by endotoxin in experimental otitis media with effusion in mice. *Acta Otolaryngol* 2008;128:750-755.
- [11] Kariya S, Okano M, Higaki T, Makihara S, Haruna T, Eguchi M, et al. Neutralizing antibody against granulocyte/macrophage colony-stimulating factor inhibits otitis media.

Laryngoscope. 2012 Nov 21. doi: 10.1002/lary.23795. [Epub ahead of print]

[12] Mills KH. TLR-dependent T cell activation in autoimmunity. *Nat Rev Immunol* 2011;11:807-822.

[13] Boyd JH. Toll-Like Receptors and Opportunities for New Sepsis Therapeutics. *Curr Infect Dis Rep* 2012;14:455-461.

[14] Mullarkey M, Rose JR, Bristol J, Kawata T, Kimura A, Kobayashi S, et al. Inhibition of endotoxin response by e5564, a novel Toll-like receptor 4-directed endotoxin antagonist. *J Pharmacol Exp Ther* 2003;304:1093-1102.

[15] Rossignol DP, Lynn M. TLR4 antagonists for endotoxemia and beyond. *Curr Opin Investig Drugs* 2005;6:496-502.

[16] Kalil AC, LaRosa SP, Gogate J, Lynn M, Opal SM; Eritoran Sepsis Study Group. Influence of severity of illness on the effects of eritoran tetrasodium (E5564) and on other therapies for severe sepsis. *Shock* 2011;36:327-331.

[17] Si Y, Zhang ZG, Huang CY, He JF, Feng LQ, Chen YB, Chen T, Huang X. [Differential expression of toll-like receptors in chronic suppurative otitis media and cholesteatoma]. *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* 2012;47:388-393. [Article in Chinese]

Figure legend:

Fig 1: The expressions of toll-like receptors 2 and 4 in patients with chronic otitis media and with cholesteatoma. Positive immunostaining of toll-like receptors (arrow) is observed both in chronic otitis media and in cholesteatoma. The normal control middle ear subjects show no positive cells. (TLR-2, toll-like receptor 2; TLR-4, toll-like receptor 4; Bar, 50  $\mu$ m)

Fig 2: Positive immunohistochemical staining for toll-like receptors is observed in middle ear mucosa of chronic otitis media and cholesteatoma epithelium. (A) toll-like receptor 2 in cholesteatoma, (B) toll-like receptor 2 in chronic otitis media, (C) toll-like receptor 4 in cholesteatoma, (D) toll-like receptor 4 in chronic otitis media. (Bar, 50  $\mu$ m)

Fig.1

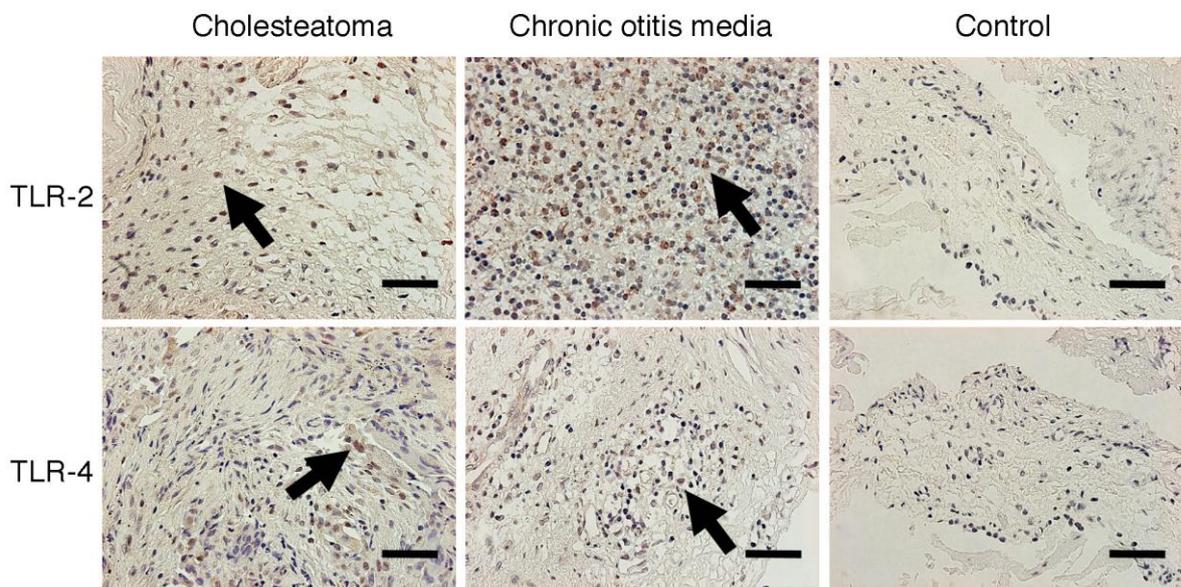
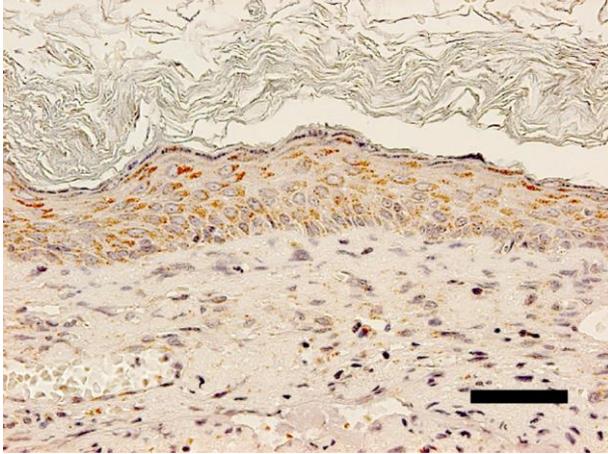
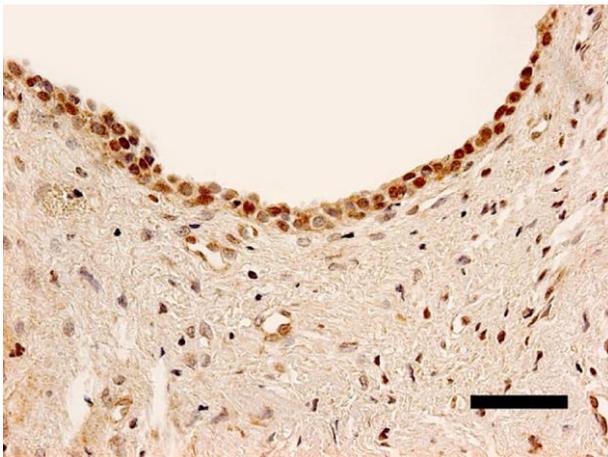


Fig.2

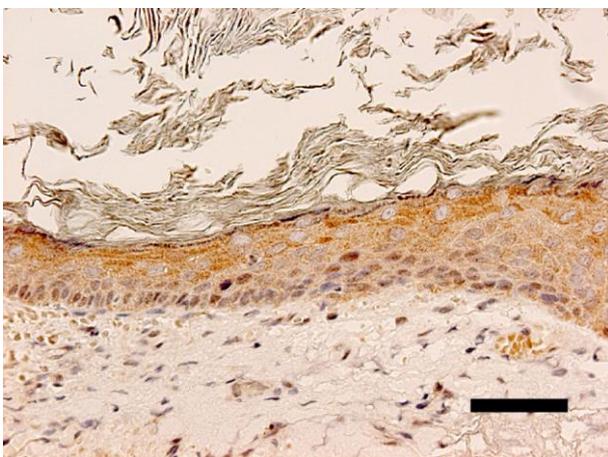
A



B



C



D

