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Original Article

Comparison of Endoplasmic Reticulum Stress Messenger Ribonucleic Acid Expression Between Chronic Otitis Media With and Without Cholesteatoma

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BACKGROUND: We evaluated and compared the role of endoplasmic reticulum stress in chronic otitis media with cholesteatoma and chronic otitis media without cholesteatoma.

METHODS: The messenger ribonucleic acid expression of endoplasmic reticulum stress was measured and compared between chronic otitis media with cholesteatoma and chronic otitis media without cholesteatoma according to the presence or absence of bacteria, type of hearing loss, ossicle destruction, and facial canal dehiscence.

RESULTS: The expression of immunoglobulin heavy chain-binding protein messenger ribonucleic acid was higher in the chronic otitis media without cholesteatoma group than in the chronic otitis media with cholesteatoma group, and Protein kinase RNA (PKR)-like endoplasmic reticulum kinase and activating transcription factor 6 messenger ribonucleic acid expression were higher in the chronic otitis media with cholesteatoma group than in the chronic otitis media without cholesteatoma group.

CONCLUSION: Endoplasmic reticulum stress messenger ribonucleic acids were expressed in both chronic otitis media with cholesteatoma and chronic otitis media without cholesteatoma. The levels of expression of endoplasmic reticulum stress messenger ribonucleic acids differed according to clinical features, suggesting that different endoplasmic reticulum stress pathways are involved in the pathophysiology of different types of chronic otitis media.

KEYWORDS: Cholesteatoma, Chronic otitis media, ER stress

INTRODUCTION

Otitis media refers to an inflammatory disease occurring within the middle ear cavity. After inflammatory changes occur in the middle ear cavity, other factors such as host immune response and biochemical factors mediate the course of the disease and may affect recurrence and chronicity.[1,2] Chronic otitis media (COM) is defined as otitis media persisting for more than 3 months. Chronic otitis media is divided into subtypes with or without cholesteatoma. Chronic otitis media without cholesteatoma (COMC) results in perforation of the tympanic membrane, and chronic otitis media with cholesteatoma (CholeOM) shows cholesteatoma irrespective of perforation of the tympanic membrane.[3,4] The COMC and CholeOM are characterized by differing pathophysiology of middle ear inflammation and clinical manifestations.

The endoplasmic reticulum (ER) is an important organelle responsible for protein and lipid synthesis, calcium storage, and signal transduction.[5,6] Cells produce proteins to perform their functions, and this production is adjusted selectively and finely according

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to the environment. The ER is responsible for the "quality control" of proteins, which are transported to the appropriate location after undergoing structure formation and transformation. Endoplasmic reticulum stress, an increase in unfolded proteins in the ER, is caused by alterations in the homeostatic network of proteins.[7,8]

When proteins rapidly flow into the ER and the homeostasis of ER is disturbed by physiological and pathological causes, protein folding may cause misfolding or unfolding, because the ER has limited protein-folding capacity. Unfolded proteins cause increased ER stress due to excessive increases in protein synthesis, inhibition of protein maturation, dysfunction of chaperone molecules, overexpression of abnormal proteins associated with disease, reduction of calcium concentration in the ER, oxidative stress, and viral infection. Cells are part of a series to address ER stresses, called the unfolded protein response (UPR).[9,10] In general, the UPR of eukaryotes is mediated with sensor proteins located on the membrane of the ER, such as IRE1α/β (inositol-requiring endonuclease), PERK (PKR-like ER kinase), and ATF6a/beta (activating transcription factor 6).[11] Spliced XBP1 (XBP1s) is involved in overall ER stress as a signaling substance of IRE1, CHOP (C/EBP-homologous protein) as a final stage of signaling of PERK, and BiP (immunoglobulin heavy chain-binding protein) as a chaperone.

In recent studies, chronic ER stress has been associated with degenerative neurological diseases, cancer, metabolic diseases, and inflammatory diseases. The role of ER stress in various diseases, especially inflammatory diseases, has not yet been investigated in association with COM with or without cholesteatoma, and there have been no studies in patients with COM. Endoplasmic reticulum stress may be involved in the progression of inflammation and disease in COM. The expression of ER stress may change depending on the presence of cholesteatoma and the pathophysiology and clinical features of otitis media.

The purpose of this study was to evaluate the expression of ER stress according to the presence of cholesteatoma and clinical features.

MATERIAL AND METHODS

Study Design

Patients diagnosed with COM who visited Kyung Hee University Hospital from January 2015 to December 2019 were included. Granulation tissue or cholesteatoma was collected either intraoperatively or at an outpatient clinic after adequate irrigation of the external auditory canal. Patients were excluded if sufficient specimens could not be obtained, specimens were contaminated, or they did not consent to the study. Granulation tissue was obtained from 34 patients with COM, and cholesteatoma samples were obtained from 23 patients with COM. All patients involved in this study provided written informed consent. Granulation tissue from 34 patients with COM and cholesteatoma from 23 patients with COM were sampled. Specimens were used only when the patient fully understood the purpose of the experiment and then voluntarily agreed to participate. Specimens collected in sterilized tubes were frozen in liquid nitrogen and stored at −70°C until the experiment. Clinical records were retrospectively reviewed for age, sex, date of onset, affected side, microbial culture results, audiologic results, and operation findings. Sensorineural hearing loss was identified if the bone conduction threshold was more than 30 dB and the air-bone gap was less than 10 dB, while the conductive hearing loss was identified if the air-bone gap was more than 10 dB and the bone conduction threshold was less than 25 dB.[9] The study protocol was approved by the Clinical Research Ethics Committee of Kyung Hee University Hospital (Approved No: IRB 2017-12-030 & 2020-05-080). Student's *t*-test and Mann–Whitney *U*-test were used by IBM Statistical Package for the Social Sciences version 20.0 (IBM SPSS Corp.; Armonk, NY, USA). *P*-values less than .05 were considered significant.

Ribonucleic Acid Extraction and Real-Time Polymerase Chain Reaction

Total ribonucleic acid (RNA) was purified from homogenized tissue samples using TRIzol reagent. First-strand complementary deoxyribonucleic acid (cDNA) was synthesized from 1 μg aliquots of total RNA using a reverse transcription system with random hexamers (Promega, Madison, Wis, USA). Primer sequences for amplification are shown in Table 1. Real-time polymerase chain reaction (PCR) was performed on a StepOnePlus real-time PCR system with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif, USA). Each reaction mixture contained 2 μL of cDNA, 10 μL of Power SYBR Green PCR Master Mix, 2 μL of primer, and 7 μL of PCR-grade water. The amplification protocol consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. The crossing points of the target genes with β-actin were calculated using the formula $2^{-(\text{target gene} - \beta\text{-actin})}$, and relative amounts were quantified.[12]

RESULTS

Clinical Data

There were no significant between-group differences in age, sex, duration of disease, or left- and right-sided lesions ($P > .05$ each). The reoperation rate was higher in the CholeOM than in the COMC group. There were no significant differences in facial canal dehiscence or destruction between the 2 groups ($P > .05$), but the destruction of the

Table 1. Primers for Real-Time Polymerase Chain Reaction

Name	Direction	Primer Sequences	Length (bp)
β-actin	Forward	5′-GCGAGAAGATGACCCAGATC-3′	77
	Reverse	5′-GGATAGCACAGCCTGGATAG-3′	
CHOP	Forward	5′-GTACCTATGTTTCACCTCCTGG-3′	150
	Reverse	5′-TGGAATCTGGAGAGTGAGGG-3′	
sXBP1	Forward	5′-TGGATTCTGGCGGTATTGAC-3′	146
	Reverse	5′-TCCTTCTGGGTAGACCTCTG-3′	
ATF6	Forward	5′-CCTGTCCTACAAAGTACCATGAG-3′	148
	Reverse	5′-CCTTTAATCTCGCCTCTAACCC-3′	
BiP	Forward	5′-CCTGGGTGGCGGAACCTTCGATGTG-3′	358
	Reverse	5′-CTGGACGGGCTTCATAGTAGACCGG-3′	
IRE1α	Forward	5′-GCGAACAGAATACACCATCAC-3′	147
	Reverse	5′-ACCAGCCCATCACCATTG-3′	
PERK	Forward	5′-GAACCAGACGATGAGACAGAG-3′	150
	Reverse	5′-GGATGACACCAAGGAACCG-3′	

ATF6, activating transcription factor 6; BiP, immunoglobulin heavy chain-binding protein; CHOP, C/EBP-homologous protein; IRE1α, inositol-requiring enzyme 1α; PERK, PKR-like endoplasmic reticulum kinase; sXBP1, X-box binding protein 1.

Table 2. Demographic and Clinical Characteristics of Patients with COMC and CholeOM

	COMC (n = 34)	CholeOM (n = 23)	*P*
Demographics			
Age, years, mean ± SD	55.73 ± 12.43	49.65 ± 16.95	.124
Sex, male:female, n (%)	13:21 (38.2:61.8)	12:11 (52.2:47.8)	.415
Disease onset, months	10.12 ± 10.91	11.39 ± 12.04	.688
Affected side, Rt:Lt:Both, n (%)	17:17:0 (50.0:50.0:0)	13:10:0 (56.5:43.5:0)	.788
Otorrhea positive, n (%)	26 (76.5)	15 (65.2)	.383
Revision surgery, n (%)	2 (5.9)	7 (30.4)	.023*
Audiologic configuration			
PTA (AC), dB, mean ± SD	51.22 ± 19.27	53.15 ± 22.15	.730
PTA (BC), dB, mean ± SD	28.30 ± 15.41	24.05 ± 17.39	.340
Hearing loss type			
Conductive hearing loss, n (%)	20 (58.8)	17 (73.9)	.415
Sensorineural hearing loss, n (%)	11 (32.4)	5 (21.7)	.558
Normal, n (%)	3 (8.8)	1 (4.2)	.514
Surgical finding			
Ossicle destruction, n (%)	11 (32.4)	19 (82.6)	<.001*
Facial canal dehiscence, n (%)	14 (41.2)	13 (56.5)	.290

AC, air conduction; BC, bone conduction; CholeOM, chronic otitis media with cholesteatoma; COMC, chronic otitis media without cholesteatoma; PTA, pure tone audiometry.
*$P < .05$.

ossicles was more frequent in the CholeOM group ($P < .05$). Hearing tests revealed no significant differences in the degrees of hearing loss or in the numbers of patients with conductive hearing loss and sensorineural hearing loss ($P > .05$) (Table 2).

Expression of Endoplasmic Reticulum Stress Messenger Ribonucleic Acid

Expression of BiP mRNA was higher in the COMC group than in the CholeOM group, whereas the expression levels of PERK and ATF6 mRNA were higher in the CholeOM group ($P < .05$) (Figure 1). In patients with sensorineural hearing loss, there was no difference in expression of ER stress mRNA between the 2 groups ($P > .05$). However, in patients with conductive hearing loss, the levels of PERK, ATF6, and BiP mRNAs were significantly higher in the CholeOM than in the COMC group ($P < .05$) (Figure 2). The expression of ER stress mRNA did not differ significantly between groups ($P > .05$), although there was more ossicular destruction in the CholeOM than in the COMC group (Figure 3). Facial canal dehiscence and destruction did not differ significantly in the 2 groups. Moreover, ER stress and mRNA expression did not differ in these 2 groups, even in the presence of facial canal dehiscence or destruction ($P > .05$) (Figure 4).

DISCUSSION

Endoplasmic reticulum stress mRNA expression was compared in different surgical samples. The formation of granulation tissue is the most common finding in COMC. Granulation tissues are formed by inflammation of the middle ear epithelium and changes in the epithelial cells and subcutaneous tissues of the middle ear mucosa. In the acute phase and subacute phase of otitis media, cellular components are abundant, but in the chronic phase, fibrous tissue is abundant.[13] Cholesteatoma is a phenomenon in which keratinized squamous epithelium grows in the middle ear, mastoid, and vertebral bodies. Chronic otitis media with cholesteatoma is a disease in which keratin accumulates into the middle ear cavity of the mucous membrane and that involves significant disease progression between COM with papillomas and COM with no papules. For these reasons, clinical features are different between CholeOM and COMC. Keratin accumulation, along with desquamation of the epidermis, leads to the destruction of the surrounding tissue.[14,15] In this study, the expression of BiP mRNA was high, and expressions of PERK and ATF6 mRNA were higher in the CholeOM group than in the COMC group. PKR-like endoplasmic reticulum kinase is a type I transmembrane

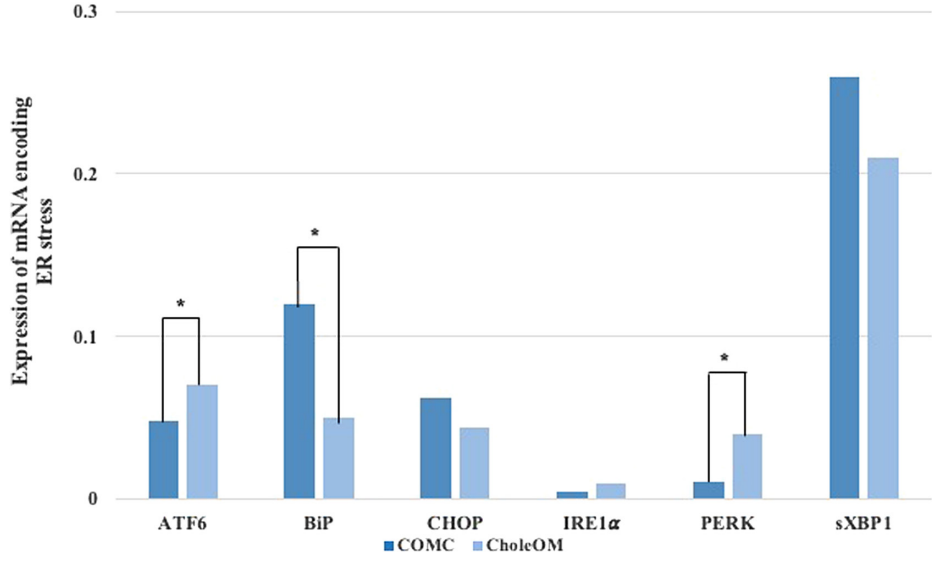

Figure 1. Expression of mRNA encoding ER stress in COMC and CholeOM. CholeOM, chronic otitis media with cholesteatoma; COMC, chronic otitis media without cholesteatoma; ER, endoplasmic reticulum; mRNA, messenger ribonucleic acid.

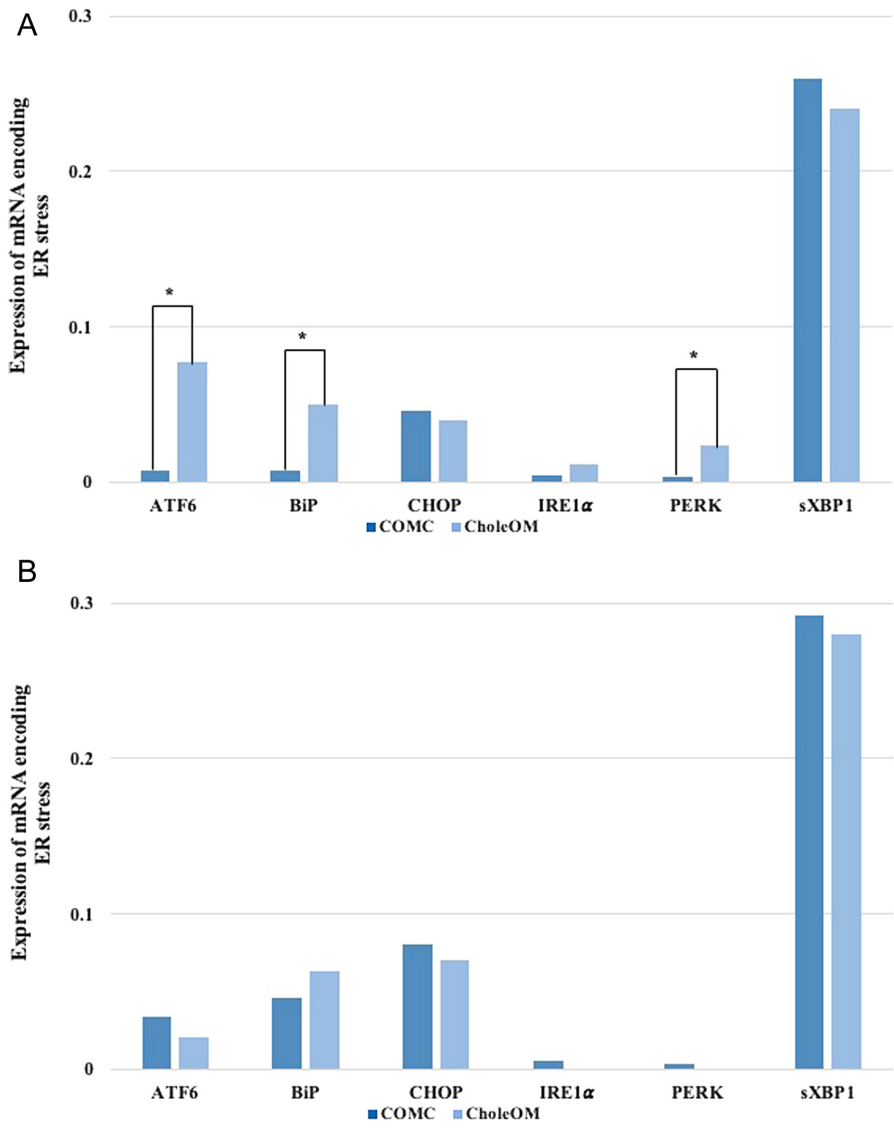

Figure 2. Expression of mRNA encoding ER stress in patients with (A) conductive hearing loss and (B) sensorineural hearing loss. ER, endoplasmic reticulum; mRNA, messenger ribonucleic acid.

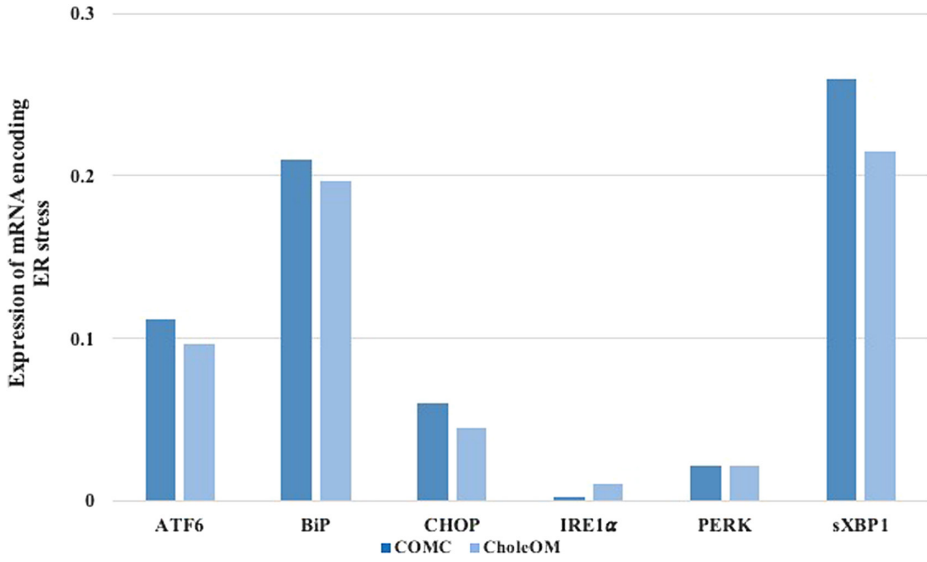

Figure 3. Expression of mRNA encoding ER stress in patients with ossicle destruction. ER, endoplasmic reticulum; mRNA, messenger ribonucleic acid.

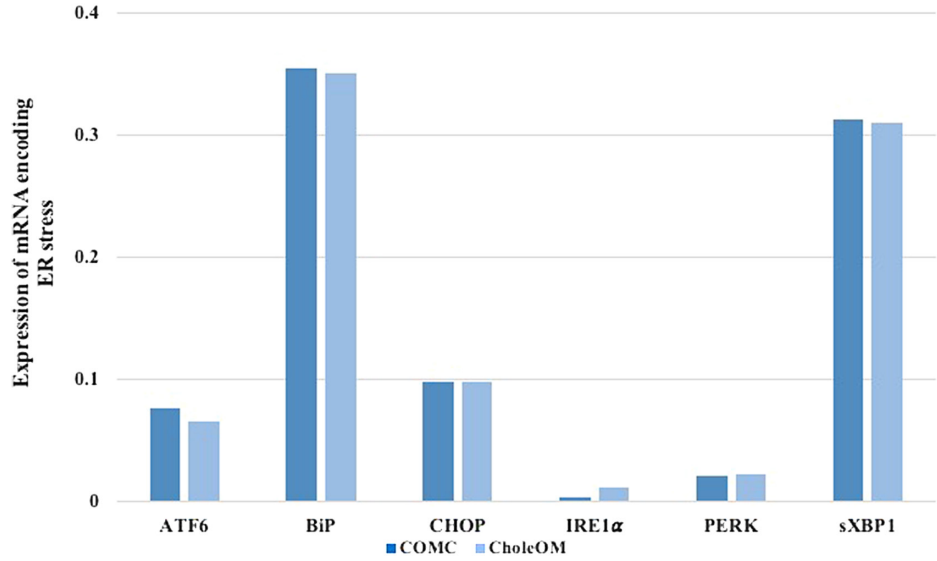

Figure 4. Expression of mRNA encoding ER stress in patients with facial canal dehiscence. ER, endoplasmic reticulum; mRNA, messenger ribonucleic acid.

protein kinase that is activated through autophosphorylation after increased stress in the ER and is known to inhibit the general protein translation process by phosphorylating serine 51 of the eukaryotic initiation factor 2 alpha subunit, a type II transmembrane protein in the membrane of the ER. PKR-like endoplasmic reticulum kinase increases the transcription of chaperone molecules and ER-related degradation-related genes. In this study, we found that factors related to translational attenuation and transcriptional induction, rather than ER-associated degradation, were more significant in the CholeOM group than in the COMC group.

Otorrhea is the most common symptom in patients with COM. Most COM is accompanied by intermittent irritation, which may be persistent in acute otitis media with acute infection. In other studies, the incidence of otorrhea in CholeOM is lower than in COMC, and CholeOM patients do not report otorrhea symptoms in the early phases of the disease.[16-19] In this study, the incidence of otorrhea in the CholeOM group was lower than that of the COMC group, but the difference was not significant. In the otorrhea-positive group, the expressions of PERK and BiP mRNA were higher in the CholeOM group than in the COMC group. Immunoglobulin heavy chain-binding protein is bound to the domain of the ER of 3 IRE1, PERK, and ATF6 detectors under stress-free conditions. When unfolded or misfolded proteins accumulate in the ER, abnormal proteins bind to the biopolymer from the ER stress sensor. Immunoglobulin heavy chain-binding protein degradation induces activation of the vesicle stress sensor. The most common symptom of COM is otorrhea due to bacterial infection. In this study, 65%-76% of bacteria were detected. In the CholeOM group, the expressions of PERK and ATF6 mRNA were high and 2 out of 3 ER stress sensors were increased. Therefore, we hypothesized that BiP mRNA increased when formed.

The hearing loss associated with COM is usually conductive hearing loss. Complications associated with myringitis include mixed hearing loss or sensorineural hearing loss in some cases.[20] In this study, there were no differences in the expression of ER stress mRNA between the 2 groups according to sensorineural hearing loss. However, PERK, ATF6, and BiP mRNA expressions were significantly higher in the CholeOM group than in the COMC group when patients reported conductive hearing loss. There were no differences in the amounts of 6 ER stress mRNA in cases of sensorineural hearing loss, and we were unable to determine why ER stress mRNA expression is increased only in cases of conductive hearing loss. When it was not known whether a lesion is mild or moderate, the expression of PERK and ATF6 mRNA was high in CholeOM and increased in PERK and BiP mRNA in the bacteria culture-positive group.

In this study, we did not study all ER stress mRNAs but only selected ER stress mRNAs, which are representative of ER stress. We found that, in the molecular signaling pathway of ER stress, ER stress mRNAs associated with attenuation and transcriptional induction are increased. However, increases in PERK and BiP mRNA in cholesteatoma compared with granulation tissue were associated with the development of cholesteatoma, possibly associated with the clinical characteristics of the patients. However, there were no differences between the 2 groups in ossicular destruction or facial canal dehiscence, or in other clinical features. There was no difference in ER stress mRNA expression between the 2 groups. Although ER stress has been studied in various diseases, ER stress has not been studied in the CholeOM group, but also in the COMC group. Therefore, the results of this study suggest detailed roles and target genes for each of the 6 ER stress mRNAs in COM and what roles they play in COM with or without cholesteatoma.

This study had several limitations. First, normal middle ear mucosa could not be obtained due to ethical reasons. Therefore, the levels of ER stress mRNAs could not be compared in normal and OM mucosa. Second, we did not include any children with COM, suggesting that the effect of age-dependent ER mRNA expression was not considered. Finally, this study investigated the effects of ER stress responses on mRNA, but not protein, expression. The Western blotting analysis is therefore needed to compare protein expression in these groups of patients

Six ER stress mRNAs were expressed in both granulation tissue and cholesteatoma. The expression of BiP mRNA was high in the COMC

group, and the expressions of PERK and ATF6 mRNA were high in the CholeOM group. The expression levels of ER stress mRNA were different according to clinical features.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of Kyung Hee University Medical Center (Approval No: 2017-12-030).

Informed Consent: Written informed consent was obtained from all patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – S.G.Y.; Design – D.W.K., S.G.Y.; Supervision – S.G.Y.; Funding – S.G.Y., S.S.K.; Materials – Y.J.K., Y.I.K.; Data Collection and/or Processing – Y.I.K., S.H.K.; Analysis and/or Interpretation – Y.J.K., S.S.K., Y.I.K., S.H.K.; Literature Review – S.G.Y.; Writing Manuscript– D.W.K., S.G.Y.; Critical Review – S.G.Y.

Declaration of Interests: The authors declare that they have no conflict of interest.

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