Partial Epithelial–Mesenchymal Transition Was Observed Under p63 Expression in Acquired Middle Ear Cholesteatoma and Congenital Cholesteatoma

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Introduction: Partial epithelial–mesenchymal transition (p-EMT) is a process by which epithelial cells partially lose their intercellular adhesion and change to obtain migration ability. The transcription factor p63 regulates the expression of cadherin family and induces epithelial cell proliferation. In this study, we hypothesized that p-EMT under p63 expression may be a key factor in epithelial cell growth in middle ear cholesteatoma.

Methods: Specimens were surgically excised from patients with congenital cholesteatoma (CC) (n = 48), acquired middle ear cholesteatoma (AC) (n = 120), and normal skin tissue (n = 34). We analyzed immunohistochemically for the EMT marker (N-cadherin), adherence junction marker (E-cadherin), and tight junction marker (claudin-1, claudin-4, occludin). We also examined the labeling index (LI) of p63 and Proliferating cell nuclear antigen (PCNA) (late S phase marker), and Snail expression as a mobility marker.

Results: The expression of p63 (CC 51.0 ± 7.4%, AC 50.0 ± 5.9%) was significantly higher in the thickened epithelium of CC and AC compared with normal skin tissue ($p < 0.0001$). The loss of E-cadherin was observed (CC 50.0%, AC 55.8%) but the expression patterns in the tight junction were almost normal. N-cadherin was partially detected in the basal and upper layer of epithelium in CC and AC. In contrast to that of normal skin tissue, the LI of PCNA was significantly higher in AC ($p < 0.0001$). The positive rate of Snail was significantly higher in CC ($p < 0.0001$).

Conclusion: This study indicates that p-EMT via the p63 signaling pathway might plays an essential role in epithelial growth in AC and CC formation, although tight junction formation and terminal differentiation were not affected in those processes. **Key Words:** Acquired middle ear cholesteatoma—Congenital cholesteatoma—E-cadherin—Immunohistochemistry—p63—Partial EMT.

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Middle ear cholesteatoma is a type of chronic otitis media that typically expands into the middle ear, resulting in bone destruction and hearing loss (1,2). In previous studies, our research team and others have indicated that the pathological status of cholesteatoma was a benign hyperkeratinizing epithelial cyst rather than a tumor; however, an increasing number of stem and progenitor cells were detected in the epithelium and papillary ingrowth (3,4).

Epithelial mesenchymal transition (EMT) is a process by which the loss of epithelial cell–cell adhesions that promote a mesenchymal phenotype is observed, which enable migration and invasion ability (5). EMTs are thought to be associated with tumor progression and to occur during cancer metastasis (5). Cells undergoing EMT often display cadherin switching, in which they downregulate one cadherin and induce expression of another; for example, from E-cadherin (E-cad) to N-cadherin (N-cad) (6). This so-called ''cadherin switch'' changes the cell–cell adhesion molecules relative to those of its tissue of origin and has been proposed to be required for a cell undergoing EMT to separate from its neighbor cells (7). Tight junction components, claudin (CLD) and occludin (OCL), are another mode of cell–cell adhesion molecules and it has been shown that CLD1 promotes EMT in human bronchial epithelial cells (8) and the reduction of CLD4 induces EMT (9). However, in recent years many studies have failed to detect EMT,

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or have argued that EMT is not fully required for metastasis, leading to significant controversy in the field of oncology (10,11). An increasing number of studies have shown that during developmental and wound-healing processes, cells undergo EMT and transition to a partial state; downregulating just a subset of epithelial characteristics and increasing only some mesenchymal traits, such as invasive motility (6). Under a partial EMT (p-EMT) status, EMT-like cells maintained the expression of basal epithelial cell markers and did not express most of the transcription factors considered to be EMT master regulators (12).

A master regulator of epidermal gene transcription is p63, and it plays an essential function in controlling epidermal development (13,14), and cell proliferation and stemness (15,16). We previously showed an increase in the expression of p63 in a cholesteatoma matrix (17). p63 is also known as one of the regulators of various cell matrix and cell–cell adhesion complexes in the epidermis and controls cadherin family expression, inducing epithelial proliferation in human epithelial cells (18).

For this study, we hypothesized that p-EMT under p63 expression may be one of the key factors in the proliferative activities of epithelial cells in middle ear cholesteatoma. To address this hypothesis, we first analyzed the positive rates of p63 in the congenital cholesteatoma (CC) group, the acquired cholesteatoma (AC) group, and the normal skin group. We then addressed the expression level of tight junction markers (CLD1, CLD4, and OCL), the adherence junction marker E-cad, and the EMT marker N-cad in the CC, AC, and normal skin groups. Understanding these p-EMT and associated signaling events under physical factors can improve therapeutic outcomes in patients with middle ear cholesteatoma.

METHODS

Patients and Specimen Harvest

Specimens from 48 ears in the CC (36 male and 12 female; average age 6 yr; range 2–13 yr) and 120 ears in the AC groups (74 male and 46 female; average age 42 yr; range 10–74 yr) a, confirmed by histopathologic examination, were obtained in these studies (Table 1). From all the patients in both groups, epithelium and granulation tissues were harvested during surgery; they were harvested from the site of cholesteatoma that included epithelium and connective tissues. All of the patients were treated surgically at the Department of Otorhinolaryngology, Jikei University Hospital, between April 2016 and September 2018. Cholesteatoma tissues were harvested from the patients during surgery. In 34 of the ears of the study subjects with cholesteatoma (21 male and 13 female; average age 54 yr; range 24–86 yr), a small piece of normal skin tissue from the edge of a retroauricular incision was harvested during surgery under the agreement.

This study protocol was approved by the Human Ethics Review Committee of the Jikei University School of Medicine and signed, informed consent was obtained from all the patients or their guardians for this study (approval number is 27-344 8229).

Tissue Preparation

Specimens were obtained from all 168 patients and used for immunohistochemistry. Each of the specimens was fixed overnight in 10% buffered formalin at 4°C and embedded in paraffin. Serial sections were cut at a 5 μm thickness and placed onto 3-aminopropyltriethoxysilane-coated glass slides. When we performed immunohistochemistry we prepared the serial section of each specimen. We performed hematoxylin and eosin (H&E) staining in adjacent section to determined epithelial and subepithelial regions. The serial sections were processed for an immunohistochemical analysis of p63, CLD1, CLD4, OCL, E-cad, N-cad, Snail, and Proliferating cell nuclear antigen (PCNA).

Immunohistochemistry

For the detection of p63, CLD1, CLD4, and OCL, an enzyme immunohistochemistry was performed on the paraffin sections of tissue as described previously (17,19–21). Paraffin sections were deparaffinized with toluene and rehydrated by serially graded ethanol solutions. In the case of OCL detection, each section was pretreated with proteinase K (Dako, Glostrup, Denmark) dissolved in phosphate-buffered saline (PBS) (10 μg/ml) at room temperature for 15 minutes. For the detection of CLD1 and p63, the sections were autoclaved in 0.01 M citrate buffer (pH 6.0) at 120°C to retrieve the antigen for 10 minutes. For the detection of CLD4, the sections were autoclaved in Tris/EDTA buffer (pH 9.0) at 120°C for 10 minutes. After inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methanol, the sections were preincubated with 500 μg/ml normal goat IgG (Sigma) and 1% bovine serum albumin (BSA) (Sigma) in PBS for 1 hour to block nonspecific reaction with the first antibody. The sections were then reacted overnight with the first antibody in 1% BSA in PBS, washed three times with 0.075% Brij 35 in PBS, and reacted with horseradish peroxidase (HRP)-goat anti-rabbit IgG (Medical & Biological Laboratories Co., Japan) or HRP-goat anti-mouse IgG (Chemicon International, Inc., CA) for 1 hour. After the slides were washed with 0.075% Brij 35 in PBS, HRP sites were visualized

TABLE 1. *Cases and positive rate of E-cad, N-cad, Snail, and mean of p63 LI, PCNA LI in normal skin, acquired middle ear cholesteatoma and congenital middle ear cholesteatoma specimens*

	Number (Male/Female)	Age (Yr)	E-cad Positive Cases (%)	N-cad Positive Cases (%)	Snail Positive Cases (%)	p63 LI (n)	PCNA LI (n)
Skin	34 (21/13)	54	34/34 (100)	0/34 (0)	1/34 (2.9)	41.1 ± 4.6 (n = 34)	27.5 ± 8.7 (n = 34)
Congenital Cholesteatoma	48 (36/12)	6	24/48 (50.0)[b]	28/48 (58.3)[b]	31/48 (64.6)[b]	51.0 ± 7.4 (n = 48)[b]	32.0 ± 12.6 (n = 48)
Acquired Cholesteatoma	120 (74/46)	42	67/120 (55.8)[b]	93/120 (77.5)[b]	40/120 (33.3)[a]	50.0 ± 5.9 (n = 120)[b]	49.6 ± 8.6 (n = 120)[b]

[a] $p = 0.0001$.
[b] $p < 0.0001$.
E-cad indicates E-cadherin; LI, labeling index; N-cad, N-cadherin; PCNA, Proliferating cell nucelar antigen.

TABLE 2. *List of primary antibodies for immunohistochemistry*

Antibody	Antigen; Company, Cat. no.	Working Dilution
Mouse monoclonal anti-p63	4A4; Abcam, ab735	1:100
Rabbit polyclonal anti-Claudin1	Claudin1; Thermo Fisher, #51-9000	1:200
Rabbit polyclonal anti-Claudin4	Claudin4; abcam, ab53156	1:250
Mouse monoclonal anti-Occludin	OC-3F10; Thermo Fisher, #33-1500	1:200
Mouse monoclonal anti-E-cadherin	NCH-38; Dako, M3612	1:100
Rabbit polyclonal anti-N-cadherin	D4R1H; Cell signaling, #13116	1:50
Rabbit polyclonal anti-Snail	SNAI1; LSBio LS-B11351	1:50
Mouse monoclonal anti-PCNA	PC10; Dako, M0879	2.0 μg/ml

with three, 3′-diaminobenzidine-4HCl (DAB) and H_2O_2. For negative control, normal rabbit IgG (Sigma) or normal mouse IgG (Sigma) was used instead of the first antibodies, respectively, in every experiment. The antibodies used in this study are listed in Table 2.

Identification of EMT Marker

To identify the EMT marker, we carried out fluorescence immunohistochemistry in the sections of specimens of all groups as described previously (17). For the detection of E-cad and N-cad, the sections were autoclaved in 0.01 M citrate buffer (pH 6.0) at 120°C to retrieve the antigen for 10 minutes. After immersion with the single or mixed first antibody, the sections were incubated with the secondary antibodies (Alexa Fluor 488-goat anti-mouse IgG (Thermo Fisher Scientific #A-11001) (1:1,000) and Alexa Fluor 555-goat anti-rabbit IgG (Thermo Fisher Scientific #A-21428) (1:500) for 1 hour. After washing three times with 0.05% Tween 20 in PBS, the sections were counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Dako). For every experimental run, negative control samples were prepared by reacting the sections with normal mouse IgG or normal rabbit IgG instead of the first antibody. The antibodies used in this study are listed in Table 2.

Identification of Proliferating Cells and Migrating Cells

The nuclear antigen associated with cell proliferation was immunohistochemically detected using the anti-PCNA antibody and migration activity was detected using anti-Snail antibody. For this purpose, sections were first deparaffinized and then the slides autoclaved in 10 mM citrate buffer (pH 6.0) for 15 minutes at 120°C, according to the protocol described by Ehara et al. (22) and incubated with mixed first antibody. Then the sections were incubated with the secondary antibodies (Alexa Fluor 488-goat anti-mouse IgG (1:1,000) and Alexa Fluor 555-goat anti-rabbit IgG (1:500) for 1 hour. After washing three times with 0.05% Tween 20 in PBS, the sections were counterstained with DAPI. As a negative control, some sections were incubated with normal mouse IgG instead of anti-PCNA antibody and normal rabbit IgG instead of anti-Snail antibody. The antibodies used in this study are listed in Table 2.

Quantitative Analysis

For the quantitative analysis of p63 and PCNA, more than 1,000 cells were counted in the epithelial region in three or four random fields at 400 × magnification on a section of a specimen and the number of positive cells was expressed as a percentage of positive cells per total number of counted cells (labeling index [LI]; mean ± SD). The specimen that had less than 1,000 counted cells was excluded in this analysis. For the quantitative analysis of CLD1, CLD4, and OCL, images of the immunostainings were captured using an Axio Cam camera and AxioVision software (Carl Zeiss) under light microscopy and the positive region of the epidermis at three locations of each section was measured using ImageJ software from the National Institutes of Health. The staining intensities of E-cad, N-cad, and Snail in the epithelial region were graded as (+) positive or (−) negative compared with the background staining with normal rabbit IgG and normal mouse IgG.

Statistical Analysis

The percentages of specimens expressing E-cad, N-cad, and Snail were compared among the three groups using the Fisher's exact test. The LIs of p63, CLD1, CLD4, OCL, and PCNA were expressed as mean ± SD. Differences between the groups were examined for statistical significance using the two-way analysis of variance (ANOVA) test followed by Tukey's post hoc tests for normally distributed data. A p value of less than 0.005 denoted the presence of a statistically significant difference. All analyses were performed using a statistical software package (JMP version 13; JMP pro 14.0.0; SAS Institute Japan, Tokyo, Japan). Precise values for p are given in the results for all significant differences, as stipulated in the guidelines for O&N publications.

RESULTS

Expression of p63 and Tight Junction Markers (Claudin-1, Claudin-4, Occludin)

In the normal skin specimens, p63-positive cells were detected primarily in the basal layer and the expression level was slightly weak; however, p63-positive cells were detected in the basal and upper layers of the thickened wall of the cholesteatoma matrix in the CC and AC specimens (Fig. 1). p63 LI in the CC group (51.0 ± 7.4%, n = 48) and AC group (50.0 ± 5.9%, n = 120) was significantly higher than that for the normal skin group (41.1 ± 4.6%, n = 34; one-way ANOVA $F(2, 199) = 113.86$, $p < 0.0001$ with Tukey's multiple comparison test) (Fig. 2A). According to the tight junction molecules, CLD1 and CLD4 were detected in all layers of the epithelium in the CC and AC specimens and the localization of positive cells was almost the same as that for the normal skin group (Fig. 1). Another tight junction molecule (OCL) was strongly detected at the apical cell border of the epithelium in the CC and AC groups and positive lesion was almost the same as that for the normal skin group (Fig. 1). The LI of CLD1, CLD4, and OCL in

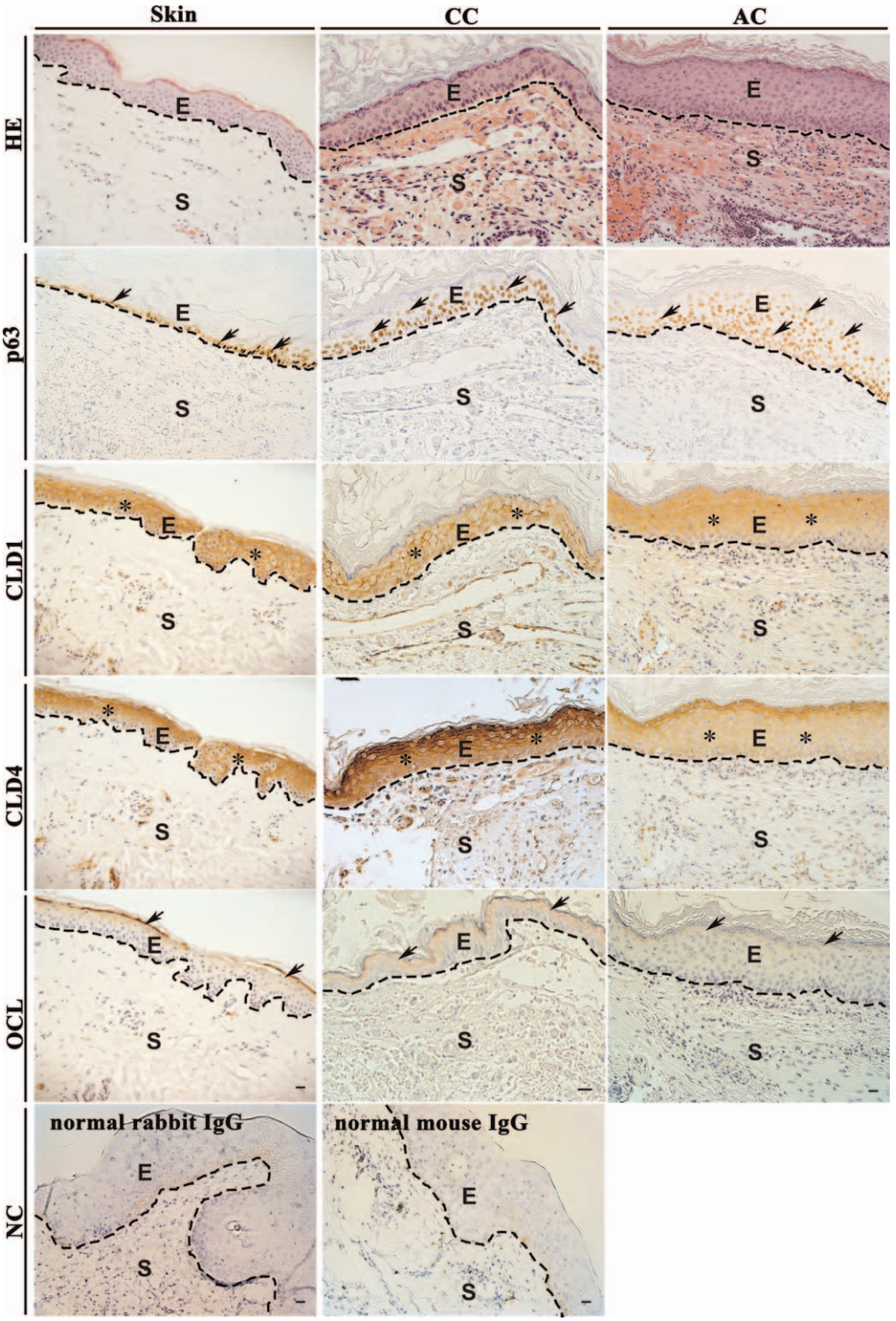

FIG. 1. Upper column: H&E staining of normal skin (skin), congenital cholesteatoma (CC), and acquired middle ear cholesteatoma (AC). Immunohistochemical detections of p63, claudin1 (CLD1), claudin4 (CLD4), and occludin (OCL) in paraffin sections of skin, CC and AC. p63-positive cells were detected in the basal and upper layer in CC and AC. In skin, p63-positive cells were detected mainly in the basal layer and expression level was slightly weak. CLD1 positive region was detected almost all layer in the epithelium of skin, CC and AC. CLD4 positive region was detected almost all layer in the epithelium of skin, CC and AC. OCL positive region was detected strongly at the apical cell border in the epithelium of skin, CC and AC. For the negative control (NC) of CLD1 and CLD4, the section was reacted with normal rabbit IgG instead of the first antibody, no staining was found. For the negative control of p63 and OCL, the section was reacted with normal mouse IgG instead of the first antibody, no staining was found. Scale bars: 20 μm, *Arrows*: positive cells, *Asterisks*: positive layers, Dashed lines: basement membrane, E: epithelial region, S: subepithelial region.

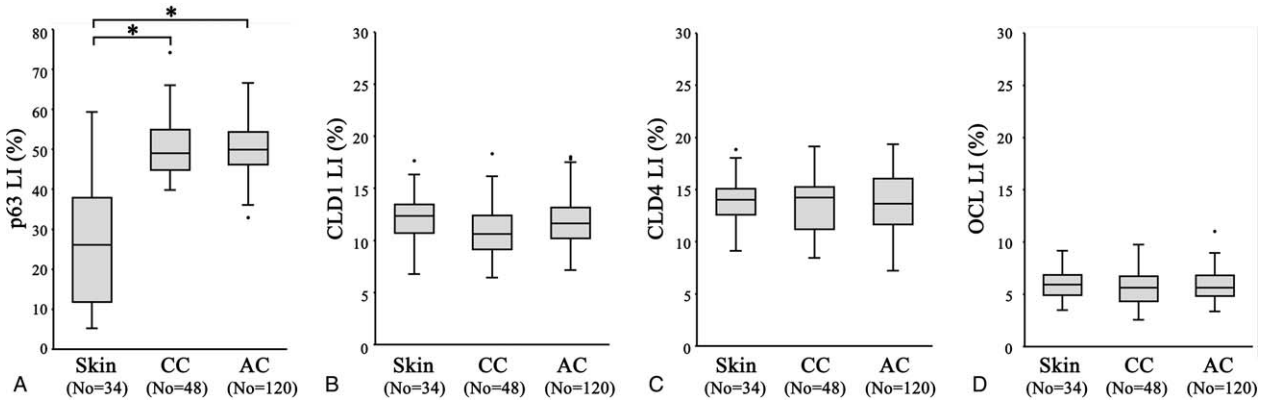

FIG. 2. Labeling index (LI) of p63, claudin1 (CLD1), claudin4 (CLD4), and occludin (OCL) in normal skin (skin), congenital cholesteatoma (CC) and acquired middle ear cholesteatoma (AC). Data are mean ± SD. *A,* Box plots; labeling index (LI) of p63. *B,* Box plots; labeling index (LI) of CLD1. *C,* Box plots; labeling index (LI) of CLD4. *D,* Box plots; labeling index (LI) of OCL. $^*p < 0.0001$.

the CC and AC groups was almost the same as that for the normal skin group (CLD1: 10.9 ± 2.5%, n = 48, CC group, 11.8 ± 2.3%, n = 120, AC group, 12.3 ± 2.3%, n = 34, normal skin group; one-way ANOVA F (2, 199) = 3.87, $p = 0.0225$ with Tukey's multiple comparison test, CLD4: 13.6 ± 2.7%, n = 48, CC group, 13.6 ± 2.8%, n = 120, AC group, 14.0 ± 2.2%, n = 34, normal skin group; one-way ANOVA F (2, 199) = 0.20, $p = 0.8221$ with Tukey's multiple comparison test, OCL: 5.8 ± 1.9%, n = 48, CC group, 6.0 ± 1.4%, n = 120, AC group, 5.9 ± 1.4%, n = 34, normal skin group; one-way ANOVA F (2, 199) = 0.09, $p = 0.9154$ with Tukey's multiple comparison test) (Fig. 2, B–D).

Expression of the EMT Marker (N-cadherin) and Adherence Junction Marker (E-cadherin)

In the epithelial layer of the normal skin tissues, E-cad-positive cells were detected in all layers of the epithelium and N-cad-positive cells were not detected (Fig. 3). However, in the thickened epidermal layer of the CC and AC group tissues, the number of E-cad-positive cells dramatically decreased in the basal layer and upper basal layer (Fig. 3). N-cad-positive cells were detected mainly in basal layer of the epithelium in the CC group and partially expressed in the upper and basal layer of the epithelium in the AC group tissues (Fig. 3). The percentage of E-cad-positive specimens was significantly higher in the normal skin group (100.0%, 34/34 specimens) than in the CC group (50.0%, 24/48, $p < 0.0001$, Fisher-Exact-test, Table 1) and the AC group (55.8%, 67/120, $p < 0.0001$, Fisher exact test, Table 1). Furthermore, 28 of 48 specimens (58.3%) were positive for N-cad in the CC group and 93 of 120 specimens (77.5%) were positive in the AC group, while none of the 34 (0%, 0/34) specimens were scored as N-cad-positive in the normal skin group. This indicated that the incidence of N-cad-positive specimens was significantly higher in the CC and AC groups than in the normal skin group ($p < 0.0001$, Fisher exact test, Table 1).

Analysis of Epithelial Cell Proliferation and Migration Activity

An immunohistochemical analysis for PCNA was performed to evaluate the effects of EMT on the proliferative activity of epithelial cells, and migration activity of epithelial cells was evaluated by detecting Snail. In the epithelium of the normal skin specimens, PCNA-positive cells were found only in the basal layer and Snail-positive cells were detected scarcely (Fig. 4A). Interestingly, in the CC group tissue specimens, PCNA-positive cells were found in the suprabasal layers and Snail-positive cells were found in the basal layers (Fig. 4A). In the AC group tissue specimens, PCNA-positive cells were found in the suprabasal and upper layers and Snail-positive cells were detected scarcely in any layer (Fig. 4A). When the sections were reacted with normal mouse IgG instead of the first antibody, no staining was found (data not shown). The PCNA LI was significantly higher in the AC group than in the normal skin group and CC group (AC group 49.6 ± 8.6%, n = 120 compared with normal skin group 27.5 ± 8.7%, n = 34 and CC group 32.0 ± 12.6%, n = 48; two-way ANOVA F (2, 199) = 100.71, $p < 0.0001$ with Tukey's multiple comparison test) (Fig. 4B, Table 1). Snail-positive rate of the CC group (64.6%, 31/48) was significantly higher than that of normal skin group (2.9%, 1/34, $p < 0.0001$, Fisher exact test, Table 1) and AC group (33.3%, 40/120, $p = 0.0001$, Fisher exact test, Table 1).

DISCUSSION

First, we showed an increase in the expression of p63 in a cholesteatoma matrix of CC and AC. p63 is known to suppress E-cad expression in the epidermis and induce epithelial proliferation in human epithelial cells (18). Indeed in this study, we also noted that the expression level of E-cad was lower in the basal and upper layers of the epithelium of the CC and AC group specimens, which was almost as same as p63-positive region. We suggested that the expression level of E-cad might be lower under

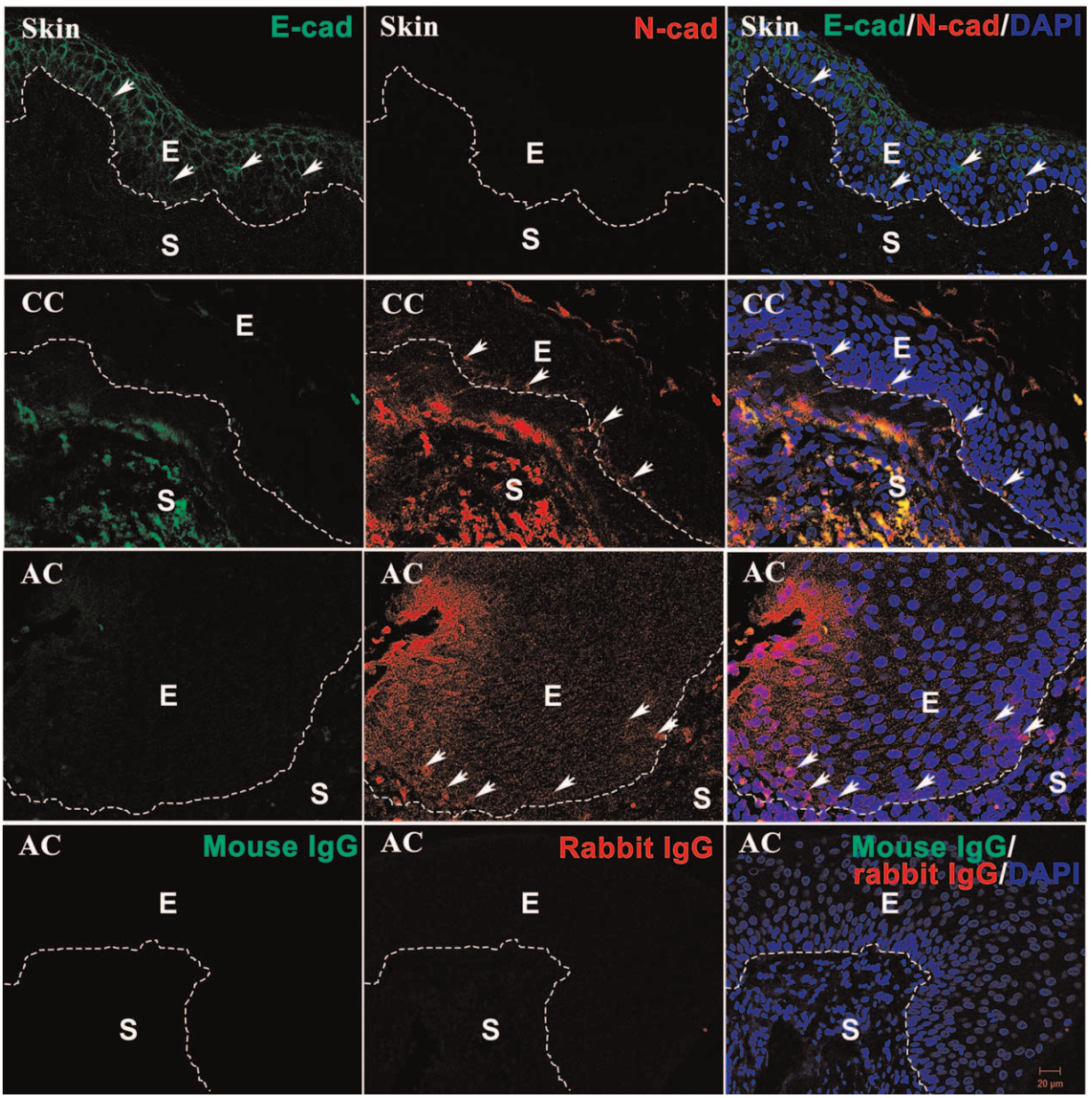

FIG. 3. Double immunofluorescence detection of E-cadherin (E-cad, green) and N-cadherin (N-cad, red) in normal skin (skin), congenital cholesteatoma (CC), and acquired middle ear cholesteatoma (AC). The nuclei were stained with DAPI (blue). Staining for E-cad was reduced in epithelial region in CC and AC. N-cad was partially expressed in the basal layer in CC and basal and upper layer in AC. For the negative control of E-cad and N-cad the section was reacted with normal mouse IgG instead of E-cad and normal rabbit IgG instead of N-cad, no staining was found. Scale bar: 20 μm, *Arrows*: positive cells, Dashed lines: basement membrane, E: epithelial region, S: subepithelial region.

p63 expression in the basal and upper layers of the epithelium the CC and AC group specimens. However, N-cad was expressed mainly in the basal layer and partially in the upper layer of the CC and AC group specimens. During p-EMT, it is thought that epithelial cells lose just a subset of epithelial characteristics and increasing only some mesenchymal traits. N-cad E-cad, OCL and cytokeratins are the most commonly used markers for epithelial cells and N-cad and vimentin for mesenchymal cells (23). The cadherin switch occurred partially during p-EMT and both E-cad (−)/N-cad (+) cells and E-cad (−)/N-cad (−) cells were typically detected (5). Therefore, our results indicate that p-EMT might occur in the cholesteatoma matrix. A p-EMT state has been noted in association with many developmental, wound healing, fibrosis, and cancer processes (24–27), evident through the existence of intermediate hybrid epithelial and mesenchymal phenotypes (28). Especially in neck cancer tissues, tumor cells formerly under a p-EMT state divide into bipotential

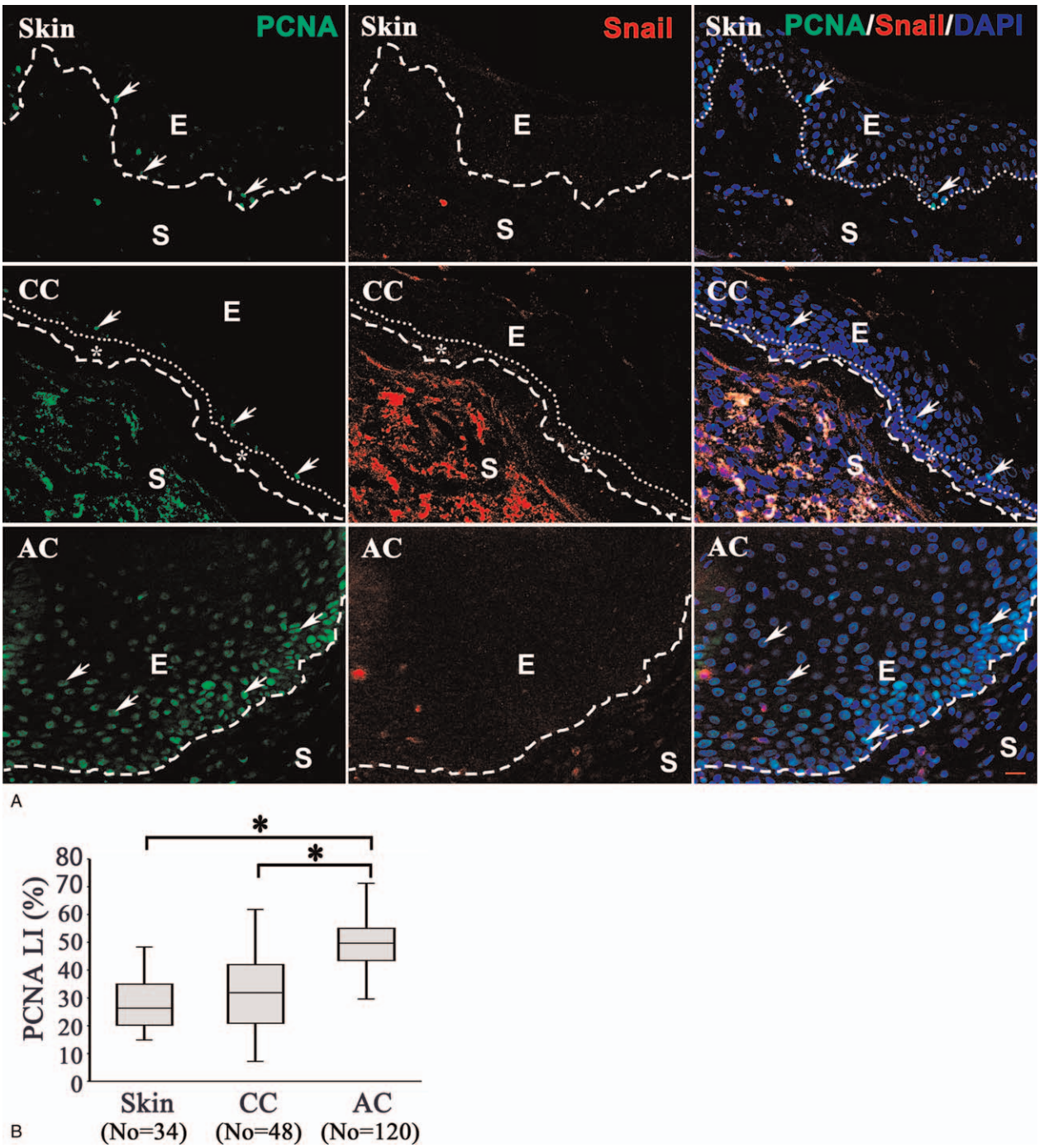

FIG. 4. *A*, Double immunofluorescence detection of PCNA (green) and Snail (red) in normal skin (skin), congenital cholesteatoma (CC), and acquired middle ear cholesteatoma (AC). The nuclei were stained with DAPI (blue). PCNA-positive cells (*arrows*) were detected in the upper layers in CC and basal and upper layers in AC. Snail-positive cells were detected in basal layers in CC group. Scale bar: 20 μm, *Arrows*: positive cells, Dashed lines: basement membrane, Dotted lines: under PCNA positive layer, E: epithelial region, S: subepithelial region. *B*, Labeling index (LI) of PCNA in skin, CC and AC. Box plots were showing the LI of PCNA of the epithelium in each group. Data are mean ± SD. $^{*}p < 0.0001$.

cells that have basal and mesenchymal characters and induce invasion (12). Indeed, in the previous studies we demonstrated an increase of CK-14 (basal cell marker)-positive cells in the cholesteatoma matrix (17). We next hypothesized that p-EMT could activate epithelial cell proliferation in the cholesteatoma matrix. Regarding proliferation, high PCNA LI was observed in the AC group compared with the normal skin group but not in CC

group. Interestingly, the PCNA LI of CC group was almost the same as that of normal skin group. The localization of PCNA-positive cell is almost the same as in the N-cad positive region in the AC group but the localization of PCNA-positive cell in CC group was different from N-cad positive region. In the CC group the localization of Snail-positive cell is almost the same as in the N-cad positive region. Our study showed CC group was significantly higher in Snail expression rate compared with normal skin group and AC group. It is known that Snail is a major determinant of the mesenchymal movement (29) and recent study indicated that Snail levels in directing control the movement and the metastatic behavior of tumor cells (30). Temporal bone histopathological studies of CC demonstrate two distinct pathological types, closed type CC and open type CC (31). Almost all of CC is a closed type CC and it is known as a keratotic cyst in the anterior mesotympanum, which is easily to remove; however, we previously demonstrated advanced cholesteatoma spreading from the tympanic cavity into the mastoid cavity in 55.6% ears of the closed type of cholesteatoma (32). Indeed, we analyzed advanced cholesteatoma spreading from the tympanic cavity into the mastoid cavity in 56.25% (27/48) ears of CC group in this study (data not shown). On the other hand, an open type CC, in which there is no containment of the keratotic debris and the cholesteatoma matrix is in direct continuity with middle ear mucosa, is difficult to extirpate and more likely to be associated with residual disease (31). According to these facts, we concluded p-EMT could activate epithelial cell proliferation in AC group and epithelial cell movement in the cholesteatoma matrix in CC group. N-cad is known to control the motility, migration, and proliferative activity of cancer cells (33), which supports our results.

We also demonstrated immunohistochemistry for tight junction molecules and hypothesized that overexpressed CLD1 and reduced CLD4 could promote p-EMT in the cholesteatoma matrix. In a recent study, the reduction of CLD1 expression was indicated in middle ear cholesteatoma tissues by RT-PCR and the authors concluded that increased permeability of the cholesteatoma epithelium is compatible with the acid lysis hypothesis of bone resorption in cholesteatoma (34). However, CLD1 is known to be expressed most widely in mammalian tissues including epithelial cells, endothelial cells, and fibroblasts (35,36), so we analyzed the expression pattern of tight junction molecules, including CLD1, in the matrix of cholesteatoma tissues and performed a quantitative analysis. Against our expectations, the expression level and localization of CLD1, CLD4, and OCL in the CC and AC groups were the same as that of normal skin group. CLD1 is a p63 direct target gene in epithelial development and a p63 deficiency leads to the inhibition of CLD1 (37). CLD1 and CLD4 are controlled in part by the Sp1-containing critical promoter region (38). However, Kakuki et al. (39) indicated that a knockdown of ΔNp63 had no change of tight junction proteins, OCL, CLD1, CLD4, and CLD7 in head and neck SCC. On the other hand, Kaneko et al. (40) recently indicated that a knockdown of p63 by siRNAs of TAp63 and ΔNp63 induced the expression of CLD1 and CLD4 with an increase of Sp1 activity. They concluded that p63 negatively regulated tight junction proteins and their function in the nasal epithelium. In this way, the role of p63 against CLDs is controversial, so the direct effect of overexpressed-p63 against CLDs and OCL expressions should be analyzed further.

Taken together, our results indicate the possibility that during the epithelial growth process of cholesteatoma, a reduction of E-cad might occur initially under p63 expression in the cholesteatoma matrix and N-cad expression induced in basal cells to then invade the middle ear region. Controling p-EMT through the downregulation of p63 could be a promising strategy for treating middle ear cholesteatoma.

CONCLUSION

In conclusion, p-EMT under the p63 signaling pathway plays an essential role in epithelial cell growth in CC and AC formation, although tight junction formation and terminal differentiation are not affected during these processes. The downregulation of p63 may suppress growth activity and lead to the normal differentiation of epithelial cells of the cholesteatoma matrix. Understanding these p-EMT and associated signaling events can improve therapeutic outcomes in patients with middle ear cholesteatoma.

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