Received: 22 March 2022 | Revised: 13 June 2022 | Accepted: 17 June 2022

DOI: 10.1002/lio2.856

ORIGINAL RESEARCH

Laryngoscope Investigative Otolaryngology

Immunohistochemical localization of D-β-aspartic acid in congenital and acquired middle ear cholesteatoma

Shiori Kitaya MD[1,2] | Ryoukichi Ikeda MD, PhD[1,3] | Jun Suzuki MD, PhD[1] | Hidetoshi Oshima MD, PhD[3] | Yuri Nomura MD, PhD[3] | Yusuke Kusano MD[1,3] | Nobuo Ohta MD, PhD[2] | Tetsuaki Kawase MD, PhD[1] | Kazue Ise PhD[4] | Kazuhiro Murakami MD, PhD[5] | Yasuhiro Nakamura MD, PhD[5] | Hironobu Sasano MD, PhD[6] | Yukio Katori MD, PhD[1]

[1]Department of Otolaryngology, Head and Neck Surgery, Tohoku University Hospital, Sendai, Japan

[2]Division of Otolaryngology, Tohoku Medical and Pharmaceutical University Hospital, Sendai, Japan

[3]Center for Otologic Surgery, Sen-en Rifu Hospital, Rifu, Miyagi, Japan

[4]Technical Services Division, Tohoku Medical and Pharmaceutical University, Sendai, Japan

[5]Division of Pathology, Tohoku Medical and Pharmaceutical University Hospital, Sendai, Japan

[6]Department of Pathology, Tohoku University School of Medicine, Sendai, Japan

Correspondence
Dr. Nobuo Ohta, Division of Otolaryngology, Tohoku Medical and Pharmaceutical University Hospital, 1-12-1, Fukumuro Miyaginoku, Sendai 983-8512, Japan.
Email: noohta@hosp.tohoku-mpu.ac.jp

Abstract

Objective/Hypothesis: Middle ear cholesteatoma is characterized by abnormal growth of the keratinizing squamous epithelium of the temporal bone. D-β-aspartic acid is the major isomer of D-aspartic acid found in elderly tissue. We assessed the immunoreactivity to k-β-aspartic acid of congenital and acquired middle ear cholesteatomas.

Study Design: Case–control studies.

Material and Methods: Tissue samples were collected from 21 patients comprising 21 ears with congenital middle ear cholesteatoma and 26 patients comprising 29 ears with acquired type. Their clinical and histopathological features were investigated. We divided the middle ear cholesteatoma samples into three layers: the perimatrix, matrix, and cystic contents. The patterns of immunoreactivity to D-β-aspartic acid expression were then assessed immunohistochemically.

Results: Two patterns of immunoreactivity to D-β-aspartic acid were detected in middle ear cholesteatoma: infiltrative and diffuse. In congenital middle ear cholesteatoma, D-β-aspartic acid expression was observed throughout all the layers (perimatrix, matrix, and cystic contents), and immunoreactivity to D-β-aspartic acid was dramatically strong in all layers. The expression levels of D-β-aspartic acid to the cystic content and perimatrix were significantly higher in congenital middle ear cholesteatoma than in the acquired type.

Conclusions: This study showed the expression levels of D-β-aspartic acid in middle ear cholesteatoma to differ significantly between congenital and acquired middle ear cholesteatoma. Our results indicate that overexpression of D-β-aspartic acid is likely to be involved in the pathogenesis of cholesteatoma, and we speculate that D-β-aspartic acid could be a novel biomarker for, and a therapeutic target in, congenital and acquired middle ear cholesteatoma.

Level of Evidence: 4

KEYWORDS
acquired middle ear cholesteatoma, congenital middle ear cholesteatoma, D-β-aspartic acid, matrix, perimatrix

1 | INTRODUCTION

Middle ear cholesteatoma is a chronic middle ear disease that is characterized by abnormal growth of the keratinizing squamous epithelium in the temporal bone.[1] This pathological condition erodes nearby bone structures, leading to hearing impairment, facial paralysis, and labyrinthine fistular and intracranial complications.[2] The pathological features of middle ear cholesteatoma include squamous epithelium, granulation tissue, and keratinaceous debris.[3] The components of middle ear cholesteatoma are divided into three layers: the perimatrix (lamina propria), the matrix, and cystic contents. Middle ear cholesteatomas are classified into three categories: the congenital type, which is specific to childhood; the acquired type, which affects both children and adults; and unclassifiable cholesteatomas whose precise origin cannot be detected.[4] The mean age of patients and extent of bone destruction are thought to differ between the congenital type and the acquired type. The exact pathogenic molecular mechanisms behind the formation and propagation of middle ear cholesteatoma are still not clear. The currently prevalent hypothesis for the mechanism of acquired cholesteatoma is that the keratinocytes of the middle ear become hyperproliferative. The most widely accepted mechanism for the congenital type is the epidermoid cell rest theory: remaining epidermoid cells, termed epibranchial placodes, are sited behind the intact tympanic membrane and fail to involute.[5]

D-β-aspartic acid has been found in proteins obtained from various human tissues in elderly people: lenses,[6] skin,[7] the aorta,[8] glandular tissue,[9] the brain,[10] and ligaments.[11] The presence of D-β-aspartic acid at various ages of the living body is considered to result from the racemization of L-β-aspartic acid in proteins.[9] D-β-aspartic acid in the proteins of living creatures is therefore regarded as a useful marker of the aging process.[6] The racemization of amino acids is accelerated by ultraviolet irradiation. D-β-aspartic acid-containing proteins are seen in certain ultraviolet-exposed tissues: in cataracts,[12] pinguecula,[13] and in actinic keratosis of the skin.[14] Thus, D-β-aspartic acid is also considered a useful index of history of ultraviolet irradiation. The immunohistochemical localization of D-β-aspartic acid in various parts of the body is currently a focus of major research efforts. However, the difference in D-β-aspartic acid expression between acquired and congenital cholesteatoma has hitherto not been studied. The present study investigates the immunohistochemical localization of D-β-aspartic acid in congenital and acquired middle ear cholesteatoma and explores its correlation with certain clinical and examination findings.

2 | MATERIALS AND METHODS

2.1 | Patients

A retrospective survey of medical records in Sen-En Rifu Hospital included patients with previously clinically diagnosed congenital and acquired middle ear cholesteatoma. The patient selection algorithm is shown in Figure 1. Overall, 34 cases of acquired middle ear cholesteatoma underwent surgery from April 2016 to December 2018. Five cases were excluded because three of the histopathological diagnoses were of chronic otitis media, one case had no cholesteatoma tissue (only a cavity problem), and the other case consisted of an outpatient biopsy. This study identified 21 patients (14 men and 7 women subjects aged 3–66 years, average 13.3 ± 15.6 years), comprising 21 ears (13 right ears and 8 left ears) with congenital middle ear cholesteatoma from April 2014 to October 2021 and 26 patients (15 men and 11 women subjects aged 22–80 years, average 49.4 ± 17.5 years), comprising 29 ears (12 right ears and 17 left ears) with acquired middle ear cholesteatoma. Histopathological diagnosis of middle ear cholesteatoma was made at Tohoku University Hospital's Division of Pathology. Pure-tone audiometry, computed tomography, and blood examinations were performed before surgery. Self-reported and clinical data were obtained from a questionnaire and medical records. The human ethical and clinical trial committee at Tohoku Medical and Pharmaceutical University approved this survey (2017-2-058). We obtained written consent for publication of the clinical findings, image and pathological examinations from patients.

2.2 | The classification and staging of middle ear cholesteatoma

We classified middle ear cholesteatoma into two general categories: congenital and acquired based on the EAONO/JOS system 2017 Classification and Staging of Middle Ear Cholesteatoma proposed by the European Academy of Otology and Neuro-Otology.[4] Congenital middle ear cholesteatoma is generally an expanding cystic mass with keratinizing squamous epithelium located medial to the intact tympanic membrane, seems to be present at birth, but is usually diagnosed during in early childhood with no prior history of otorrhea, perforation, or previous ear surgery. Acquired middle ear cholesteatoma is not present at birth, and is assumed to develop from a retraction pocket of the pars flaccida, pars tensa, or both and from basal cell invasion through the basilar membrane and secondary to eustachian tube dysfunction. The staging of middle ear

cholesteatoma was evaluated by otolaryngologists also based on the EAONO/JOS system 2017 Classification and Staging of Middle Ear Cholesteatoma.[4]

2.3 | Measuring methods

2.3.1 | Analysis of cholesteatoma samples

Tissue samples of congenital and acquired middle ear cholesteatoma were collected from 21 patients from April 2014 to October 2021 and 26 patients from April 2016 to December 2018 who had undergone middle ear cholesteatoma surgery at Sen-en Rifu Hospital.

2.3.2 | Immunohistochemistry to detect D-β-aspartic acid

Four-micrometer sections were taken from paraffin-embedded tissue blocks, deparaffinized, and rehydrated. The sections were then heated (Autoclave, 121°C) in Antigen Retrieval Solution (pH 9.0) for 5 min for antigen retrieval. Endogenous peroxidase activity was blocked with

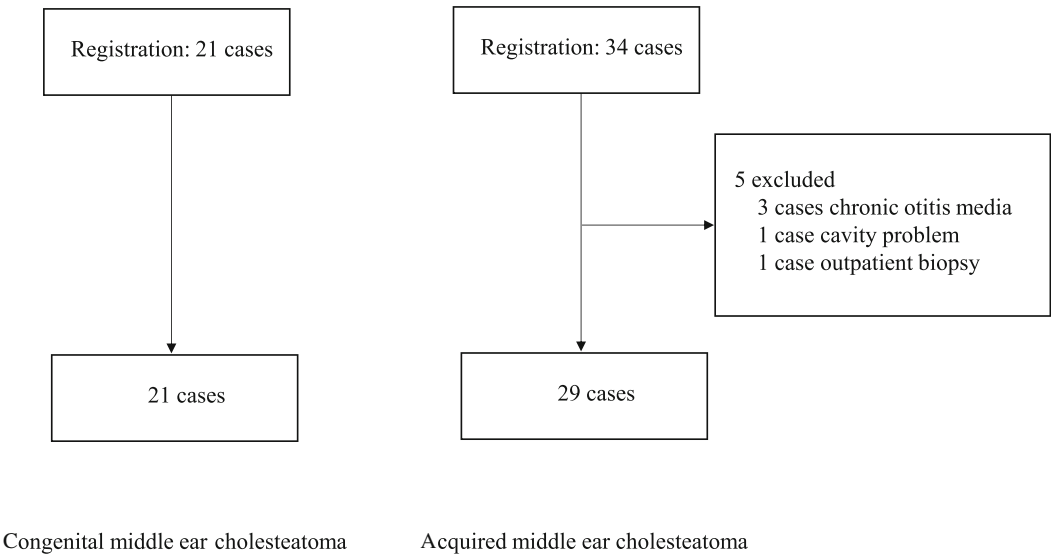

FIGURE 1 Patient selection algorithm for acquired middle ear cholesteatoma

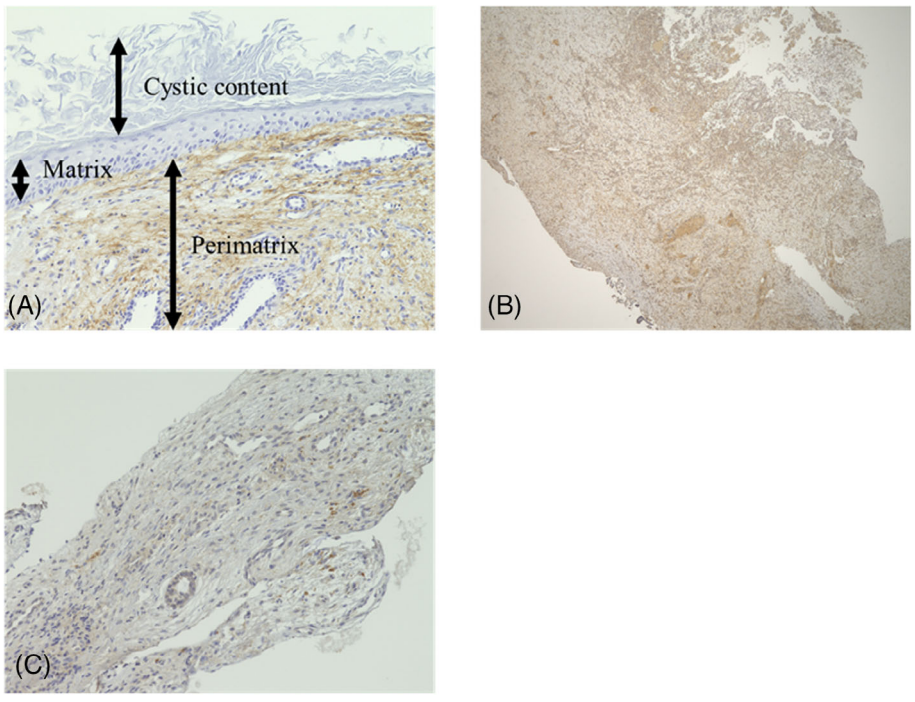

FIGURE 2 Pattern of immunoreactivity (B: diffuse, C: infiltrative). (A) Middle ear cholesteatoma consists of three layers: the perimatrix, the matrix, and cystic contents. The perimatrix is the most peripheral layer of the middle ear cholesteatoma. It consists of an abundance of granulation tissue containing collagen fiber, fibrocytes, and inflammatory cells such as lymphocytes, histiocytes, plasma cells, and neutrophil leucocytes. The matrix consists of hyperproliferative stratified keratinizing squamous epithelium. The cystic contents comprised abundant laminated layers, free-flowing or aggregated, desquamated, anucleate keratin mixed with sebaceous materials. (B) The diffuse pattern consisted of D-β-aspartic acid strongly expressed throughout each layer, including cystic content, matrix and perimatrix. (C) The infiltrative group consisted of partially-observed D-β-aspartic acid in varying degrees.

TABLE 1 The characteristics of clinical observations of congenital middle ear cholesteatoma

Case no.	Gender	Age	Side	Cystic content	D-β-asp matrix	Peri matrix	Otorrhea	Sniffing habit	Air conduction (dB)	Bone conduction (dB)	A-B gap (dB)	Stage	Recontraction	Eosinophil count (μl)	Allergy
1	M	5	R	2	2	2	−	−	65	5	60	II	IVil	81.7	−
2	M	4	R	2	2	2	−	−				II	IIIil	60.3	BA
3	F	20	L	2	2	2	−	−	15		15	II	IIIc	60.0	−
4	M	34	L	2	2	2	−	−	33.3	8.3	25	II	wo	111.4	−
5	F	38	L	2	2	2	−	−	50	40	10	II	IIIc	72.2	−
6	M	5	R	2	2	2	−	−	23.3	13.3	10	II	IIIiM	427.5	−
7	M	6	L	2	2	2	+	−	60	26.7	33.3	II	IVc	63.8	−
8	M	10	R	2	2	2	−	−	40	5	35	II	IVc	179.7	−
9	M	5	L	2	2	2	−	−	33.3	0	33.3	II	IVc	263.3	BA
10	F	10	L	None	None	2	−	−	31.7	6.7	25	II	IVil	99.9	AR
11	M	4	L	2	None	None	−	−	20		20	I	I	207.2	−
12	F	7	L	2	2	2	−	−	50	1.7	48.3	II	IVil	367.9	−
13	M	3	R	2	2	2	−	−	10		10	I	I	77.3	−
14	M	12	R	2	2	2	−	−	35	−5	40	II	wo	302.7	−
15	M	3	L	2	2	2	−	−	20		20	II	I	400.5	−
16	M	6	R	2	2	2	−	−	16.7		16.7	II	IIIc	552.0	AR
17	F	12	L	None	None	2	−	−	38.3	10	28.3	II	IVc	110.4	−
18	F	22	L	2	None	None	−	−	63.3	11.7	51.6	II	IVc	110.9	−
19	M	4	R	2	2	2	−	−	15		15	II	I	135.5	−
20	M	66	L	2	None	None	−	−	25	13.3	11.7	II	I	58.7	−
21	F	4	L	2	2	2	+	−	71.7	3.3	68.4	III	IIIc	245.1	−

Notes: Gender: M, male; F, female. Operation side: R, right; L, left. Expression types of D-β-aspartic acid in congenital middle ear cholesteatoma: 1 infiltrative, 2 diffuse; not listed: none (no sections of each layer were observed in specimens). Complaint of otorrhea and habit of sniffing: −, not noted; +, noted; not listed: not listed in the medical record.
Abbreviations: AR, allergic rhinitis; BA, bronchial asthma.

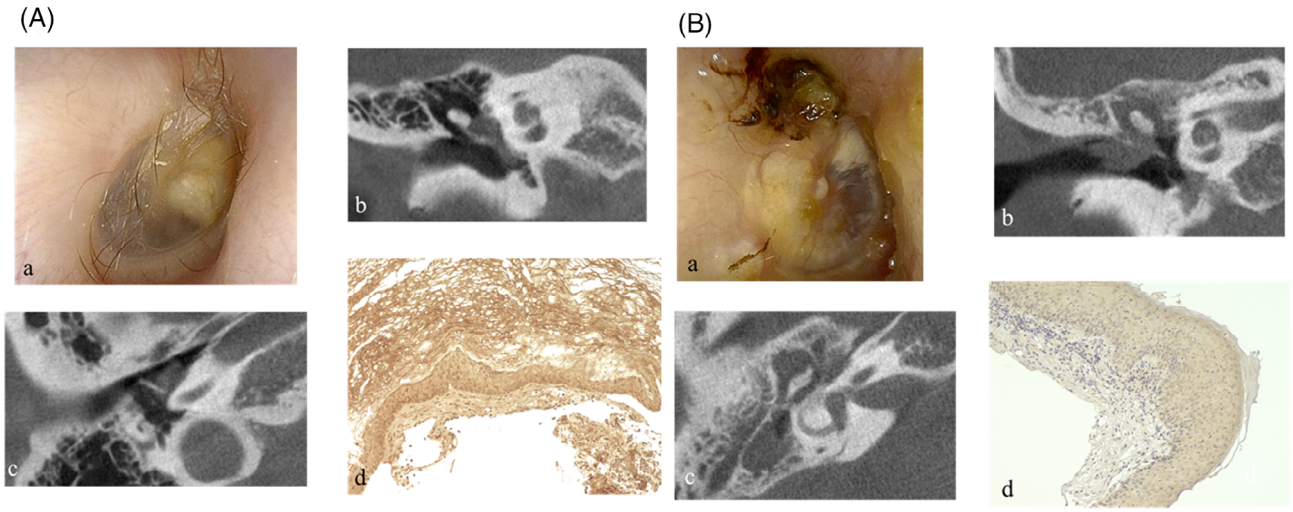

FIGURE 3 (A) Congenital cholesteatoma affecting a 20-year-old man (Case 3) was referred to our department because of antibiotic-resistant right hearing impairment. (a) Endoscopic findings of the right external auditory canal and tympanic membrane: A white mass was observed at the anterior superior quadrant of the tympanic membrane. (b and c) Axial and coronal computed tomography of the right temporal bone: A mass was observed in the anterior tympanic cavity. (d) Typical immunohistochemical localization of D-β-aspartic acid: The immunoreactivity to D-β-aspartic acid was very highly and significantly strong in all layers (cystic content, matrix and perimatrix). (B) Acquired cholesteatoma affecting a 25-year-old man (Case 2) referred to our department because of antibiotic-resistant right middle ear effusion. (a) Endoscopic findings of the right external auditory canal and tympanic membrane: Large epitympanic erosion and crust. (b and c) Axial and coronal computed tomography of the right temporal bone: Mass in the attic with blunting of the scutum. (d) Representative immunohistochemical localization of D-β-aspartic acid: The expression of D-β-aspartic acid was observed throughout all layers, especially significantly strong in the matrix.

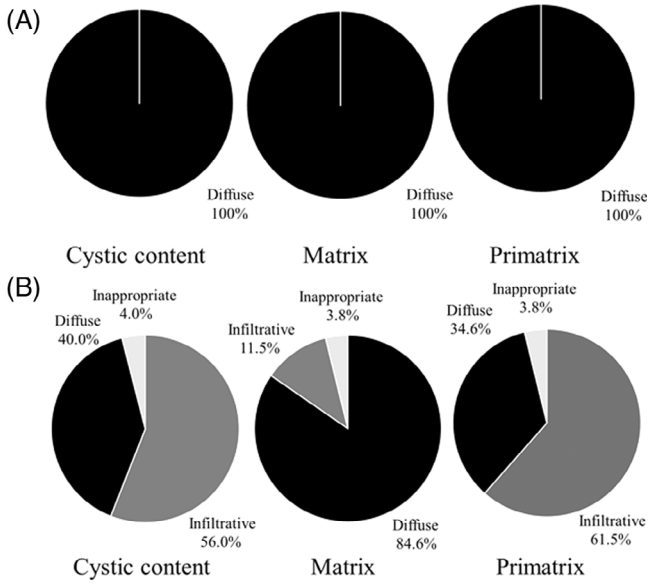

FIGURE 4 (A) Expression of D-β-aspartic acid (a: cystic content, b: matrix, c: perimatrix) in congenital middle ear cholesteatoma. Immunoreactivity to D-β-aspartic acid was significantly strongly observed throughout all the layers. (B) Expression of D-β-aspartic acid (a: cystic content, b: matrix, c: perimatrix) in acquired middle ear cholesteatoma. Immunoreactivity to D-β-aspartic acid was observed throughout all the layers, but significantly strongly in the matrix.

H_2O_2 3% in absolute methanol at room temperature for 10 min. All sections were preincubated in Normal Goat Serum (Vector Laboratories ImmPRESS HRP REAGENT Kit Anti-Rat: MP-7444) at room temperature for 20 min to block nonspecific background staining. The sections were then treated with a polyclonal antibody (diluted 1:800) against D-aspartic acid (Advanced Targeting Systems: AB-T047), dissolved in phosphate-buffered saline (PBS) containing 1% normal bovine serum albumin, and kept at 4°C overnight. The sections were then incubated with a secondary antibody (Anti-Rat HRP Reagent [Vector Laboratories ImmPRESS HRP Reagent Kit Anti-Rat: MP-7444]) for 30 min at room temperature, and finally incubated with diaminobenzidine (DAB) in PBS and counterstained with hematoxylin.

2.3.3 | Assessment of slides and evaluation of immunoreactivity

Immunostained sections were assessed under a Keyence microscope with a ×4, ×20, or ×40 eyepiece reticle. We evaluated immunoreactivity to D-β-aspartic acid in congenital and acquired middle ear cholesteatoma, divided into three layers: the perimatrix, the matrix, and cystic contents. The perimatrix is the most peripheral layer of middle ear cholesteatoma. It consists of an abundance of granulation tissue containing collagen fiber, fibrocytes, and inflammatory cells such as lymphocytes, histiocytes, plasma cells, and neutrophil leucocytes.[15] The matrix consists of hyperproliferative stratified keratinizing squamous epithelium.[1] The cystic contents comprise abundant laminated layers, free-flowing or aggregated, and desquamated anucleate keratin mixed with sebaceous materials.[3] We also divided all the cases into two groups according to degree of immunoreactivity: infiltrative and diffuse types. (Figure 2) In the diffuse pattern group, D-β-aspartic acid

TABLE 2 The characteristics of clinical observations of acquired middle ear cholesteatoma

Case no.	Gender	Age	Side	D-β-asp keratin	Matrix	Peri matrix	otorrhea	Sniffing habit	Air conduction (dB)	Bone conduction (dB)	A-B gap (dB)	stage	Recontraction	Eosinophil count (μl)	Allergy
1	M	75	R	2	2	2	+	−	78.3	56.7	21.6	II	IIIiM	342.38	−
2	F	78	R	1	2	1	−	−	38.3	36.7	1.6	I	I	99.45	AR、CRS
3	M	44	L	None	2	1	+	−	56.7	30	26.7	II	IIIc	882.5	AR
4	M	24	L	1	1	2	+	+	31.7	18.3	13.4	II	IIIiR	178.71	−
5	F	32	R	1	None	None	+	+	30	15	15	II	IIIiR	133.05	−
6	F	24	L	2	2	2	+	−	22.5	8.5	14	II	IIIc	319.55	−
7	M	40	R	2	None	None	+	+	25	30	−5	II	I	558.4	−
8	M	39	L	1	2	1	+	+	30	10	20	II	I	470.02	−
9	M	72	L	2	None	None	+	−	38.3	23.3	15	II	IIIc	81.12	−
10	M	45	R	1	2	2	−	−	28.3	5	23.3	II	IIIc	229.36	BA
11	M	45	L	1	2	1	+	−	26.7	10	16.7	II	I	229.36	BA
12	F	40	L	None	2	2	+	−	15	8.3	6.7	I	I	188.77	BA
13	M	80	L	1	2	1	+	−	100	68.3	31.7	III	wo	78.39	−
14	F	22	L	2	2	1	−	+	38.3	25	13.3	II	IIIc	91.91	−
15	M	42	L	1	2	1	+	+	6.7	11.7	−5	I	IIIiR	530.64	−
16	M	31	R	None	2	2	−	−	28.3	11.6	16.7	II	IIIiM	160.94	−
17	F	53	L	1	2	2	+	−	61.7	13.3	48.4	II	IIIc	161.46	−
18	M	66	L	2	2	2	+	−	51.7	28.3	23.4	II	IIIc	52.03	−
19	M	48	L	1	1	1	−	+	31.7	10	21.7	II	IIIc	168.36	−
20	M	28	L	None	None	None	+	+	38.3	18.3	20	II	IViM	180.67	AR
21	F	66	R	2	2	1	+	−	50	41.6	8.4	II	I	137.8	BA
22	F	28	L	2	2	1	+	+	23.3	13.3	10	I	I	299.39	AR
23	M	37	R	1	2	1	−	+	53.3	8.3	45	II	IIIc	240.21	−
24	M	69	R	2	2	1	−	−	46.7	18.3	28.4	III	IVc	177.84	ACOS
25	F	68	R	2	2	2	+	−	30	15	15	II	I	29.92	−
26	F	63	L		2	1	−	−	41.7	30	11.7	II	IVc	139.08	−
27	F	62	R	1	2	1	−	−	73	46.7	26.3	II	IVc	130.9	−
28	F	50	L	1	1	1	−	−	18.3	11.7	6.6	II	I	211.75	−
29	M	63	R	1	2	1	+	−	83.3	60	23.3	III	IVc	88.83	−

Notes: Gender: M: male, F: female. Operation side: R: right, L: left. Expression types of D-β-aspartic acid in congenital middle ear cholesteatoma: 1: infiltrative, 2: diffuse; not listed: none (no sections of each layer were observed in specimens). Complaint of otorrhea and habit of sniffing: −: not noted, +: noted, not listed: not listed in the medical record.

Abbreviations: ACOS, asthma-COPD overlap syndrome; AR, allergic rhinitis; BA, bronchial asthma; CRS, chronic rhinosinusitis.

TABLE 3 Patient characteristics and the relationship between D-β-asp expression type and clinical findings in acquired middle ear cholesteatoma

		Congenital	Acquired	*p*-Value
Gender		0.33 ± 0.48	0.41 ± 0.5	.39
Age		13.33 ± 15.61	49.45 ± 17.83	<.001
Side		0.38 ± 0.5	0.59 ± 0.5	.13
D-β-asp	Cystic content	2	1.42 ± 0.5	<.001
	Matrix	2	1.88 ± 0.33	.22
	Perimatrix	2	1.36 ± 0.49	<.001
Otorrhea		0.1 ± 0.3	0.66 ± 0.48	<.001
Sniffing habit		0	0.34 ± 0.48	.02
Air conduction		35.83 ± 18.65	41.28 ± 21.64	.43
Bone conduction		10 ± 11.46	23.56 ± 16.97	<.001
A-B gap		34.28 ± 18.09	17.72 ± 12.07	<.001
stage		1.95 ± 0.38	1.97 ± 0.5	.67
Eosinophil count		189.9 ± 144.91	227.34 ± 183.12	.34
Allergy		0.19 ± 0.4	0.31 ± 0.47	.24

Notes: The expression levels of D-β-aspartic acid of cystic content and perimatrix were significantly higher in congenital middle ear cholesteatoma than in acquired middle ear cholesteatoma.

was strongly expressed throughout each layer. In the infiltrative group, D-β-aspartic acid was observed partially and in varying degrees. (Figure 2) Regarding the classification of diffuse group and infiltrative type, those with a stained area of D-β-aspartic acid of 70% or more of the section were defined as diffuse type, and those with less than 70% were defined as infiltrative type. We were not able to assess immunoreactivity to poor-quality or necrotic specimens. The histology data were randomized, and blinded evaluation of data was conducted by three experienced otolaryngologists. They classified each case into three groups for each layer, and adopted a majority decision if they disagreed.

2.3.4 | Analysis of blood samples

We collected blood samples before each operation and determined the correlation between blood eosinophil count and immunoreactivity to D-β-aspartic acid.

2.4 | Statistical analysis

We used one-way analysis of variance (ANOVA) and the Mann–Whitney *U* test to compare the statistical differences between groups. The analysis was performed using SPSS version 27 statistical software (IBM, Chicago, IL). Differences with a *p*-value of <.05 were regarded as significant.

3 | RESULTS

3.1 | Immunohistochemical localization of D-β-aspartic acid in congenital middle ear cholesteatoma

The expression of D-β-aspartic acid in congenital middle ear cholesteatoma was investigated in 21 samples from 21 patients (Table 1). Figure 3A shows all the different examination findings of typical cases of congenital cholesteatoma. D-β-aspartic acid expression was observed throughout all the layers. Furthermore, the immunoreactivity to D-β-aspartic acid was dramatically strong in all layers in all cases, regardless of clinical or laboratory findings. (Figure 4A).

3.2 | Immunohistochemical localization of D-β-aspartic acid in acquired middle ear cholesteatoma

The expression of D-β-aspartic acid in acquired middle ear cholesteatoma was investigated in 29 samples from 26 patients (Table 2). Figure 3B shows all the different examination findings in typical cases of acquired cholesteatoma. D-β-aspartic acid expression was observed throughout all layers; however, dramatically strong immunoreactivity to D-β-aspartic acid was detected only in the matrix, unlike with congenital middle ear cholesteatoma. The ratio in the diffuse type of matrix was higher than in the other two layers: immunoreactivity was seen in the matrix in 22 cases (84.6%), in the cystic contents in 10 cases (40.0%), and in the perimatrix in 9 cases (34.6%) (Figure 4B).

3.3 | Clinical significance of D-β-aspartic acid expression in congenital and acquired middle ear cholesteatoma

We evaluated patients' clinical data to prescribe the pathological significances in D-β-aspartic acid levels in congenital and acquired middle ear cholesteatoma (Table 3). Patients with congenital middle ear cholesteatoma were significantly younger than those with the acquired type (13.33 ± 15.61 vs. 49.45 ± 17.83 years). The expression levels of D-β-aspartic acid in the cystic content and perimatrix were significantly higher in the congenital than in the acquired type.

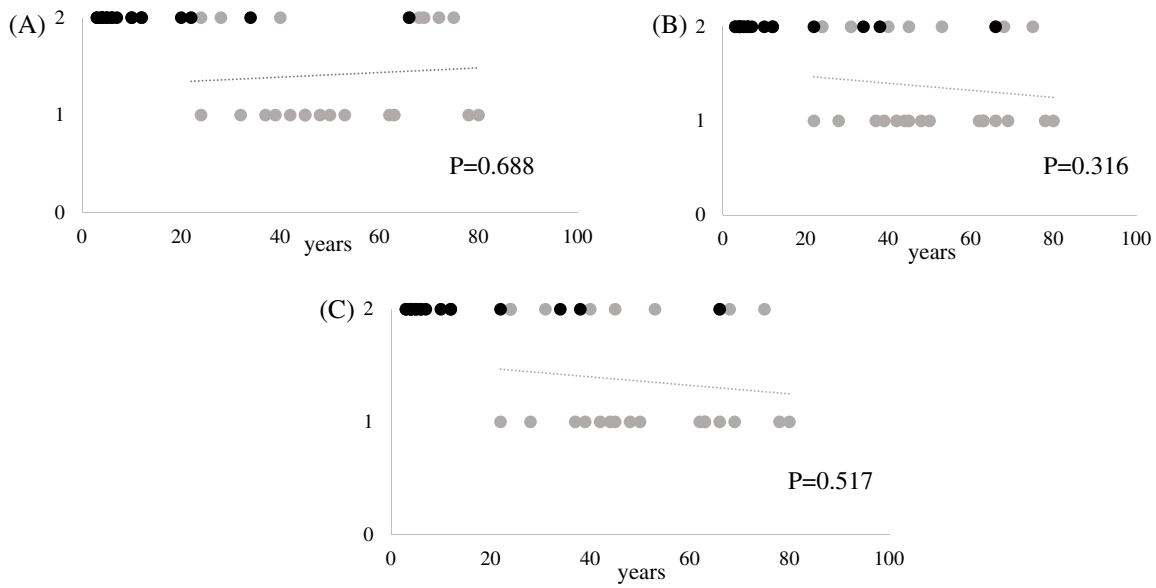

FIGURE 5 Expression of D-β-aspartic acid (A: cystic content, B: matrix, C: perimatrix) in congenital and acquired middle ear cholesteatoma. Expression types of D-β-aspartic acid: 0 negative, 1 infiltrative, 2 diffuse. Black points: congenital middle ear cholesteatoma; gray points: acquired middle ear cholesteatoma. The expression levels of D-β-aspartic acid in individual layers of acquired middle ear cholesteatoma and congenital cholesteatoma did not significantly correlate with age.

The acquired-type patients had significantly more complaints of otorrhea than the congenital-type patients (0.1 ± 0.3 vs. 0.66 ± 0.48). Bone conduction, as measured by pure tone audiometry tests carried out before surgery, was significantly higher in acquired middle ear cholesteatoma patients than in congenital middle ear cholesteatoma patients (10 ± 11.46 vs. 23.56 ± 16.97 dB). In contrast, air-bone gap, as tested by pure tone audiometry, was significantly higher in congenital middle ear cholesteatoma patients than in acquired middle ear cholesteatoma patients (34.28 ± 18.09 vs. 17.72 ± 12.07 dB).

Between congenital and acquired middle ear cholesteatoma patients, there was no significant difference in gender, left or right side, habit of sniffing before surgery, air conduction as measured by pure tone audiometry before surgery, stage of middle ear cholesteatoma, blood eosinophil count, presence of a medical history of any of the allergic diseases, allergic rhinitis, bronchial asthma, or chronic sinusitis. We also investigated the relationship between the expression levels of D-β-aspartic acid in cystic content, matrix and perimatrix in acquired middle ear cholesteatoma and the age of the subjects. The expression levels of D-β-aspartic acid in congenital and acquired middle ear cholesteatoma did not significantly correlate with age (Figure 5).

4 | DISCUSSION

Amino acids contain one or more asymmetric tetrahedral carbon atoms that make D-enantiomer and L-enantiomer structures. The chemical and physical properties of L-amino acids and D-amino acids are the same. However, their optical characteristics are different.[9] Fujii et al. reported D-aspartic acid formation to be accompanied by isomerization from natural α-aspartic acid to abnormal β-aspartic acid via a succinimide.[6] This leads to the formation of four isomers: the normal L-α-aspartic acid, the biologically uncommon L-β-aspartic acid, D-α-aspartic acid, and D-β-aspartic acid in proteins. D-β-aspartic acid is normally rare but is the major isomer found in elderly tissues.[16] The presence of D-β-aspartic acid is thought to be the result of racemization of L-β-aspartic acid in proteins.[9] D-β-aspartic acid in the proteins of living creatures is considered to be a novel marker of damage caused by aging.[6] Ultraviolet (UV) irradiation of the skin is closely related to the formation of D-β-Asp in the elastic fibers of the skin.[17] In the present study, D-β-aspartic acid expression was detected all the layers of middle ear cholesteatoma in the matrix, despite the middle ear cavity not having been exposed to UV irradiation. These results indicate that the racemization of L-amino acids may occur for reasons other than aging or UV irradiation. Furthermore, contrary to our expectations, in congenital middle ear cholesteatoma, immunoreactivity to D-β-aspartic acid was very marked in all the layers in all the samples. In most cases, these remaining epidermoid cells are resorbed at around the 33rd week of gestation. However, a congenital middle ear cholesteatoma may form if epidermoid cells fail to involute due to persistent inflammation.[18] On the other hand, the currently prevalent hypothesis for acquired middle ear cholesteatoma is that negative pressure or dysfunction of the Eustachian tube causes a deepening retraction pocket that, when obstructed, prevents the clearing of desquamated keratin from the recess, resulting in a cholesteatoma. Local infection and inflammation lead to the disruption of self-cleaning systems, causing cell debris and keratinocytes to accumulate inside the retraction pocket, possibly inducing the immigration of immune cells, Langerhans' cells, T-cells, and macrophages. An imbalance is created and a vicious circle of epithelial proliferation, keratinocyte

differentiation and maturation, prolonged apoptosis, and disruption of self-cleaning systems is established. The inflammatory stimulus might induce epithelial proliferation along with the expression of lytic enzymes, inflammatory mediators, and various cytokines. These mediators and cytokines might lead to the activation and maturing of osteoclasts, with the consequence of degradation of the extracellular bone matrix and hyperproliferation, bone erosion, and finally, progression of the disease. Our results suggest a difference in the mechanism of origin and development between congenital middle cholesteatoma and acquired middle ear cholesteatoma.

Our study has several limitations. First, the number of cholesteatoma patients recruited in this study was relatively small because the samples were collected from only a single center. Second, we have not determined which protein contains D-β-aspartic acid in congenital and acquired middle ear cholesteatoma. Identifying this protein may lead to elucidation of the pathogenesis of middle ear cholesteatoma. Further research with many more samples is required to reveal the mechanism of expression of D-β-aspartic acid in middle ear cholesteatoma.

5 | CONCLUSION

In summary, we observed that the expression pattern of D-β-aspartic acid is different for congenital and acquired middle ear cholesteatoma. Our results indicate that overexpression of D-β-aspartic acid is likely involved in the pathogenesis of cholesteatoma, and we speculate that D-β-aspartic acid could be both a novel biomarker and a therapeutic target for congenital and acquired middle ear cholesteatoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Shiori Kitaya https://orcid.org/0000-0002-7238-1338
Ryoukichi Ikeda https://orcid.org/0000-0003-0076-6092
Nobuo Ohta https://orcid.org/0000-0002-2821-6976

REFERENCES

1. Semaan MT, Megerian CA. The pathophysiology of cholesteatoma. *Otolaryngol Clin North Am*. 2006;39(6):1143-1159.
2. Xie S, Xiang Y, Wang X, et al. Acquired cholesteatoma epithelial hyperproliferation: roles of cell proliferation signal pathways. *Laryngoscope*. 2016;126(8):1923-1930.
3. Castle JT. Cholesteatoma pearls: practical points and update. *Head Neck Pathol*. 2018;12(3):419-429.
4. Yung M, Tono T, Olszewska Olszewska E., Yamamoto Y., Sudhoff H., Sakagami M., Mulder J., Kojima H., Incesulu A., Trabalzini F., Ozgirgin N. EAONO/JOS joint consensus statements on the definitions, classification and staging of middle ear Cholesteatoma. *J Int Adv Otol* 2017: 13:1–8.
5. Michaels L. Origin of congenital cholesteatoma from a normally occurring epidermoid rest in the developing middle ear. *Int J Pediatr Otorhinolaryngol*. 1988;15:51-65.
6. Fujii N, Harada K, Momose Y, Ishii N, Akaboshi M. D-amino acid formation induced by a chiral field within a human lens protein during aging. *Biochem Biophys Res Commun*. 1999;263(2):322-326.
7. Fujii N, Tajima S, Tanaka N, Fujimoto N, Takata T, Shimo-Oka T. The presence of D-beta-aspartic acid-containing peptides in elastic fibers of sun-damaged skin: a potent marker for ultraviolet-induced skin aging. *Biochem Biophys Res Commun*. 2002;294(5):1047-1051.
8. Powell JT, Vine N, Crossman M. On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta. *Atherosclerosis*. 1992; 97(2–3):201-208.
9. Kaji Y, Oshika T, Takazawa Y, Fukayama M, Takata T, Fujii N. Localization of D-beta-aspartic acid-containing proteins in human eyes. *Invest Ophthalmol Vis Sci*. 2007;48(9):3923-3927.
10. Shapira R, Chou CH. Differential racemization of aspartate and serine in human myelin basic protein. *Biochem Biophys Res Commun*. 1987; 146(3):1342-1349.
11. Ritz-Timme S, Laumeier I, Collins M. Age estimation based on aspartic acid racemization in elastin from the yellow ligaments. *Int J Leg Med*. 2003;117(2):96-101.
12. Masters PM, Bada JL, Zigler JS Jr. Aspartic acid racemisation in the human lens during ageing and in cataract formation. *Nature*. 1977;268:71-73.
13. Kaji Y, Oshika T, Amano S, Okamoto F, Koito W, Horiuchi S. Immunohistochemical localization of advanced glycation end products in pinguecula. *Graefes Arch Clin Exp Ophthalmol*. 2006;244:104-108.
14. Kaji Y, Oshika T, Nejima R, Mori S, Miyata K, Fujii N. Immunohistochemical localization of D-β-aspartic acid-containing proteins in pterygium. *J Pharm Biomed Anal*. 2015;116:86-89.
15. Louw L. Acquired cholesteatoma pathogenesis: stepwise explanations. *J Laryngol Otol*. 2010;124(6):587-593.
16. Fujii N, Ishibashi Y, Satoh K, Fujino M, Harada K. Simultaneous racemization and isomerization at specific aspartic acid residues in alpha B-crystallin from the aged human lens. *Biochim Biophys Acta*. 1994;1204(2): 157-163.
17. Miura Y, Fujimoto N, Komatsu T, et al. Immunohistochemical study of chronological and photo-induced aging skins using the antibody raised against D-aspartyl residue-containing peptide. *J Cutan Pathol*. 2004;31(1):51-56.
18. Levenson MJ, Michaels L, Parisier SC, Juarbe C. Congenital cholesteatomas in children: an embryologic correlation. *Laryngoscope*. 1988;98: 949-955.

How to cite this article: Kitaya S, Ikeda R, Suzuki J, et al. Immunohistochemical localization of D-β-aspartic acid in congenital and acquired middle ear cholesteatoma. *Laryngoscope Investigative Otolaryngology*. 2022;7(4): 1155-1163. doi:10.1002/lio2.856