B-ENT, 2013, **9**, 241-246

E-cadherin expression in acquired cholesteatoma

M. Erdoglija[1], N. Milanovic[1], M. Colic[2] and M. Jovic[3]

[1]Department of Otorhinolaryngology, Otology Unit, Military Medical Academy, Belgrade, Serbia; [2]Institute of Medical Research, Division of Clinical Immunology, Military Medical Academy, Belgrade, Serbia; [3]Institute of Pathology, Military Medical Academy, Belgrade, Serbia

Key-words. E-cadherin, cholesteatoma matrix, antigen-presenting cells, Langerhans cells, immunohistochemistry

Abstract. *E- cadherin expression in acquired cholesteatoma. Objectives*: The epithelial cell adhesion molecule E-cadherin is important for maintenance normal tissue architecture and for cell-cell communication and immune cell migration. E-cadherin is also present in cholesteatomas. This study determined E-cadherin expression in acquired cholesteatomas and analyzed its expression according to cholesteatoma clinical and histological characteristics.
Methods: We investigated E-cadherin expression in 30 samples from operated patients with acquired middle ear cholesteatomas that were classified according to their clinical and histological characteristics. E-cadherin expression in cholesteatoma was determined immunohistochemically. A semi quantitative method was used to determine the index of expression of E-cadherin and t-tests, Mann-Whitney U tests and Spearman correlation analysis were used for statistical analysis.
Results: We found significant expression of E-cadherin on CD1, CD3 total, CD4 ($p<0.05$), high expression of E-cadherin on CD8 total and CD19/CD38 lymphocytes ($p<0.01$) and very high expression of E-cadherin on mast cells and antigen-presenting cells, including Langerhans cells ($p<0.005$). We graduated results as no statistically significant ($p>0.05$), statistically significant ($0.05>p>0.01$), highly statistically significant ($0.01>p>0.005$) and very highly statistically significant ($p<0.005$).
Conclusion: E-cadherin expression was the same in the cholesteatoma matrix in all samples. There were no differences in expression according to the clinical and histological characteristics of the cholesteatomas.

Introduction

E-cadherin is an intercellular adhesion molecule on the epithelial cells of the cholesteatoma matrix that is involved in immune cell migration and in communication between cells in the cholesteatoma during the immune response. The E-cadherin/beta-catenin complex plays a crucial role in epithelial cell-cell adhesion and assures the integrity of the tissue architecture.[1-3] E-cadherin is expressed in the immune cells that infiltrate into the cholesteatoma perimatrix and takes part in the immune cell migration to cholesteatoma development. The aim of this study was to determine expression of E-cadherin in cholesteatoma and to analyze its expression according to the clinical and histological characteristics of the cholesteatomas. This study did not investigate or analyze E-cadherin expression in normal epithelium.

Materials and methods

Patients

We investigated samples from 30 subjects, 7 children and 23 adults who underwent primary surgery for acquired middle ear cholesteatomas in our clinic from 1998 to 2005. We performed canal wall up tympanoplasty in all pediatric patients (range 10-16 years old) and in most adults (19 patients). We performed canal wall down tympanoplasty in 4 adults, in 2 reoperations of recurrent cholesteatoma after canal wall up tympanoplasty and in 2 reoperations of recurrent cholesteatoma after canal wall down tympanoplasty (Table 1). We used high resolution temporal bone CT to establish the diagnosis of recurrent cholesteatoma before reoperation. Currently, improvements in MRI techniques have led to more accurate details of the cholesteatoma

Presented at the 1st Congress of the Confederation of European Oto-Rhino-Laryngology, Head and Neck Surgery in Barcelona, July 2-6, 2011.

Table 1

Patient age and surgery type

Patient age, years	Canal wall down tympanoplasty	Canal wall up tympanoplasty
10-16	0	7
18-68	4	19
Reoperation	2	2

using delayed contrast enhanced T1 weighted imaging and diffusion-weighted imaging.[4]

The cases were first categorized according to the degree of bone destruction (16 patients with more and 14 patients with less bone destruction), the presence of infection (22 patients with and 8 patients without infection) and cholesteatoma extension (20 patients with more and 10 patients with less cholesteatoma extension). We also investigated the correlations between E-cadherin expression and the histological characteristics of the cholesteatomas to determine whatever there were any differences in E-cadherin expression according to the degree of keratinization, inflammation or extracellular matrix proliferation. This study also looked at possible the correlations between the immunohistochemical and the clinical characteristics of cholesteatoma.

Cases were categorized according to clinical findings i.e. having cholesteatoma induced erosion of 2 or 3 auditory ossicles, leading to ossicular discontinuity along with erosion of wall of the middle ear or having less bone destruction with no ossicle damage or one auditory ossicle damage because of the cholesteatoma process. A positive microbial swab before or during surgery indicated the presence of bacterial infection in the cholesteatoma while a negative swab indicated absence of infection. In the group categorized as having more cholesteatoma extension, cholesteatoma was found in all three compartments of the middle ear; in contrast, cholesteatoma extension in one or two compartments of the middle ear defined cases with less cholesteatoma extension.

Immunohistochemistry

E-cadherin expression was investigated using immunohistochemistry. Samples of cholesteatoma from all 30 cases were soaked in formalin, embedded in paraffin and cut into 4 mm sections using a microtome. All samples were taken from tissue representing complete resection of the cholesteatoma. The slides were deparaffinized, hydrated and washed with TRIS-buffered saline plus 0.5% Tween 20 (TBST buffer). Samples were then subjected to microwave treatment for 30 min in citrate buffer (pH = 6.0) to retrieve the antigens. Endogenous peroxidase activity was blocked using water plus 3% H202 for 30 min. Finally, the cells were characterized using mouse monoclonal antibodies that react with the following human proteins: CD1a, CD3, CD19, CD38 and CD68 (Dako); CD8, c-kit, CD117, HLA-DR (Serotec); CD4 (Novocastra); and E-cadherin (Santa Cruz Biotechnology).

DAKO Envision (K5007) was used to visualize bound antibodies. Diaminobenzidine was applied for 10 min as a chromogen. The slides were counterstained with Mayer's hematoxylin, washed in water, dehydrated in increasing concentrations of ethanol, cleared in xylene and mounted in Canada balsam.

Statistical analysis

The number of immune cells that showed positive staining with the cell-specific antibodies was reported quantitatively. The number of different types of immune cells in one high-powered microscope field (HPF) is shown in Table 2. The histological characteristics of cholesteatomas were scored using semi-quantitative method to obtain "indexes" for the characteristics. These indexes were then summarized and scored mean estimated score. Keratinisation was scored as follows: no keratinisation 0; low level of keratinisation 1; moderate level of keratinisation 2; high level of keratinisation 3. The mean estimated score of keratinisation for all samples was 2.10 ± 0.77.

Inflammation/inflammatory cell infiltration was scored as follows: no inflammatory cell infiltration 0; low level of inflammatory cell infiltration i.e. fewer then 5 cells in one HPF, 1; moderate level of inflammatory cell infiltration, i.e. 5-20 cells in one HPF, 2; high level of inflammatory cell infiltration, i.e. more then 20 cells in one HPF, 3. The mean

Table 2

Summary of the estimated number of immune cells in a 1-mm² high power microscope field

Immune cells	Number of immune cells/mm² (average ± SD)
CD1a	74.18 ± 55.13
CD3 total	102.14 ± 74.16
CD3 ep.	21.02 ± 14.27
CD4	70.20 ± 54.25
CD8 total	43.54 ± 32.26
CD8 ep.	8.91 ± 5.18
CD19/CD38	40.68 ± 32.68
CD68	53.99 ± 32.92
c-kit	23.05 ± 10.71
HLA-DR total	93.43 ± 50.68
HLA-DR ep.	41.21 ± 23.79

CD: cluster of differentiation receptor on lymphocytes or other immune cells; ep.: epithelial; HLA-DR: human leukocyte antigen; c-kit: cytokine receptor expressed on the surface of mast/stem cells; SD: standard deviation; CD19/CD38: B lymphocytes identified by a combination of anti-CD19 and anti-CD38 antibodies.

estimated index of inflammation/inflammatory cell infiltration for all samples was 1.55 ± 0.74.

Extracellular matrix proliferation (collagen) was scored as follows: no collagen 0; low level of collagen expression 1; moderate level of collagen expression 2; high level of collagen expression 3. The mean estimate index of extracellular matrix proliferation for all samples was 1.31 ± 0.60.

Finally E-cadherin expression was scored as follows: no expression, 0; low level of expression, 1; moderate level of expression, 2; high level of expression, 3. We used the independent group t-test and the Mann-Whitney U test to statistically analyze the results, and standard statistical methods to calculate arithmetical mean, Spearman correlation analysis was used to test for correlation between two cholesteatoma characteristics. A p value less than 0.05 was considered to be significant in all statistical analyses, while a p value less then 0.05 and more then 0.01 was considered to be highly significant result and a p value less then 0.005 was considerate to be very highly significant result.

Results

E-cadherin immunoreactivity was observed as yellow-brown staining of the E-cadherin protein in the cholesteatoma matrix between keratinocytes

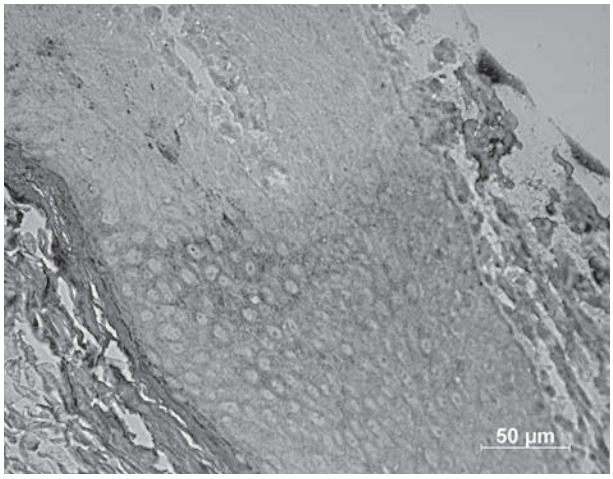

Figure 1

Expression of E-cadherin in the cholesteatoma matrix, showing areas in which staining is intense and barely detectable.

(Figure 1). Figures 2, 3 and 4 show representative examples of low, moderate and high E-cadherin expression.

E-cadherin expression in the cholestatoma for all samples was as follows: no expression 2 samples; low expression 19 samples; moderate expression 5 samples; and high expression 4 samples. The semi-quantitative mean score for E-cadherin expression was 1.40 ± 0.83 in cholesteatomas showing more bone destruction, and 1.31 ± 0.63 in those showing less bone destruction. Similar results were found for cases with or without infection and with more or less cholesteatoma extension (Table 3). The mean score for E-cadherin expression for all cases was 1.37 ± 0.73.

There were no statistically significant correlation between of E-cadherin expression and keratinisation, inflammation or extra cellular matrix proliferation ($p > 0.05$), (Table 4). In addition there is no statistically significant correlation between E-cadherin expression and epithelial CD3- and CD8-positive lymphocytes and macrophages ($p > 0.05$). There was a significant correlation between E-cadherin expression and the presence of other types of lymphocytes ($p > 0.05$) and very highly significant correlation between E-cadherin expression and mast cells and antigen-presenting cells, including Langerhans cells ($p < 0.005$), (Table 5).

Discussion

There were no differences in the expression of E-cadherin according to the degree of bone

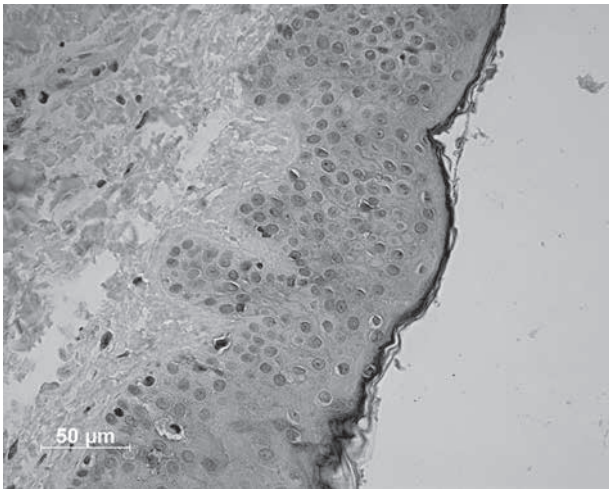

Figure 2
Low E-cadherin expression, which was the most frequent finding in the cholesteatomas analyzed in this study.

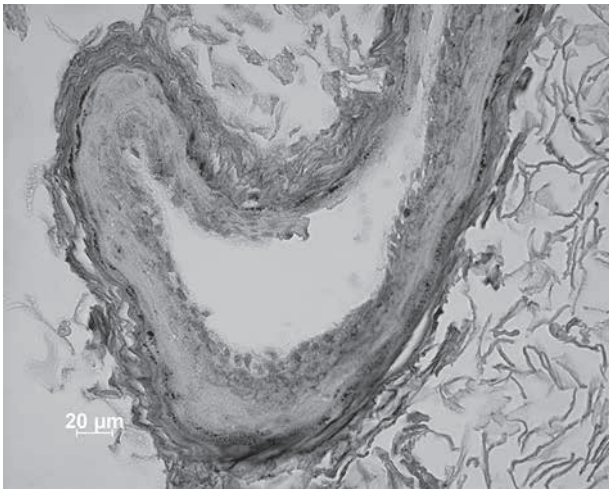

Figure 3
Moderate E-cadherin expression in the basal and suprabasal layers of a cholesteatoma.

destruction, the presence of bacterial infection or the degree of cholesteatoma extension in this study. In addition, there were no statistically significant correlations between E-cadherin expression and the histological characteristics of cholesteatoma. Naim *et al*.[5] reported about irregular tissue architecture in the E-cadherin-beta-catenin system in external auditory canal cholesteatoma compared with normal epithelium that might result in unregulated desquamation and the accumulation of keratinocytes and other cellular debris. The present study did not compare the cholesteatoma samples with samples taken from normal epithelium, but we

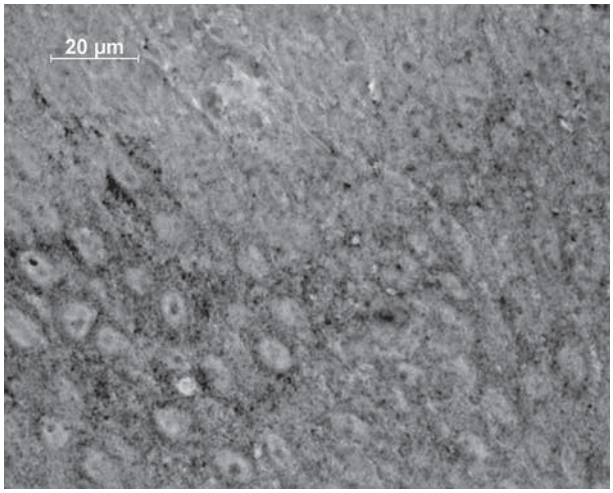

Figure 4
High E-cadherin expression on epithelial cells in the cholesteatoma matrix.

found that epithelial E-cadherin expression was the same in all examined cholesteatomas. Thus, in contrast to tumour cells, the cells in cholestatomaa appear to have maintained normal intercellular communications. Recent studies revealed a reduced (<35%) membranous staining of E-cadherin in stages IV and V nasopharyngeal carcinoma compared with normal nasopharyngeal epithelium. This loss of E-cadherin expression in nasopharyngeal carcinomas could be involved in tumour aggressiveness, especially in high-grade invasive tumours that show metastasis.[6-8] E-cadherin may also important for tumour prognosis. Davies *et al*.[8] recently reported that E-cadherin expression is induced in well-differentiated ovarian cancers.

E-cadherin expression was correlated strongly with the number of mast cells and antigen-presenting cells, including Langerhans cells in the cholesteatomas in our study. Increased expression of E-cadherin on antigen-presenting cells and mast cells suggests that the adhesion molecule E-cadherin plays a crucial role in the early immune response in cholesteatoma development. Antigen presenting cells internalize antigens and display a portion of the antigen on the cell surface, while Langerhans cells are dentritic cells in the skin and mucosa that contain large Birbeck granules. These cells are present in all layers of cholesteatoma matrix, but are most prominent in the middle layers of stratum spinosum. Langerhans dentritic cells in tissue are involved in antigen capture, uptake and processing. Once dentritic cells arrive in the secondary lym-

Table 3

Semi-quantitative index of E-cadherin expression according to cholesteatoma clinical characteristics

Cholesteatoma characteristics	Group of patients	Index (average ± SD)	Statistical significance
Bone destruction	A more	1.31 ± 0.63	P >0.05
	B less	1.40 ± 0.83	
Infection	C presence	1.35 ± 0.75	p >0.05
	D absence	1.38 ± 0.70	
Cholesteatoma extension	E more	1.28 ± 0.75	p >0.05
	F less	1.50 ± 0.71	

SD: standard deviation; p: p value.

Table 4

Correlation between E- cadherin expresiion and cholesteatoma histological characteristics of cholesteatoma

Index	Index of keratinizations	Index of inflammatory process	Index of extracellular matrix proliferation
Index of E-cadherin expression	r = -0.11 p>0.05	r = 0.18 p>0.05	r = 0.16 p>0.05

r: relation between two characteristics; p: p value.

Table 5

Correlation between E- cadherin expression and cholesteatoma immunohistochemical characteristics of cholesteatoma

Index of E-cadh. expr.	CD3 total	r = 0.40	p<0.05 *
Index of E-cadh. expr.	CD3 ep.	r = 0.21	p>0.05 NS
Index of E-cadh. expr.	CD4	r = 0.35	p<0.05 *
Index of E-cadh. expr.	CD8 total	r = 0.44	p<0.01 **
Index of E-cadh. expr.	CD8 ep.	r = 0.23	p>0.05 NS
Index of E-cadh. expr.	CD19 / CD38	r = 0.43	p<0.01 **
Index of E-cadh. expr.	HLA-DR total	r = 0.51	p<0.005 ***
Index of E-cadh. expr.	HLA-DR ep.	r = 0.45	p<0.01 **
Index of E-cadh. expr.	CD68	r = 0.22	p>0.05 NS
Index of E-cadh. expr.	CD1a	r = 0.32	p<0.05 *
Index of E-cadh. expr.	c-kit	r = 0.48	p<0.005 ***

CD: cluster of differentiation receptor on lymphocytes or other immune cells; HLA-DR: human leukocyte antigens; c-kit: cytokine receptor expressed on the surface of mast/stem cells; r: relation between two characteristics; p: p value; NS: non-significant; *: statistically significant correlation; **: highly statistically significant; ***: very highly statistically significant correlation.

phoid tissue, however, they lose these properties while gaining the capacity to interact with naïve T-cells. Langerhans cells also stimulate and activate T-cells in the immune response in cholesteatoma. Through cell-to-cell interaction, Langerhans cells play a key role in skin-related disorders, including cholesteatoma.[9,10] Mast cells from the progenitor cells of the bone marrow are found in cholesteatoma perimatrix, under the epithelium and close the blood vessels, nerves and lymph tissue.[11] Mast cells are round cells with many lipid organelles and granules in the cytoplasm that are rich in histamine and heparin. Histamine dilates postcapillary venules, activates the endothelium, and increases blood vessel permeability. Some cytokines from lymphocytes, such as IL-3, activate mast cells to release the granules.[11] Mast cells produce cytokines, leukotrienes, prostaglandins, thrombin activates factor, and other factors involved in cholesteatoma immunopathology. Antigen-presenting cells are mostly detected in the suprabasal layer of the cholesteatoma matrix. In our study, mast cells were detected in the

suprabasal layer, too, which is unusual. Albino et al. reported that 19-34% of mast cells were within the suprabasal layer of the cholesteatoma matrix, a phenomenon observed only in grossly inflamed tympanic membrane specimens but not in other control tissues.[11] E-cadherin is responsible for cell-to-cell communication and probably for mast cell migration Naim *et al*.[12,13] reported negative immunostaining for beta-catenin in the suprabasal layers of external auditory canal cholesteatomain contrast to the expression of E-cadherin we found in this study. We found no differences in E-cadherin expression according to the cholesteatoma patient age, in agreement with Welkoborsky *et al*.,[14] who found no differences in cell adhesion molecule expression in pediatric and adult cholesteatoma patients.

Conclusion

All 30 of the cholesteatomas we analyzed showed E-cadherin expression. There were no differences in E-cadherin expression according to the clinical and histological characteristics of the cholesteatoma. We found that E-cadherin was expressed at similar levels in the epithelial cells of all of the cholesteatomas, implying normal intercellular communication. E-cadherin plays an important role in immune response, but these results show that its expression is not correlated with the clinical or histological characteristics of cholesteatomas.

Aknowledgement

I wish to thank to all my colleagues and nurses in ENT Clinic, Institute of Pathology and Institute of Medical Research in Military Medical Academy for helping me in this investigation.

References

1. Shih HC, Shiozawa T, Miyamoto T, Kashima H, Feng YZ, Kurai M, Konishi I. Immunohistochemical expression of E-cadherin and beta-catenin in the normal and malignant human endometrium: an inverse correlation between E-cadherin and nuclear beta-catenin expression. *Anticancer Res*. 2004;24(6):3843-3850.
2. Takeichi M, Watabe M, Shibamoto S, Ito F, Oda H, Uemura T, Shimamura K. Dynamic control of cell-cell adhesion for multicellular organization [Article in English, French]. *C R Acad Sci III*. 1993;316(9):813-821.
3. Jacob R, Welkoborsky HJ, Mann W. Epithelium-stroma interaction in cholesteatoma of the middle ear [in German]. *Laryngorhinootologie* 2001;80(1):11-17.
4. Vercruysse JP, De Foer B, Somers T, Casselman J, Offeciers E. Magnetic resonance imaging of cholesteatoma: an update. *B-ENT*. 2009;5(4):233-240.
5. Naim R, Sadick H, Schafer C, Hormann K. External auditory canal cholesteatoma: analysis of the integrity of tissue structure. *Int J Mol Med*. 2004;14(4):601-604.
6. Zheng Z, Pan J, Chu B, Wong YC, Cheung AL, Tsao SW. Downregulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. *Hum Pathol*. 1999;30(4):458-466.
7. Zvrko E, Mikic A, Vuckovic Lj, Knezevic M, Djukic V. The study of E-cadherin expression in glottic laryngeal squamous cell carcinoma [in Serbian]. *Serbian Journal of Experimental and Clinical Research*. 2009;10(3):89-94.
8. Davies BR, Worsley SD, Ponder BA. Expression of E-cadherin, alpha-catenin and beta-catenin in normal ovarian surface epithelium and epithelial ovarian cancers. *Histopathology*. 1998;32(1):69-80.
9. Takahashi S, Nakano Y. Immunohistochemical demonstration of Langerhans'cells in cholesteatoma using an antiserum against S-100 protein. *Arch Otorhinolaryngol*. 1989;246(1):48-52.
10. Hussein MR, Saved RH, Abu-Dief EE. Immune cell profile in invasive cholesteatomas: preliminary findings. *Exp Mol Pathol*. 2010;88(2):316-323.
11. Albino AP, Reed JA, Bogdany JK, Sasson J, Parisier SC. Increased numbers of mast cells in human middle ear cholesteatoma: implications of treatment. *Am J Otol*. 1998; 19(3):266-272.
12. Naim R, Riedel F, Bran G, Sadick H, Hormann K. Cell adhesion proteins in external auditory canal cholesteatoma. *HNO*. 2004;52(12):1083-1087.
13. Naim R, Riedel F, Bran G, Hormann K. Expression of beta catenin in external auditory canal cholesteatoma. *Bio factors*. 2003;19(3-4):189-195.
14. Welkoborsky HJ, Jacob RS, Hinni ML. Comparative analysis of the epithelium stroma interaction of acquired middle ear cholesteatoma in children and adults. *Eur Arch Otorhinolaryngol*. 2007;264(8):841-848.

Dr. Milan Erdoglija
Department of Otorhinolaryngology, Otology Unit
Military Medical Academy
Crnotravska 17
11040 Belgrade, Serbia
Tel.: +381 11 3608524
Mobile phone: +381 65 8316009
E-mail: milan.erdoglija@gmail.com