

Comparative immunohistochemical study of acquired cholesteatoma in children and adults

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Objective

To compare the histopathological structure and immunohistochemical characteristics of acquired cholesteatoma in children and adults.

Patients and methods

This study was carried out on 40 patients presenting with cholesteatomatous middle-ear disease. Twenty patients were of a pediatric age group (<18 years) and the rest were adults (>18 years). Patients were admitted to the ENT Department of Alexandria University Hospital. All cholesteatoma specimens were collected intraoperatively and preserved for histopathological examination and immunohistochemical technique using the epidermal growth factor (EGF) monoclonal antibody.

Results

Histopathological examination of the submitted specimens showed that strips of stratified squamous epithelium with the underlying tissues were fibrous in adults, whereas cellular inflammatory infiltrates were observed in children. The degree of fibrosis was significantly higher in the adult group, whereas the pediatric group had higher inflammatory infiltrate. Immunohistochemical examination indicated significantly higher expression of EGF in children compared with adults both in the matrix and in the perimatrix of acquired cholesteatoma.

Conclusion

Children with acquired cholesteatoma had higher inflammatory infiltration and significant expression of EGF in both the matrix and the perimatrix with less fibrosis compared with adults, explaining the possible pathogenesis of aggressive behavior of cholesteatoma in children.

Keywords:

cholesteatoma, epidermal growth factor, immunohistochemistry

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Introduction

Cholesteatoma occurs when stratified squamous keratinizing epithelium accumulates in the middle ear or other pneumatized portion of the temporal bone. The term aural distinguishes this type of cholesteatoma from a similar pathologic entity that occurs outside the temporal bone. Cholesteatoma can be classified into congenital or acquired [1]. The annual incidence of cholesteatoma is about 3/100 000 in children and 9.2/100 000 in adults [2].

Cholesteatoma appears as a benign keratinizing squamous cell cyst made up of three components: desquamated keratin, matrix, and perimatrix. The matrix contains keratinizing squamous epithelium lining a cyst-like structure. The perimatrix known as lamina propria is the peripheral part of cholesteatoma that consists of granulation tissue and cholesterol granules [3].

Aural cholesteatoma is characterized by hyperproliferation of keratinizing squamous epithelium in the middle ear cleft. Recent research has attributed an

important role in keratinocyte proliferation to numerous growth factors and cytokines, and one of these is the epidermal growth factor (EGF) [4–7].

EGF, a single-chain polypeptide consisting of 53 amino acids, can stimulate the growth and differentiation of a variety of mammalian cells, including epithelial cells. Its expression in keratinocytes may be considered a marker indicating the state of proliferation and terminal differentiation [8].

Childhood cholesteatoma has long been known to be more aggressive than the adult form, with a poorer clinical prognosis, because of the higher rates of residual and recurrent disease compared with adults, possibly because of anatomic and physiologic differences. Eustachian tube anatomy and dysfunction

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predispose children to more frequent infections and retraction pockets, and well-pneumatized mastoids in children allow for more extensive disease compared with more sclerotic mastoid bones. Another explanation could be the higher proliferative activity of cholesteatoma keratinocytes in children compared with adults [9–11].

In this study, we attempted to investigate the histologic and cellular phenomena underlying the development of cholesteatoma and its differential aggressiveness between children and adults. Using immunohistochemistry, we attempted to elucidate the site and degree of EGF localization in acquired cholesteatoma in adults and children.

Patients and methods

This study was carried out on 20 children (<18 years) and 20 adults (1<8 years) with acquired cholesteatoma admitted to the Department of Otorhinolaryngology of Alexandria University Hospital between July 2012 and September 2013. All patients with congenital cholesteatoma and recurrent cholesteatoma were excluded from this study.

These patients were evaluated by a detailed assessment of history, a thorough otological examination, an audiological assessment, and computed tomography scan of petrous bone.

Tympanomastoidectomy was performed for all patients and cholesteatomas were exenterated. Specimens were harvested after surgical procedures and sent to the laboratory for pathological analysis of the degree of inflammation, fibrosis, localization, and degree of staining of EGF.

Biopsies were cut into 5 µm thick-sections from formalin-fixed paraffin-embedded blocks of specimens. Sections were stained with the following stains:

- (1) Conventional H&E stain for histopathological examination under light microscopy.
- (2) Masson trichrome stain for better evaluation of fibrosis.
- (3) Immunohistochemical staining for the EGF monoclonal antibody.

A primary polyclonal antibody was used, which is an EGF as a proliferation marker of cholesteatoma. The expression of EGF was visualized using the peroxidase–antiperoxidase technique, which was

performed according to the manufacturer's protocol (Lab Vision Corporation/Neo Markers, Fremont, California, USA).

Specimens were immediately fixed in 10% buffered formalin for 2 days, dehydrated through a graded ethanol series, and embedded in paraffin. Dewaxed paraffin sections (5 µm thicknesses) were subjected to the peroxidase–antiperoxidase technique. Then, the specimen was mounted in coated slide and stained by polyclonal antibodies directed EGF as the primary antibodies. They were then deparaffinized in xylene (two changes, 10 min each). The sections were then rehydrated by washing in descending grades of alcohol (two changes, 5 min each) and finally brought to water for another 5 min. Sections were not allowed to dry from this point on. After deparaffinization and hydration, sections were incubated with 10% normal goat serum to block nonspecific binding for 5 min. Blocking of endogenous peroxidase activity was performed by incubation in a 0.3% H₂O₂ solution in water for 10 min. This also reduced any nonspecific background staining. Sections were then incubated at 40°C overnight with a mixture of primary antibodies.

The next day, slides were brought to room temperature, and then subsequently the secondary antibody was applied for 30 min. After washing, the linking agent was used in a humidity chamber at room temperature unless otherwise specified. Two consecutive washes in PBS were performed between two successive steps unless otherwise stated (PBS was prepared with 0.4 g potassium dihydrogen orthophosphate and 7.2 g sodium phosphate bibasic anhydrous in 1000 ml distilled water, 0.01 mol/l, pH 7.2). Then, sections were subjected to pretreatment for epitope retrieval; antigen retrieval was performed by microwave heating in 10 mol/l citrate buffer. The sections were reacted with an extra-avidin conjugate such as chromogen for 10 min in the dark. The sections were well rinsed in tap water and lightly counterstained with Harris hematoxylin. Slides were dehydrated in ascending grades of alcohol and then cleared in xylene, and coverslips were added.

Control staining was performed by substituting normal serum immunoglobulin G for the primary antibodies. All specimens were also stained with hematoxylin and eosin for a conventional histological examination.

Statistical analysis

The Mann–Whitney *U*-test was used for the statistical analysis and a *P*-value of less than 0.05 was defined as a statistically significant difference.

Results

Forty operative specimens were obtained and examined histopathologically and immunohistochemically for the role of EGF in cholesteatoma in two groups of patients. One group included 20 pediatric patients; their ages ranged from 8 to 18 years, with an average of 13.63 ± 3.53 years. The other group was the adult group and included 20 patients with an age range between 21 and 48 years, and an average age of 30.63 ± 9.08 years. In the pediatric group, 65% were males, whereas 35% were females. However, in the adult group, 40% were males and 60% were females.

Histopathological examination

Microscopic examination of the collected specimens showed strips of stratified squamous epithelium devoid of rete ridges. The contents of the cysts were lamellar keratin with numerous ghost cells (keratinized denuded cells with an unstained, shadowy center).

The perimatrix was fibrous and showed a chronic nonspecific inflammatory cellular infiltrate, mostly lymphohistiocytic.

Scoring of the grade of fibrosis in children and adult cases was performed, where the adult cases showed a significantly higher grade of fibrosis compared with the pediatric cases ($P=0.001$) (Fig. 1a and b). The mean score of fibrosis was 44.9 ± 3.02 in the adult cases, whereas in the pediatric cases, it was 15.05 ± 3.07 .

However, the intensity of the inflammatory infiltrate in the pediatric group was higher than that observed in the adult group (Fig. 1).

Immunohistochemical examination

All the specimens collected were stained immunohistochemically for EGF expression. EGF immunoreactivity was nuclear. Positivity was observed in both groups.

No significant difference in EGF immunoreactivity was found between the matrix and the perimatrix in each group.

In the matrix

EGF immunoreactivity was significantly higher in pediatric patients compared with the adult patients ($P<0.001$). The level of EGF staining in the matrix in children ranged between 32.0 and 45.0, with a mean of 37.7 ± 3.31 , whereas in adults, the range was 20.0–30.0, with a mean of 25.25 ± 2.61 (Fig. 2) (Table 1).

In the perimatrix

EGF positivity in the perimatrix of the pediatric cases was significantly higher than that found in the adult cases ($P<0.001$). EGF positivity in children ranged between 33.0 and 49.0, with a mean of 38.8 ± 4.82 , whereas in adults, it ranged between 22.0 and 29.0, with a mean of 25.40 ± 2.11 (Fig. 3) (Table 1).

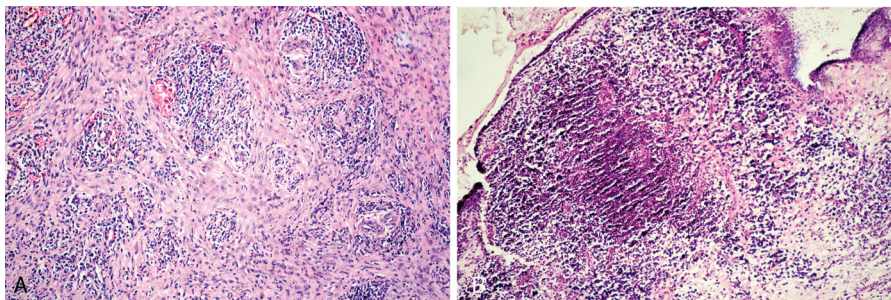
Discussion

Cholesteatoma is characterized by cellular hyperproliferation. The pathobiologic cause for the observed increase in the proliferation of cholesteatoma is not completely understood.

Acquired cholesteatoma is more aggressive in children. The reason for the difference in behavior is still unclear. Several studies have sought a physiopathological explanation for this finding. According to Quaranta *et al.* [12], the explanation lies in the cholesteatoma perimatrix. Following up this hypothesis, they reported that the number of mononuclear cells' infiltration (plasmocytes, lymphocytes, macrophages, granulocytes, and giant cells) in the perimatrix was greater in children than in adults, with evidence of enzyme–collagenase activity.

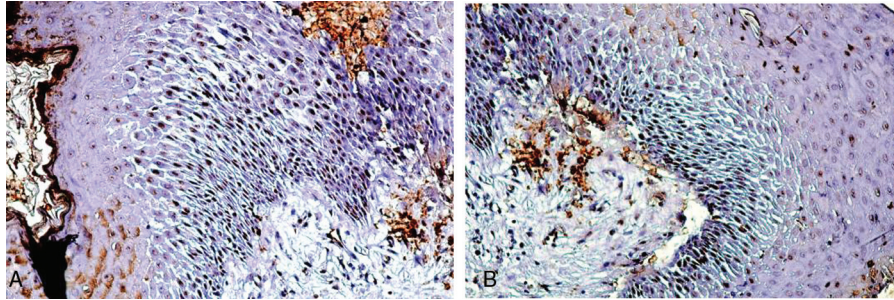
Analysis of the expression of MIB1 (a monoclonal antibody marker of cell proliferation) in child and

Figure 1



Cholesteatoma in (left) adult group and (right) pediatric group. Note the higher grade of fibrosis in the adult group as compared to the pediatric case as well as the greater intensity of the inflammatory Cellular infiltration in the pediatric case as compared to the adult case (H & E, $\times 200$).

Figure 2



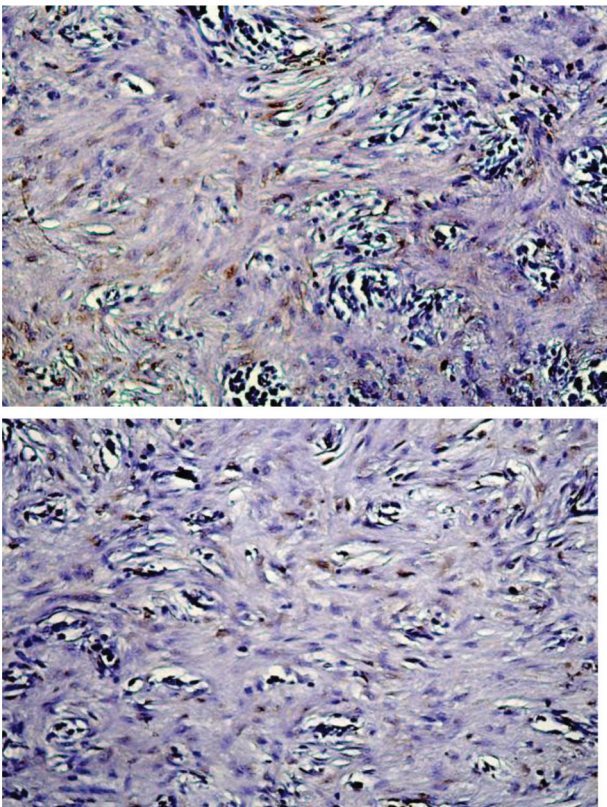
Note the difference in the numbers of positive cells in the matrix of cholesteatoma in (left) children and (right) adults. (EGF monoclonal antibody, streptavidin-peroxidase technique, $\times 100$).

Table 1 Comparison between epidermal growth factor expression levels in children and adult cases of cholesteatoma

	Children (n=20)	Adults (n=20)	Z	P
Matrix				
Minimum–minimum	32.0–45.0	20.0–30.0	13.202*	<0.001
Mean \pm SD	37.70 \pm 3.31	25.25 \pm 2.61		
Median	37.0	25.0		
Perimatrix				
Minimum–minimum	33.0–49.0	22.0–29.0	11.389*	<0.001
Mean \pm SD	38.80 \pm 4.82	25.40 \pm 2.11		
Median	37.50	25.0		

Z, Z for Mann–Whitney U-test; * $P\leq 0.05$, Statistically significant.

Figure 3



Two cases of cholesteatoma. Note the high EGF staining in the prematrix of cholesteatoma in (upper) children as compared to (lower) adults. (EGF monoclonal antibody, streptavidin-peroxidase technique, $\times 200$).

adult cholesteatoma and within the external auditory canal skin was carried out; the proliferation index was normal in the latter, elevated in the adults, and relatively higher in children [13].

We used histopathological and immunohistochemical examinations to evaluate the degree of inflammation, fibrosis, and localization, and the degree of expression of EGF in the matrix and perimatrix in pediatric and adult cholesteatoma.

In the present study, the examined histopathological specimens of adult cholesteatomas showed a significantly higher grade of fibrosis compared with the pediatric cases ($P=0.001$), whereas the intensity of the inflammatory infiltrate in the pediatric cases was higher than that observed in the adult cases. The higher degree of fibrosis in the adult cholesteatomas specimens indicates that the adult cholesteatoma is more in the reparative process and less invasive, whereas the inflammatory process is more exacerbated in children, a characteristic that could be responsible for the higher degree of aggressiveness of the pediatric cholesteatomas.

Several studies have focused on the correlation between the histopathological structure of cholesteatoma and its aggressive behavior in children. They compared the histopathological components, perimatrix thickness,

and degree of inflammation between pediatric and adult cholesteatomas. Dornelles Cde *et al.* [14] reported no differences in the histological components of acquired cholesteatomas in adults and children. These results were not similar to the results in the present study.

Welkoborsky *et al.* [15] reported no significant differences between pediatric and adult cholesteatomas on the cellular level, concluding that the aggressive behavior of pediatric cholesteatoma may be explained by middle-ear ventilation disturbance or the decreased calcium salt content of pediatric bone.

Different growth factors and cytokines have been identified to be involved in the pathogenesis of cholesteatoma. EGF, transforming growth factor- α , and interleukin 1 seem to be responsible for the increased proliferation rate of keratinocytes [12–15].

EGF stimulate the proliferation and differentiation of epidermal cells, fibroblasts, and endothelial cells. EGF are present in all layers of the matrix and perimatrix in the cholesteatoma.

There have been several researches on the relation between EGF overexpression and growth of cholesteatoma. Yetiser *et al.* [16] showed that 75% of cholesteatoma keratinocytes express EGFR as opposed to only 10% of normal canal keratinocytes, concluding that the aggressive and destructive behavior of cholesteatoma is likely to be mediated by cytokines and EGF.

Also, Goto [17] proved that the EGF content of cholesteatoma is higher than that of normal skin and EGF in-situ may be related strongly to the growth and bony destruction of cholesteatoma. He also found that the difference in EGF immunoreactivity between active and inactive cholesteatoma was greater in the fibroblast in the subcutaneous tissue of cholesteatoma than in the epidermis. This is the reason why the activity of cholesteatoma exists in the subcutaneous tissue. These results suggest that EGF plays an important role in accelerating the growth of cholesteatomas.

In this study, we investigated the differential distribution of EGF using immunohistochemistry in the matrix and the perimatrix of cholesteatoma in both adult and pediatric patients in an attempt to find clues to explain the aggressive behavior of pediatric cholesteatoma.

EGF-positive staining was present in both groups in this study; however, there was a significantly higher level in cholesteatomas in children than in adults. The results from the current study suggest that EGF influence the development of cholesteatoma in the two groups, but to a higher degree in children, and may be responsible for the more aggressive behavior of cholesteatoma in children.

Our results are not in agreement with the findings obtained by Alves *et al.* [18], who studied the presence of EGFR in samples of acquired aural cholesteatoma collected from 50 (35 adults and 15 children) patients and correlated the expression of this receptor with patients' ages; they found no statistically significant differences in age-related variances in EGFR expression.

Liu *et al.* [19] studied the effects of EGF on the proliferation of keratinocytes and EGF-mediated signaling pathways underlying the pathogenesis of cholesteatoma and found that EGF led to the activation of the EGFR/PI3K/Akt/cyclinD1 signaling pathway, which played a critical role in EGF-induced cell proliferation, suggesting that inhibition of this pathway may be used for the development of potential therapeutic targets for intratympanic drug therapy for cholesteatoma.

Conclusion

The degree of fibrosis was significantly higher in adult specimens compared with pediatric specimens. This may indicate that adult cholesteatoma is more in reparative process and is less invasive. EGF expression was significantly higher in the matrix and perimatrix of pediatric cholesteatomas compared with adult cholesteatomas. This may be correlated to the aggressiveness of pediatric cholesteatomas.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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