Expression of Epidermal Growth Factor Receptor in Human Middle Ear Cholesteatoma

Ying-Che Hsu, Kuen-Yao Ho, Chee-Yin Chai, Ka-Wo Lee, Ling-Feng Wang, Shu-Chuan Wu, Wen-Wei Kuo, and Shin-Meng Tsai
Departments of Otolaryngology, Pathology, Surgery, and Public Health, Kaohsiung Medical University, Kaohsiung, Taiwan.

Middle ear cholesteatoma is characterized by the presence of keratinizing epithelium, which may be a congenital remnant or due to migration from the eardrum epithelium into the mesotympanum. Cholesteatoma epithelium has different characteristics to normal epidermal epithelium, in that it is hyperproliferative and has locally invasive growth and induced resorption of auditory ossicles and surrounding bone tissue [1]. Recent research has attributed an important role in keratinocyte proliferation to numerous growth factors and cytokines, one of which is epidermal growth factor (EGF) and its receptor, EGF-receptor (EGFR). EGFR is a 170 kd to 180 kd glycoprotein with an intrinsic tyrosine-specific protein kinase that is stimulated by binding its stimulator (EGF) and transforming growth factor-alpha (TGF-α) [2]. The density of EGFR in a cell is related to the state of differentiation and the ability of the keratinocytes to differentiate [3]. EGFR expression in keratinocytes may be considered a marker indicating that the cells are in a state of proliferation and poor terminal differentiation [4]. Using immunohistochemistry, we investigated the presence of EGFR in cholesteatoma and delineated the role that EGFR may play in the growth of cholesteatoma.

Key Words: epidermal growth factor receptor, middle ear cholesteatoma

Materials and Methods

Cholesteatoma specimens were obtained from 29 patients (mean age, 38 years; range, 19–51 years) who underwent middle ear surgery at the Department of Otolaryngology,
Kaohsiung Medical University Hospital, Taiwan. Skin from the postauricular area of 34 patients (mean age, 43 years; range 19–67 years) who underwent middle ear surgery for chronic otitis media was collected during the same period to provide a control group. Immediately after surgery, tissue specimens were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin, and examined histopathologically.

**Immunohistochemical staining for EGFR**

Specimens were fixed in 10% buffered formalin solution overnight, subjected to dehydration and paraffin embedding, and sliced into 4 µm sections. After deparaffinization and washing, endogenous peroxidase in sections was inhibited using 0.3% methanolic hydrogen peroxide and then blocked with 10% normal horse serum and 1% bovine serum albumin for 30 minutes. After a brief rinse, the sections were incubated with EGFR monoclonal antibody (1:20, BioGenex Laboratories Inc, San Ramon, CA, USA) at room temperature for 120 minutes. The sections were washed in phosphate-buffered saline, incubated for 30 minutes with biotinylated anti-mouse immunoglobulin (Ig) G, and then treated with avidin-biotin complex for 30 minutes. Finally, 3,3'-diaminobenzidine was applied as a chromogen and sections were visualized after counterstaining with Mayer’s hematoxylin. EGFR labeling (in brown color) was considered positive if there was at least one EGFR-positive cell in each epithelial layer. EGFR expression in breast cancer cells was used as a positive control and the omission of primary antibody was used as a negative control. The difference in distribution patterns in the basal, parabasal, and upper layers of the epithelial tissue between the two groups was assessed using the Chi-squared test.

**RESULTS**

EGFR-positive cells in breast cancer sections were brown in color. Two patterns of immunoreactivity were recognized: cell-membrane staining and granular cytoplasmic staining (Figure 1). There was no recognizable brown color in sections where the primary antibody had been omitted.

EGFR expression in cholesteatoma epithelium was mainly associated with the cell membrane and was localized in the basal, parabasal, and upper layers (Figure 2), similar to the pattern in the positive control (Figure 1). Of the 29 cases of cholesteatoma, 23 (79%) were positive for EGFR expression in the basal layer of the epithelium. Nineteen cases (66%) were positive for EGFR in the parabasal layer and 18 cases (62%) in the upper layer. The immunoreactivity was most intense in the basal layer, and decreased in distribution towards the upper layer (Table 1).

EGFR expression in postauricular skin was localized in the basal to upper layers (Figure 3). Of the 34 normal postauricular skin samples, 29 (85%) were positive for EGFR expression in the basal layer, 27 (79%) in the parabasal layer, and 27 (79%) in the upper layer (Table 2). The distribution of EGFR-positive cells decreased from the basal layer towards the superficial layer, but no difference between the parabasal and upper layers was observed.

**Figure 1.** (A) Epithelial growth factor receptor (EGFR) immunostaining in breast cancer (positive control). Positive staining is judged by the brown color in sections. Immunoreactive sites are recognized in the cell membrane (arrow) and granular cytoplasm. (B) When primary antibody for EGFR is omitted in the same section, there is no recognizable brown color. (Original magnification ×220.)
There was EGFR expression in more cells in normal postauricular skin than in cholesteatoma skin, but there was no statistically significant difference in each layer between the two groups (Table 3).

**Discussion**

Histologically, cholesteatoma is composed of an accumulation of desquamated keratin arising from squamous epithelium. The growth of cholesteatoma seems not to cease and may be recurrent for many years postoperatively unless the cholesteatoma is totally eradicated. The clinical characteristics of cholesteatoma indicate that it has hyperproliferative ability, and autocrine growth stimulation by TGF-α and EGFR may contribute to

**Table 1. Distribution of epithelial growth factor receptor in middle ear cholesteatoma (29 cases)**

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Strongly positive/positive n (%)</th>
<th>Negative n (%)</th>
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<tbody>
<tr>
<td>Upper layer</td>
<td>18 (62)</td>
<td>11 (38)</td>
</tr>
<tr>
<td>Parabasal layer</td>
<td>19 (66)</td>
<td>10 (34)</td>
</tr>
<tr>
<td>Basal layer</td>
<td>23 (79)</td>
<td>6 (21)</td>
</tr>
<tr>
<td>Total</td>
<td>23 (79)</td>
<td>6 (21)</td>
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Figure 2. (A) Epithelial growth factor receptor (EGFR) immunostaining in cholesteatoma epithelium. Positive staining is associated with the cell membrane and is found in the basal, parabasal, and upper layers of the epithelium (arrows). (B) The same section with omission of EGFR monoclonal antibody immunostaining. (Original magnification × 220.)

Figure 3. (A) Positive immunostaining for epithelial growth factor receptor (EGFR) in normal postauricular skin. EGFR-positive cells are observed in the basal (arrow), parabasal, and upper layers of the epithelium. (B) The same section with omission of EGFR monoclonal antibody immunostaining. (Original magnification × 40.)
the unrestrained growth of cholesteatoma epithelium in the middle ear cavity [5,6]. EGFR is a transmembrane glycoprotein, with a molecular weight of about 170 kd, involved in EGF- and TGF-α-stimulated cell proliferation. It activates tyrosine protein kinase, resulting in phosphorylation of itself and other substrates, leading to cell proliferation. In human skin, EGFR is found in keratinocytes, especially in the basal layer, as well as in sebocytes, smooth muscle cells (including the arrectores pilorum), and myoepithelial cells [7]. In this study, we used immunohistochemical staining to identify EGFR distribution in cholesteatoma epithelium.

In our study, the basal layers of cholesteatoma epithelium exhibited strong expression of EGFR, with less expression in the higher layers. In the epithelium of normal skin, an intensity gradient of positive EGFR immunoreactivity was observed from the basal to higher layers. The similar expression of EGFR in cholesteatoma and postauricular skin shows that the orderly pattern of positive EGFR immunoreactivity is not deranged in cholesteatoma epithelium. Groves et al examined EGFR expression in a variety of benign and malignant epithelial neoplasms, and found that there was an ordered pattern in benign tumors but a loss of membrane labeling and cytoplasmic accumulation of EGFR in malignant tumors [8]. EGFR dysregulation may be important in the development of epithelial malignancy.

There was no significant difference in EGFR distribution in cholesteatoma and postauricular skin in our study compared with other reports in the literature. Ergun et al [5] and Uno and Saito [9] reported a significant increase in EGFR in cholesteatoma compared to postauricular skin. Bujia et al reported heterogeneity in EGFR expression in different parts of cholesteatoma [10], which suggests the presence of aberrant regulation. Later, Bujia et al used enzyme-linked immunosorbent assays (ELISAs) to confirm that the abnormal growth of cholesteatoma epithelium was reflected in aberrant expression of EGFR [11]. Relative differences have been reported in the intensity of EGFR expression in epidermal layers (basalis > spinosum > granulosum > corneum) [12]. The results of our study are different, possibly because the incubation time for sections and monoclonal antibody was longer than in previous reports: 120 minutes compared to 30 minutes [5,10]. The longer incubation time may increase the binding of monoclonal antibody to receptors in the superficial layer of normal skin, leading to changes in positive rates. We divided the intensity of immunohistochemical staining into negative and positive groups (including strongly positive immunoreactivity). Uno and Saito [9] and Bujia et al [10] included an additional subgroup for weakly or questionably positive immunoreactivity. Our study may have categorized weakly positive staining in normal skin epidermis into the positive group, resulting in no statistical difference between cholesteatoma and postauricular skin.

Kojima et al reported an increase in the rates of proliferation and apoptotic cell death in cholesteatoma epidermis [13]. The hyperproliferative epithelium of cholesteatoma may have higher metabolic status to degrade the receptor or down-regulate EGFR by cholesteatoma-produced EGF and TGF-α. The subepithelial inflammation and probable high turnover rate in cholesteatoma epithelium may also stimulate cell apoptosis. Uno and Saito subdivided cholesteatoma into “active” and “inactive” types [9], based on the thickness of the cholesteatoma epithelium, the amount of granulation in the cholesteatoma subepithelium, and the degree of otorrhea symptoms. They reported that there was stronger immunoreactivity in active than in inactive cholesteatoma. There may have been a high proportion of inactive cholesteatoma and apoptosis in our specimens, causing no significant difference in EGFR expression

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<tbody>
<tr>
<td>Upper layer</td>
<td>27 (79) 7 (21)</td>
<td></td>
</tr>
<tr>
<td>Parabasal layer</td>
<td>27 (79) 7 (21)</td>
<td></td>
</tr>
<tr>
<td>Basal layer</td>
<td>29 (85) 5 (15)</td>
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<tr>
<td>Total</td>
<td>29 (85) 5 (15)</td>
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<table>
<thead>
<tr>
<th>Epithelium</th>
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<th>Postauricular skin n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper layer</td>
<td>18 (62) 27 (79)*</td>
<td>27 (79)*</td>
</tr>
<tr>
<td>Parabasal layer</td>
<td>19 (66) 27 (79)*</td>
<td>27 (79)*</td>
</tr>
<tr>
<td>Basal layer</td>
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*p > 0.05.
between cholesteatoma and normal skin tissue.

Park et al found that deep meatal skin has different characteristics from retroauricular skin in Mongolian gerbils, with positive staining for EGFR in the suprabasal layer of experimental cholesteatoma induced by ligation of the external auditory canal [14]. There are many theories of how the epithelium grows into the middle ear, including the metaplasia hypothesis, the retraction theory, and direct ingrowth from eardrum perforation. The squamous epithelium that migrates into the middle ear cavity uses the granulation tissue and organized effusion as a bridge, resulting in migration and formation of cholesteatoma [15]. Apart from the proliferation effect of EGFR, EGFR also supports both cell-cycle progression and survival of keratinocytes [16]. EGFR may help keratinocytes to survive and proliferate in different growth conditions, such as middle ear mucosa or even bony substrate. Kojima et al used in situ hybridization to show that there was strong signal expression of EGFR mRNA in all epithelial layers of cholesteatoma, but only in part of the basal layer of normal external ear canal skin [17]. Whether the cholesteatoma epithelium has more hyperproliferative ability than normal skin, as proposed by Anniko and Mendel [1], is controversial. Shiieh et al used immunochemistry to investigate proliferating cell nuclear antigen in cholesteatoma and to evaluate its proliferative activity [18]. Their report indicates that cholesteatoma has a similar proliferative activity to normal postauricular skin, and that cholesteatoma itself is not a tumor despite its clinical behavior, which is similar to neoplastic cells.

EGFR is not the only factor involved in the development of cholesteatoma. Studies implicate a potential idiopathic response to both internal events and external stimuli with the induction of hyperproliferative cells in the cholesteatoma epithelium. Internal stimuli such as heat shock proteins or external stimuli such as cytokines released by inflammatory cells are reported to be associated with the regulation of cellular proliferation or apoptosis in cholesteatoma [19,20]. Although there was no significant difference in EGFR expression between cholesteatoma and postauricular skin in our study, other factors may be meaningful. It is hoped that further study will provide better understanding of these interactions.

REFERENCES