Investigation of Keratinized Squamous Epithelium from Mastoid Cortical Bone Dust in Patients with or without Cholesteatoma

Original Investigation

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Abstract

Objective: This study aimed to investigate squamous metaplasia in mastoid cells of patients undergoing surgery for chronic otitis media (COM) with or without cholesteatoma. Bone dust was stained with hematoxylin and eosin (H&E) for squamous cells and keratin and immunohistochemically for p63. Additionally, the feasibility of routine pathological examination of bone dust via H&E staining was evaluated for cost-effectiveness and for identifying patient groups needing advanced follow-up.

Methods: Thirty-one patients with COM were enrolled: 14 with cholesteatoma (study group) and 17 without cholesteatoma (control group). Mastoid bone dust obtained during surgery was examined specifically for the presence of squamous cells, keratin, and p63, with evaluation performed using H&E and immunohistochemical staining techniques. Findings were compared between the study and control groups.

Results: Keratin was significantly more frequent in the study group than in controls (43% vs. 6%, p=0.01). No significant differences were observed for squamous cell (p=0.43) or p63 expression (p=0.20). However, when any of the three markers were positive, a statistically significant difference was found between the groups (43% vs. 12%, p=0.049).

Conclusion: These findings suggest that the mastoid air cell systems of patients with cholesteatoma may be affected differently prior to cholesteatoma spreading to the mastoid system. This could be linked to microcirculation of inflammatory proteins, impaired aeration, and the formation of retraction pouches. These results align with the metaplasia theory as a possible explanation for the etiopathogenesis of acquired cholesteatoma.

Keywords: Cholesteatoma, otitis media, mastoid bone, squamous metaplasia, p63 protein, immunohistochemistry

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Introduction

Cholesteatoma, a disease studied for centuries, remains enigmatic. The most discussed theories on the etiopathogenesis of cholesteatoma include invagination, basal hyperplasia, migration, and squamous metaplasia. The squamous metaplasia theory, introduced by von Tröltsch in 1864, posits that middle ear mucosa transforms into squamous epithelium under pus pressure. Wendt

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(1) later suggested that this metaplasia results from chronic inflammation, and Sadé et al. (2) demonstrated pluripotent middle ear mucosal cells transforming under inflammation. Support for the theory includes biopsies showing keratinized squamous epithelium in pediatric patients with otitis media with effusion. Despite these findings, histological proof or experimental confirmation of these mechanisms contributing conclusively to cholesteatoma formation is lacking (3-7). In our study, we examined mastoid bone dust samples histopathologically and immunohistochemically (IHC) with the hypothesis that squamous epithelial tissue undergoing metaplasia within the mastoid air cell system could be a factor for recurrences in cases where cholesteatoma is limited to the middle ear. Therefore, more emphasis was placed on this theory. Moreover, if mastoid bone dust obtained during mastoidectomy is routinely subjected to histopathological examination using only hematoxylin and eosin (H&E) staining as a cost-effective and time-efficient method, patients with positive findings could be identified earlier and directed toward closer monitoring or more advanced diagnostic evaluation.

Methods

Our study is an original research designed prospectively and conducted at Dışkapı Yıldırım Beyazıt Research and Training Hospital. Ethical approval was received from Dışkapı Yıldırım Beyazıt Research and Training Hospital (decision no: 90/18 dated: 22.06.2020).

Files of all patients who were operated on due to chronic otitis media (COM) and met the inclusion criteria for the study were scanned, and the data was recorded. The content of this data included age, sex, type of surgery performed, the side of the operation, and previous otological surgical history. Informed consent, both verbal and written, was obtained from patients before surgery. Additionally, pathologies identified in the middle ear and mastoid air cell system during the operation were recorded, and histopathological evaluations of tissue samples taken during the operation were also documented.

Inclusion Criteria: Patients with no previous history of otologic surgery and who were planned to be operated on for COM with cholesteatoma (COMC) were accepted in the study group. The cholesteatoma in the patients was classified according to the STAM grading system, with involvement restricted to the T and A subzones (8). Patients with no previous history of otologic surgery and who were planned to be operated on for COM with no cholesteatoma were accepted in the control group.

Exclusion Criteria: Patients with a history of previous otologic surgery, patients who were planned to be operated on for COM without mastoidectomy, patients with cholesteatoma in mastoid air cells, patients with congenital cholesteatoma, patients who had acquired cholesteatoma due to traumatic or iatrogenic tympanic membrane perforation were excluded from the study.

Bone dust was collected during mastoidectomy using an endotracheal suction system held vertically with an exit filter to prevent dust loss (Figures 1,2). Mastoidectomies were performed as canal wall-up (CWU) or canal wall-down (CWD) procedures without cavity obliteration. Collection began only after the mastoid cortex had been washed with saline and the infusion removed via a separate suction-to avoid contamination, and it was ceased before the external

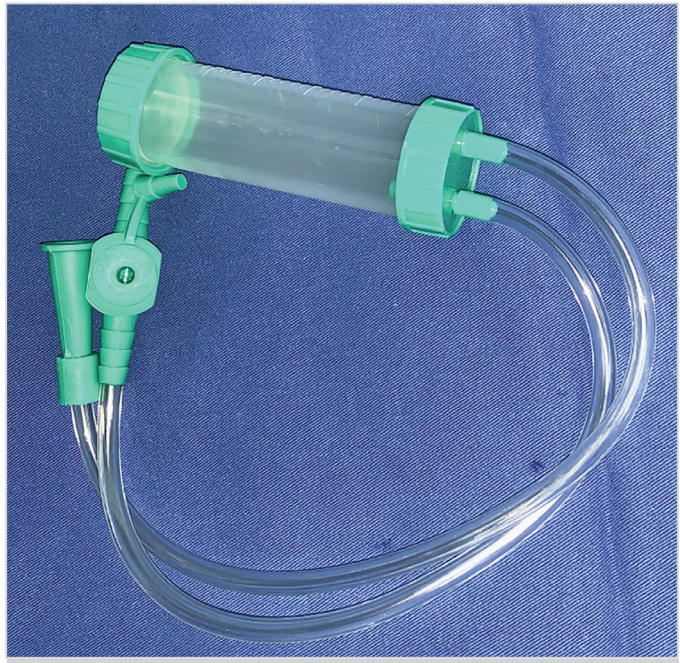

Figure 1. An illustrative image of the tracheal aspirate kit used for sample collection

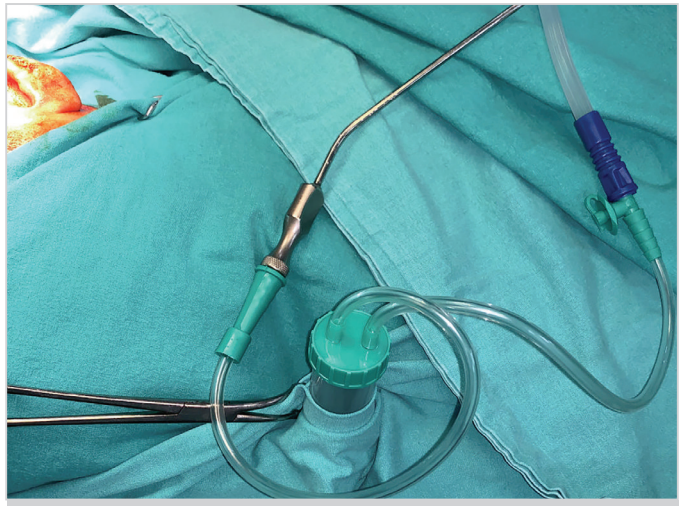

Figure 2. An illustrative image showing the stabilized position of the tracheal aspirate kit during the procedure, with the Fergusson aspirator attached at the tip

ear canal (EEC) skin incision or any intervention to the middle ear or cholesteatoma sac. The obtained bone dust was fixed in 10% neutral buffered formaldehyde and processed routinely: tissues were embedded in paraffin, sectioned at 3.5 µm, and stained with H&E using an automatic tissue staining device. All specimens were examined under a light microscope for keratin, squamous epithelium, and granulation tissue. To enhance detection of squamous epithelium, IHC staining for p63 was performed using the 4A4 clone (Ventana Benchmark XT, Roche Diagnostics, Switzerland). All mastoidectomy procedures were carried out by the same experienced surgeon. Following staining, the bone dust samples were evaluated for the presence of squamous cells (Figure 3) and keratin on H&E sections (Figure 4) and for p63 expression on IHC sections (Figure 5). Squamous cells were identified as isolated or clustered formations, while keratin was recognized by its acellular appearance and measured in micrometers. p63 expression was observed in the nuclei of squamous cells, and all parameters were recorded as either present or absent.

Statistical Analysis

Tested variables were evaluated for both groups as age, sex, IHC staining of p63, keratin presence and squamous cell presence. All the statistical analysis were run on IBM SPSS Statistics for Windows Version 26.0 software. Ordinary variables were summarized as mean ± standard deviation, median value (minimum-maximum). Groups were compared with student's t-test in ordinal variables after homogeneity tests were run and with chi-square test in nominal variables. A p-value of <0.05 was considered statistically significant.

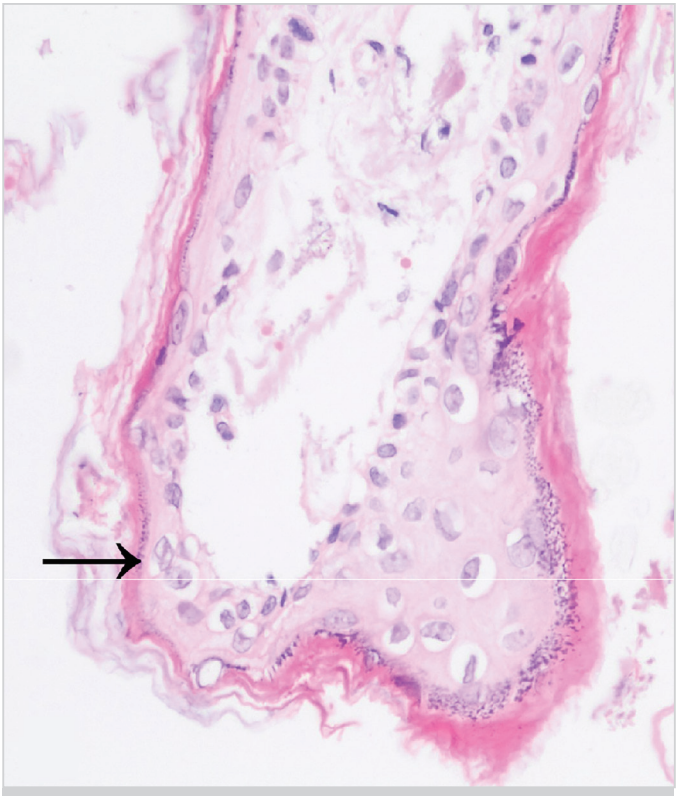

Figure 3. The images of squamous cells under a light microscope at 400 magnification field, marked with black arrow, following H&E staining
H&E: Hematoxylin and eosin

Results

Our study included 18 male and 13 female patients, totaling to 31 individuals. Mastoid bone dust was collected from all participants and subjected to histopathological examination. Based on the inclusion criteria, 14 individuals were assigned to the study group, while the remaining 17 formed the control group. The patients' ages ranged from 14 to 76 years, with a median age of 37.32±15.694 years (Figure 6).

In the study group, there were seven male patients (50%) and seven female patients (50%), with a median age of 35.14±17.244 years (Figure 6). Patient ages in this group ranged between 15 and 76 years. In the control group, there were 11 male patients (64.7%) and six female patients

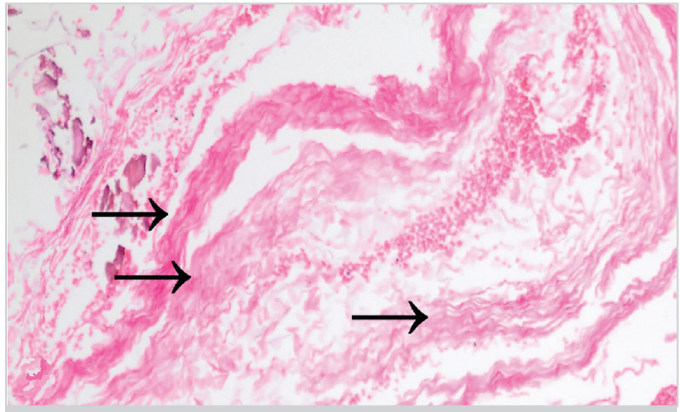

Figure 4. The image of keratin lamellae after H&E staining observed at a magnification field of ×200 under a light microscope, marked with black arrows
H&E: Hematoxylin and eosin

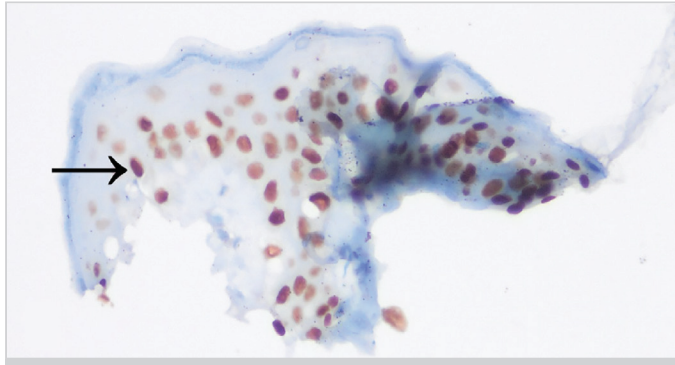

Figure 5. The image of p63 immunohistochemical staining of squamous cell nuclei at a magnification field of ×400 under a light microscope. The arrow indicates the squamous cell nucleus

Table 1. The table shows the frequency of keratin and squamous cell presence following H&E staining in the study and control groups, as well as the frequency of p63 presence after immunohistochemical staining, along with the statistical significance of intergroup comparisons

Frequency distributions of the examined parameters by groups				
Parameter		**Study group**	**Control group**	
p63	Positive	3	1	
	Negative	11	16	p=0.20
Keratin	Positive	6	1	
	Negative	8	16	p=0.014
Squamous cell	Positive	2	1	
	Negative	12	16	p=0.43
p63, keratin and squamous cell	Any one of the parameters is positive	6	2	
	All negative	8	15	p=0.049

H&E: Hematoxylin and eosin

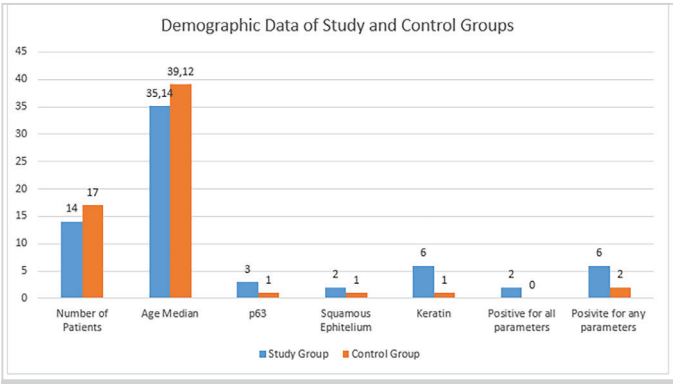

Figure 6. Comparison of demographic features and positivity rates for keratin, squamous cells, and p63 between study and control groups. Medians and positive case numbers are shown. Significant differences were found in keratin positivity and positivity for any parameter (p<0.01 and p<0.049, respectively).

(35.3%), with a median age of 39.12±14.581 years (Figure 6). Patient ages in this group ranged between 14 and 65 years. The groups were homogeneously distributed in terms of age and sex. No significant differences were observed between the groups regarding these two variables, with p-values of 0.70 and 0.41, respectively.

In the study group, 10 patients (79%) underwent CWD mastoidectomy, and four (21%) underwent CWU mastoidectomy out of a total of 14 patients. In the control group, CWD mastoidectomy was performed in five patients (29%), while CWU mastoidectomy was performed in 12 patients (71%) out of 17. A significant difference between the groups was noted (p=0.02). However, as there was no contamination during bone dust collection, we did not perform a multivariate analysis between the groups regarding surgical methods and group allocation.

In the study group, operations were performed on eight (57%) right ears and six (43%) left ears. In the control group, operations were performed on seven (41%) right ears and 10 (59%) left ears. No significant difference was found in terms of operation sides between the groups (p=0.38).

Upon evaluating the study group in terms of p63 IHC staining, while three patients (21.4%) out of 14 tested positive, 11 (78.6%) tested negative. In the control group, one patient (6%) tested positive, and 16 (94%) tested negative. There was no statistically significant difference between the groups (p=0.20, Table 1, Figure 6).

When analyzing the study group for keratin staining, six patients (43%) out of 14 had positive results, while eight (57%) had negative results. In the control group, one patient (6%) had positive staining, and 16 (94%) had negative results. Statistical analysis revealed a significant difference between the groups (p=0.01, Table 1, Figure 6).

In terms of squamous cell presence, while two patients (14%) in the study group were positive, 12 (86%) were negative. In the control group, one patient (6%) tested positive, and 16 (94%) tested negative. Statistical analysis showed no significant difference between the groups (p=0.43, Table 1, Figure 6).

If any assessed parameter (e.g., p63 IHC staining, keratin, or squamous cell presence) was positive, indicating metaplasia, six patients (43%) in the study group and two patients (12%) in the control group showed positivity for at least one parameter. Statistical analysis indicated a significant difference between the groups (p=0.049). Furthermore, two patients (14%) in the study group exhibited positivity for all parameters simultaneously, while no control group patients tested positive for all parameters (Table 1, Figure 6).

Discussion

Modern research focuses on molecular and stem cell studies to better understand the pathophysiology of cholesteatoma. In our study, we examined mastoid bone dust samples pathologically and IHC, hypothesizing that in cases where

cholesteatoma is limited to the middle ear, squamous epithelial tissue potentially undergoing metaplasia could be present in the mastoid air cell system.

In our study, a significant difference in the types of surgery performed was found between the study and control groups (p=0.02). Among the 14 patients in the study group, four underwent CWU mastoidectomy and 10 underwent CWD mastoidectomy, reflecting the more extensive surgeries required for COMC cases. To minimize epithelial contamination during CWD mastoidectomy, we avoided collecting samples while lowering the EEC's posterior wall. In CWU procedures, posterior wall incisions and drilling were deferred until the mastoid antrum was reached or mastoidectomy was completed. Consequently, mastoid bone dust collected during both types of procedures was obtained from the same sites at the same stages, eliminating contamination risk. Thus, the significant difference in surgery types did not affect study outcomes.

Cavity obliteration is a common modification in mastoid surgery, with recurrence rates of up to 19% reported in meta-analyses, possibly due to residual epithelial tissue or cholesteatoma. However, recent studies show lower recurrence in obliteration groups compared to CWU procedures, likely due to better disease clearance. While no studies have assessed bone dust for squamous epithelium or keratin in recidivism, our findings (14% squamous cell positivity, 43% keratin positivity) suggest a potential role. One study reported a 13.4% residual cholesteatoma rate with bone paté but did not confirm whether obliteration material contributed to recurrence (9).

Histological studies indicate that while all epithelial cells can synthesize keratin, only squamous transformation enables its secretion. Environmental factors and inflammation are key drivers of keratin accumulation, with disrupted homeostasis often linked to inflammatory processes (10). Supporting the metaplasia theory, Sadé et al. (2) detected keratin in 19 of 101 patients with limited cholesteatoma-and even behind intact, non-retracting tympanic membranes in 3.7% of cases. Similarly, Viswanatha et al. (11) reported three cases of cholesteatoma arising primarily in the mastoid air system without middle ear involvement.

In our study, H&E staining revealed keratin positivity in 43% of the study group versus 6% in the control group (p=0.01). Although no study has conclusively shown that keratin alone causes cholesteatoma, experimental models by Hinohira et al. (12,13) demonstrated that introducing keratin debris into epithelial cysts produced perimatrix-like granules, vascularization, and inflammatory changes, suggesting a contributory role for keratin in disease development. Notably, the absence of squamous cells or p63 staining in some keratin-positive samples may reflect sampling limitations, implying that additional sections might reveal a more complete picture of metaplastic changes.

Current theories do not fully explain the etiopathogenesis and clinical features of cholesteatoma, prompting further molecular investigation. p63, encoded by the TP63 gene, is a crucial transcription factor for epidermal development whose overexpression has been linked to squamous cell carcinomas (14). Studies have consistently shown that p63, localized in the basal layer of epithelial cells, is more prominently expressed in cholesteatoma tissues and may influence both keratin production and epithelial proliferation (15-17). Takahashi et al. (16)- Yamamoto-Fukuda et al. (17) demonstrated that keratinocyte growth factor-a key factor in cholesteatoma pathogenesis-upregulates p63, reinforcing its potential role in disease progression.

Our study is among the first to assess p63 expression in mastoid bone dust samples, offering unique insights into metaplastic changes within the mastoid air cell system that may precede cholesteatoma development. These findings highlight p63's potential as a biomarker for cholesteatoma progression and recurrence, and they open avenues for further research into its mechanistic role in this pathology.

Cholesteatoma development is widely associated with the presence of squamous cells in the middle ear, which produce keratin. However, our study revealed notable findings. Squamous cell staining was observed in two study group patients and one control, while p63 staining was positive in three study group patients and one control. Keratin positivity was more common, detected in six study group patients compared to one in the control group. Interestingly, some patients exhibited keratin without squamous cells, possibly due to sampling limitations in random sections after paraffin blocking.

When considering positivity for any parameter as an indicator of epithelial metaplasia, six study group patients and two controls tested positive, showing a statistically significant difference (p=0.049). However, simultaneous positivity for all parameters was rare, observed in only two study group patients, with none in the control group (p=0.11). These findings support the hypothesis that metaplasia can occur in mastoid air cell regions independent of cholesteatoma.

Given the potential recurrence risk in patients with any positive parameter, routine histopathological examination of mastoid bone dust using cost-effective H&E staining could help identify those requiring closer monitoring and advanced follow-ups.

A key limitation of our study is the small sample size, comprising 31 patients (14 in the study group and 17 in the control group). Larger cohorts are needed for more robust and generalizable results. Additionally, the lack of recent studies on this topic in the literature, despite older publications providing more extensive insights, highlights a

notable gap in contemporary research. This shift in research focus has limited comparisons with the current data, making it challenging to fully support our discussion. No support was received from any individual or organization for this study.

Conclusion

We evaluated p63 IHC staining, keratin, and squamous cells to identify squamous cell metaplasia in the mastoid air cell system. Our findings revealed significant differences between the study and control groups when positivity for any of these parameters was considered (p=0.049). These results indicate that the mastoid air cell system in patients with cholesteatoma undergoes alterations independent of middle ear cholesteatoma or its spread to the mastoid system.

Our findings strongly support the metaplasia theory, emphasizing its potential as a key mechanism in cholesteatoma pathogenesis. This challenges the traditional view that keratin presence is incidental and instead positions it as a possible contributing factor to recurrence risk.

Given the significant keratin positivity (p=0.01), routine histopathological examination of mastoid bone dust using H&E staining emerges as a cost-effective and time-efficient approach. This could facilitate the identification of high-risk patients requiring closer monitoring or advanced diagnostic assessments.

While our study is limited by a small sample size, the observed trends highlight the need for further research. Larger cohort studies should aim to validate these findings and elucidate the molecular mechanisms underlying squamous metaplasia and keratin accumulation. Moreover, exploring p63's role as a potential biomarker for cholesteatoma progression and recurrence could offer new insights into its invasive and recurrent nature.

In conclusion, our study underscores the importance of understanding the alterations in the mastoid air cell system to improve surgical outcomes and long-term patient management in cholesteatoma cases. Routine histopathological evaluation, alongside molecular investigations, could pave the way for tailored therapeutic approaches and enhanced recurrence prevention strategies.

Ethics

Ethics Committee Approval: Ethical approval was received from Dışkapı Yıldırım Beyazıt Research and Training Hospital (decision no: 90/18 dated: 22.06.2020).

Informed Consent: Informed consent was obtained from all patients or, in the case of minors, from their parents or legal guardians, both verbally and in writing, prior to surgery.

Authorship Contributions

Surgical and Medical Practices: H.O.O., M.D., Concept: H.O.O., M.D., Design: H.O.O., M.D., T.T.T., Data Collection and/or Processing: H.O.O., T.T.T., Analysis and/or Interpretation: H.O.O., T.T.T., Literature Search: H.O.O., M.D., Writing: H.O.O., M.D., T.T.T.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Main Points

- We found that squamous epithelial tissue or fragments, such as keratin, thought to undergo metaplasia within the mastoid air cell system could be a reason for recurrences in cases where cholesteatoma is limited to the middle ear.
- In this introductory study we aimed to investigate whether mastoidectomy dust samples should routinely undergo histopathological evaluation using hematoxylin and eosin staining alone, with a focus on cost-effectiveness and time efficiency. The goal is to identify patient groups who may require more frequent or advanced follow-up examinations.
- The study highlights the potential role of p63 as a biomarker for cholesteatoma progression, suggesting that its expression in mastoid bone dust samples could indicate early metaplastic changes and aid in risk assessment for recurrence.

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