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Original Article

Predictive Role of Ki-67 and Proliferative-Cell Nuclear Antigen (PCNA) in Recurrent Cholesteatoma

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OBJECTIVES: To investigate the potential use of Ki-67 and pronuclear cell antigen (PCNA) as indicators of recurrent cholesteatoma.

MATERIAL and METHODS: Patients who had been diagnosed with cholesteatoma and who had undergone canal wall-down mastoidectomy were included in this study. Subjects were divided into two groups: recurrent and non-recurrent (i.e., cases without recurrence for at least 2 years). Ossicular pathologies were recorded. Histopathologic specimens were stained for Ki-67 and PCNA and the percentages of stained cells were calculated.

RESULTS: Neither group demonstrated a significant difference in terms of total Ki-67 per cell, Ki-67-stained cell counts, Ki-67-staining percentages, total PCNA per cell, PCNA-stained cell counts, or PCNA-staining percentages ($p>0.05$). No significant relationship was noted between the staining percentages for either Ki-67 or PCNA and the incudostapedial involvement ($p>0.05$); however, a significant relationship was noted between Ki-67 staining and malleus involvement ($p<0.05$).

CONCLUSION: Although the recurrent and non-recurrent cholesteatoma groups showed no significant differences in terms of the percentages of stained cells for either Ki-67 or PCNA, we detected high Ki-67 staining in the malleus involvement group. We concluded that cell-proliferation markers could not be defined as indicators of recurrence of cholesteatoma, but they could be defined as indicators of destructive patterns of this disease.

KEYWORDS: Cholesteatoma, recurrent, proliferative-cell nuclear antigen, Ki-67, proliferative cells

INTRODUCTION

Cholesteatoma is a histopathologically benign, expanding, and destructive growth of the squamous epithelium. Many studies have investigated a number of different molecules and their participation in pathologic pathways; however, the etiopathogenesis of cholesteatoma remains to be established [1]. The proliferation of squamous epithelium plays a key role in the etiopathologic cascade and destructive pattern of this disease; thus, Ki-67 and pronuclear antigen (PCNA) are commonly used as cell-proliferation markers for cholesteatoma [2].

Ki-67 is a nuclear protein that occurs in proliferative cells, where it is seen essentially in the G1, S, M, and G2 phases. It is absent in the G0 phase [3, 4]. PCNA, by contrast, is a co-factor of DNA polymerase delta that participates in cell-proliferation [5]. These two proteins efficiently reflect the morphologic features of cell proliferation and are hence commonly used in mitotic index and tumor grading. The proliferative pattern of cholesteatoma is closely related with its destructive biologic behavior; thus, this process can be verified by monitoring Ki-67 and PCNA [6, 7].

The recurrence of cholesteatoma is another characteristic feature of this disease that complicates its treatment. The major prognostic factors for recurrence are the presence of ossicular erosion and a young age of the patient; these factors are also relevant to the

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destructive pattern and cell proliferation. Therefore, we considered that the Ki-67 and PCNA cell-proliferation markers might also be predictive for recurrence.

The reported recurrence rate of cholesteatoma varies from 0%-70%, depending on the different conclusions of various studies [8]. Therefore, a better estimation of the likelihood of recurrence prior to surgery could be useful for managing the follow-up process. We hypothesized that the proliferative pattern of cholesteatoma could be related to its tendency to recur based on the fact that this pattern is apparently related to the destructive character of the disease. No study in the literature has yet focused on the relationship between the proliferative pattern and recurrence of cholesteatoma. The aim of the present study was therefore to investigate whether the proliferation markers Ki-67 and PCNA could be used as indicators of the tendency for cholesteatoma recurrence.

MATERIALS AND METHODS

Patients who had been admitted to our otolaryngology clinic from 2005 to 2015 and had undergone surgery for cholesteatoma were included and retrospectively investigated. Inclusion criteria were: follow-up of at least 2 years, age between 18 and 65 years, having undergone canal wall-down surgery, and having a diagnosis of cholesteatoma based on histopathologic confirmation. Exclusion criteria were recurrent cholesteatoma, having undergone surgery at a different center, less than 2 years of follow-up, having undergone surgery other than a canal wall-down procedure, congenital cholesteatoma, and residual disease in a short period of time.

The subjects were divided into two groups: with recurrent cholesteatoma and non-recurrent cases after at least 2 years of follow-up. The demographic features of the patients and perioperative observations of ossicular involvement were recorded.

This retrospective study protocol was approved by the local ethical committee and complied with Helsinki Declaration. Informed consent was obtained from all patients.

Pathologic Evaluation

The immunohistochemical protocol was based on the streptavidin–avidin–biotin method. For each case, paraffin-embedded tissue was fixed with 10% neutral-buffered formalin and re-processed. Tissue slices (3 µm thick) were used to identify the expressions of Ki-67 and PCNA. The sections were placed on positively charged slides and kept on a slide warmer for 2 hours at 37°C for deparaffinization. The sections were then immunohistochemically stained using Ki-67 (clone 30-9) rabbit monoclonal antibody (Ventana Medical Systems, Tucson, AZ, USA), 1:100 diluted PCNA clone (EP91) rabbit monoclonal antibody (Epitomics, USA), and an Ultraview universal DAB Detection Kit (Ventana, USA), followed by processing with a BenchMark ULRTA (Ventana, USA) staining device.

The immunohistochemically stained slides were examined with a light microscope (Olympus, BX53, Olympus Corp., Tokyo, Japan). For each case, hotspot areas were identified and viewed under 400× magnification (Olympus E330, Olympus Imaging America Inc., USA). Images were transferred to a computer and visualized with Windows 7 Professional Paint software. All stained and non-stained cells were counted and compared, and the rate of staining was calculated.

Statistical Analysis

The mean, standard deviation, median, minimum, maximum, frequency, and rate were used for descriptive analysis. The distribution of variables was evaluated using the Kolmogorov–Smirnov test. The Mann–Whitney test was used to analyze independent quantitative data. Independent qualitative data were analyzed using the Chi-Square test. When use of the Chi-Square test was inconvenient, the Fischer Test was used instead. The Statistical Package for the Social Sciences (SPSS) 22.0 software (IBM Corp.; Armonk, NY, USA) was used for statistical analysis.

RESULTS

Our study included 43 patients (14 females and 29 males; mean age, 33.6±13). In total, 21 patients had recurrent cholesteatoma and 22 were non-recurrent cases. No significant differences were noted between the two groups in terms of age or gender (p=0.817; p=0.586 respectively). The incus and malleolar involvement also did not differ significantly between the two groups (p=0.887; p=1.00). Stapedial involvement was significantly higher in the recurrent cases than in the non-recurrent cases (p=0.021) (Table 1).

Table 1. Demographic features and ossicular chain involvements of recurrent and non-recurrent cases

		Recurrence (-)		Recurrence(+)		
		Mean±SD/n-%	Median	Mean.±SD/n-%	Median	p
Age		32.3±11.6	27.5	34.9±14.5	32.0	0.817[m]
Gender	Female	8	36.4%	6	28.6%	0.586[X²]
	Male	14	63.6%	15	71.4%	
Malleus	(-)	12	54.5%	11	52.4%	0.887[X²]
Erosion	(+)	10	45.5%	10	47.6%	
Incus	(-)	1	4.5%	1	4.8%	1.00[X²]
Erosion	(+)	21	95.5%	20	95.2%	
Stapes	(-)	14	63.6%	6	28.6%	0.021[X²]
Erosion	(+)	8	36.4%	15	71.4%	

[m]Mann-Whitney U test / [X²]Chi-square test SD: standart deviation

No significant differences were found between the two groups in terms of total counts of stained cells, Ki-67-stained cells, the rate of Ki-67-stained cells, PCNA-stained cells, or rate of PCNA-stained cells (p=0.091; p=0.076; p=0.395; p=0.319; p=0.096; p=0.120, respectively) (Table 2). The counts of Ki-67-stained cells were significantly higher in cases with malleus involvement than without malleus involvement (p=0.047). No significant differences were detected for malleus erosion or intact malleus in terms of the percentages of stained cells, the numbers of PCNA-stained cells, or the percentages of PCNA-stained cells (p=0.981; p=0.228; p=0.865 respectively). The cases with and without incus involvement also showed no significant differences in terms of the numbers of Ki-67-stained cells, the percentage of Ki-67-stained cells, the number of PCNA-stained cells, and the percentage of PCNA-stained cells (p=0.286; p=0.356; p=0.908; p=0,954, respectively). The cases with and without incus involvement also showed no significant differences in terms of the numbers of Ki-67-stained cells, the percentage of Ki-67-stained cells, the number of PCNA-stained cells, and the percentage of PCNA-stained cells (p=0.922; p=0.770; p=0.679; p=0.679, respectively) (Table 3).

DISCUSSION

The current medical literature contains many studies on the etiopathogenesis of cholesteatoma. Nevertheless, the exact pathologic pathway remains unclear, despite recent research that has focused on the signaling pathways and participating molecules [1]. In the clinic, surgical intervention is still considered the most effective treatment; however, recurrence of cholesteatoma remains the main weak point of surgery. This recurrence is affected by many prognostic

Table 2. Count of Ki-67 / PCNA -stained cells and staining rates in recurrent and non-recurrent cases

	Recurrence (-)		Recurrence(+)		
	Mean±s.d./n-%	Median	Mean.±s.s./n-%	Median	p
Stained cell (total)	181.9±91.4	182.5	244.0±125.2	224.0	0.091[m]
Ki-67 stained cell	58.8±37.4	51.5	81.0±41.4	77.0	0.076[m]
Ki-67 staining rate	32.6±14.5	30.6	36.9±18.3	37.8	0.395[m]
Stained cell (total)	178.2±91.2	162.5	218.8±116.7	198.0	0.319[m]
PCNA stained cell	85.9±50.7	64.5	123.6±90.5	83.0	0.096[m]
PCNA staining rate	48.3±17.0	48.3	58.1±23.9	57.3	0.120[m]

[m]Mann-whitney u test / X^2 Chi-square test
PCNA: pronuclear cell antigen

Table 3. Comparison between number of Ki-67/PCNA stained cells, staining rates and ossicular erosion

	Malleus Erosion (-)		Malleus Erosion (+)		
	Mean±s.d./n-%	Median	Mean.±s.s./n-%	Median	p
Ki-67 stained cell	61.3±42.5	50.0	79.2±36.8	69.5	0.047m
Ki- 67 staining rate	35.8±19.9	33.0	33.4±11.5	30.7	0.981m
PCNA stained cell	94.4±73.1	66.0	115.7±76.2	102.0	0.228m
PCNA staining rate	53.5±21.9	52.9	52.6±20.4	55.3	0.865m
	Incus Erosion (-)		**Incus Erosion (+)**		
	Mean.±s.d./n-%	Median	Mean.±s.d./n-%	Median	p
Ki-67 stained cell	109.5±78.5	109.5	67.7±38.5	59.0	0.286m
Ki- 67 staining rate	41.4±12.6	41.4	34.4±16.6	32.0	0.356m
PCNA stained cell	190.0±213.5	190.0	100.1±65.6	74.0	0.908m
PCNA staining rate	54.7±42.0	54.7	53.0±20.4	54.3	0.954m
	Stapes Erosion (-)		**Stapes Erosion (+)**		
	Mean.±s.d./n-%	Median	Mean.±s.d./n-%	Median	p
Ki-67 stained cell	71.1±46.7	59.0	68.3±35.3	56.0	0.922m
Ki- 67 staining rate	36.6±19.9	32.4	33.0±12.8	32.0	0.770m
PCNA stained cell	103.4±79.7	72.5	105.1±71.4	83.0	0.679m
PCNA staining rate	51.7±19.6	54.6	54.3±22.5	54.3	0.679m

[m]Mann-whitney u test
PCNA: pronuclear cell antigen

factors, including the experience and skills of the surgeon, the age of the patient, the location of the cholesteatoma, and the extent of ossicular erosion [8]. Reducing the rate of the recurrence, improving the reliability of treatment modality, and developing new approaches are the main objectives of most of the ongoing studies.

Some recent studies have focused on epidermal hyper-proliferation, bone destruction, and epithelial apoptosis associated with cholesteatoma. These studies have emphasized that hyper-proliferation of the cholesteatomal epithelium plays an important role in the pathophysiologic cascade [1, 2]. This mechanism can be verified using many markers, but Ki-67 and PCNA are the most commonly used markers for evaluating this epithelial proliferation. For example, Yamamoto–Fukuda et al. [9] used Ki-67 to demonstrate the proliferative course of cholesteatoma. Similarly, Zhang et al. [10] and Hamajima et al. [11] described the over-expression of DNA-binding/differentiation-1 and NF-kappaB in the cholesteatomal epithelium and determined that DNA-binding-1 stimulates the expression of NF-kappaB, cyclin-D1, and PCNA/Ki-67.

Both Ki-67 and PCNA have been investigated in many studies in attempts to identify the relationship between the hyper-proliferation of the cholesteatoma and its destructive pattern. These markers have also been used to demonstrate the increased turnover rate in pediatric cholesteatoma [12, 13]. Pediatric cases demonstrate a more aggressive pattern and have higher recurrence rates than is seen in adult cases [14], which could be related to cell hyper-proliferation. Bujia et al. [15] found a higher expression of Ki-67 in pediatric cases than in adult cases. By contrast, Aslıer et al. [6] found higher Ki-67 expression in adults, but they also found a significant correlation between bone erosion and Ki-67 staining, and they postulated that cell proliferation participates in this destructive pattern.

Destruction classifications, such as the Austin–Kartush classification, have been used in studies that have focused on the destructive pattern. In our study, we preferred to evaluate the ossicular chain involvement in each case. When we compared the recurrent and non-recurrent cases, we detected a significantly higher rate of stapes erosion but no significant difference between the two groups in terms of malleus and incus involvement. When we investigated the relationship between Ki-67/PCNA staining and ossicular chain erosion, we found a significant relationship only between malleus destruction and Ki-67 staining. The malleus is the strongest ossicle; hence, this finding supports the relationship between Ki-67 staining and the destructive pattern of cholesteatoma.

Among the various surgical procedures for cholesteatoma, the canal wall-down tympanoplasty method has the lowest recurrence rate [8, 16, 17]. Our aim in the present study was to evaluate the proliferative capacity of the cell while minimizing other dependent risk factors. Therefore, because of the low recurrence rate, we included only subjects who had undergone a canal wall-down procedure. One limitation of our study is that all the included patients had undergone the canal wall-down procedure and all had advanced cholesteatoma; therefore, the observation of ossicular erosion in both groups was predictable.

Cholesteatoma has a more destructive pattern in pediatric cases and recurs more often [8, 14]. The increased recurrence rate could be a reason for or a result of a more aggressive pattern; however, this issue, as well as the role of molecular factors, still requires clarification. The surgeon and the surgical approach may affect the recurrence rate, but most factors are related to the patient or to the pathologic behavior of the case.

Signaling pathways regulating apoptotic activity and cell-proliferation capacity play a major role in the identification of these patterns. For example, Choufani et al. [18] suggested a role for calcicyclin, which participates in the cell cycle, in differentiating recurrent and non-recurrent cases. Another study reported a significantly higher level of macrophage-migrating inhibitor factor in recurrent cases [19]. The only study reporting cell-proliferation markers in recurrent and non-recurrent cases was performed by Şanlı et al. [20] who investigated 19 cases of cholesteatoma in 32 patients with otitis media. Of these, eight cholesteatoma cases were recurrent and showed a significantly higher rate of Ki-67 staining when compared with the non-recurrent cases.

Our study is the first controlled study to investigate cell proliferation in the first pathology specimens of recurrent cases. We found no significant difference between recurrent and non-recurrent cases in terms of their Ki-67 and PCNA staining. However, we did not evaluate the pathologic specimens of the recurrent cases, which could be considered a limitation of our study.

CONCLUSION

Ki-67 and PCNA do not appear to have predictive roles in identifying the recurrence of cholesteatoma. Cell proliferation was not significantly different between the recurrent and non-recurrent cholesteatoma cases. However, a high level of Ki-67 immunohistochemical staining was observed in the cases with malleus involvement. Therefore, this cell-proliferation marker could be defined as an indicator of the destructive pattern of cholesteatoma.

Ethics Committee Approval: Ethics Committee approval was received for this study from İstanbul Training and Research Hospital and complied with Helsinki Declaration (03.03.2017/962).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

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