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Association of Ki-67 Level on Bone Destruction in Chronic Suppurative Otitis Media Patients with Cholesteatoma

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ABSTRACT

Background: Chronic suppurative otitis media (CSOM) with cholesteatoma is one of the most common chronic infections found in developing countries. Cholesteatoma will stimulate bone resorption from the epithelial and stromal cells resulting in cytokine and keratinocyte formation, as well as mastoid bone destruction. The Ki-67 found in the proliferating cell shows that this protein plays an important role as a cell division process marker.

Objective: Analyzing the association of Ki-67 on bone destruction in CSOM with cholesteatoma.

Method: Participants in this study were CSOM patients with cholesteatoma who underwent surgery. Participants were assessed for bone destruction based on CT scan examination and confirmed during surgery. Examination of Ki-67 levels was carried out by taking cholesteatoma tissue at the time of surgery and analyzed using the ELISA method. The results of the examination were analyzed using the Spearman's rank correlation, Pearson's correlation and logistic regression with $p < 0.05$.

Results: The value of Ki-67 levels at each degree of destruction was degree 1 of 0.301, degree 2 of 0.36 ± 0.34, degree 3 of 0.41 ± 0.07, degree 4 of 0.60 ± 0.85, degree 5 of 0.74 ± 0.12, and degree 6 equal to 0.90 ($r = 0.906$; $p = 0.001$). In addition, there was a significant relationship between symptoms duration on bone destruction ($r = 0.913$; $p = 0.001$) and levels of Ki-67 ($r = 0.887$; $p < 0.001$). The regression coefficient was 0.079 for the independent variable, bone destruction with $p = 0.009$, and the regression coefficient 0.024 for symptoms duration with $p = 0.014$.

Conclusion: Increased Ki-67 level in cholesteatoma is associated with bone destruction degree in CSOM patients with cholesteatoma.

Keywords: Bone destruction, cholesteatoma, chronic suppurative otitis media, Ki-67

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INTRODUCTION

Chronic suppurative otitis media is a chronic infection of the middle ear mucoperiosteum with perforation of tympanic membrane marked by continuous or intermittent fluid discharge (otorrhea) from the middle ear for more than 3 months. Chronic suppurative otitis media (CSOM) is a common health problem that is usually found in developing countries [1,2]. The incidence of CSOM in Indonesia remains high, as proved by the increase in patient visits with middle ear inflammation from year to year [2,3]. The reported incidence of CSOM depends on race and socioeconomic factors. The etiology and pathogenesis of otitis media are multifactorial, including genetics, infections, allergies, environmental, social, race, and eustachian tube dysfunction [1,4].

CSOM is classified into 2 types, namely CSOM without cholesteatoma and with cholesteatoma. In CSOM without cholesteatoma, the inflammation only occurs in middle ear mucosa and rarely causes dangerous infections, usually preceded by a malfunctioning of the tubes that cause an abnormality of the tympanic cavity. On the other hand, CSOM with cholesteatoma is found and often causes dangerous complication such as mastoiditis in 29.6% patients, N VII paresis in 1.94% patients, cerebral abscess in 0.78% patients, and labyrinthitis in 0.65% patients [4,5].

Cholesteatoma is defined as a disease resembling a cyst in the ear that may expand into the mastoid and destructive in nature, characterized by uncontrolled growth of squamous epithelial cells in the middle ear or temporal bone, which contains desquamated keratin and purulent material [2]. In the dangerous type CSOM or with cholesteatoma, there will be an accumulation of debris and keratinocytes which are invaded by immune system cells including Langerhans cells, T-cells, and macrophages. This process is stimulated by unbalanced epithelial proliferation, differentiation, and maturation of keratinocytes and prolongation of apoptosis. Mast cells are common in cholesteatoma tissue and contribute to chronic inflammation. Chronic inflammation derived from cholesteatoma will stimulate bone resorption with the release of Receptor Activator of Nuclear Factor κ B Ligand (RANKL) of epithelial and stromal cells, which causes the formation of the production of cytokines and keratinocytes that influence the damage the mastoid bone [6,7].

Ki-67 is a nuclear and nucleolar protein that is not only associated with a somatic proliferation of cells but is integrated as a protein regulatory association that can be assessed at each cell phase, which indicates that this protein plays an important role as a marker of cell division [8,9]. In CSOM with cholesteatoma, there is an increased ratio of RANKL/OPG in cholesteatoma tissue due to inflammation and potentiates osteoclastogenesis [6,7]. This process increases cell proliferation activity assessed by the Ki-67 antigen. Therefore, the researchers were interested in elucidating the association between Ki-67 levels with the degree of bone destruction due to cholesteatoma, thereby reducing disease severity and providing appropriate management of cholesteatoma [8-10]. Based on the description above, it is necessary to

examine the association of Ki-67 level on bone destruction in CSOM patients with cholesteatoma.

MATERLAS AND METHODS

Participant

Participants in this study were CSOM patients with cholesteatoma who met the inclusion and exclusion criteria. Participant's inclusion criteria included patients diagnosed with CSOM [2], patients underwent mastoidectomy with sufficient cholesteatoma tissue for serologic examination during surgery. Recurrent cholesteatoma patients with surgery history and and had systemic disease, such as diabetes mellitus, were excluded. Participants first received an explanation regarding the benefits and objectives of this study, and filled out the consent form before the research was carried out.

Design

A prospective study with cross-sectional design was conducted from January - December 2017, with the number of participants being 26 CSOM patients with cholesteatoma. The study was carried out in Dr. Hasan Sadikin General Hospital, Bandung, Indonesia. Participants were examined for degree of bone destruction before surgery using CT scan which was confirmed during the surgery. Canal wall-down surgery was performed by 2 standardized otologists. Furthermore, at the time of surgery, cholesteatoma tissue was taken, and the Ki-67 level was calculated.

Ethical Approval

The study received ethical approval from the Ethic Committee of Dr. Hasan Sadikin General Hospital, Bandung, Indonesia, and declared fit for research based on the Declaration of Helsinki (024 / UN6.C1.3.2 / KEPK / PN / 2016).

Bone Destruction

Assessment of the degree of bone destruction was carried out during canal wall-down surgery based on the damage to the bone tissue structure due to cholesteatoma [2]. The degree of bone destruction is divided into 6 based on its location, namely grade 1 that is a cholesteatoma found only in the middle ear (hypo- and mesoepitympanum) without any destruction of the hearing bones; grade 2 that equals to grade 1 with destruction of one or more of the hearing bones; grade 3 is a cholesteatoma found in the middle ear and mastoid cell system without destruction of the ossicles; grade 4 that equals to grade 3 with destruction of one or more of the hearing bones; grade 5 is cholesteatoma found in the middle ear, mastoid, and temporal bone; and grade 6 that equals to grade 5 with extension beyond the temporal bone [2]

Ki-67 Level

The procedure for measuring Ki-67 level was carried out using cholesteatoma tissue and MAE skin during surgery. Those materials were put on a plate and kept in a cool box at 4°C for less than 2 hours, then stored at -80°C in the Esco Lexicon II ULT freezer (Esco Technologies Inc, Hatboro, PA, USA). ELISA examination used the ELISA kit for Ki-67 (RayBiotech Inc, Georgia, USA). Readings of the absorbance value of the material was at a wavelength of 450 nm with the Humareader Single (HumaReader, Germany).

Statistical Analysis

The data were inputted first into the IBM SPSS Statistics software version 23.0 (IBM Corp., Armonk, NY, USA). The process was followed by normality test to determine the test on participant characteristic data which were grouped into 2 based on gender. The test used in data processing was independent t-test and the Mann Whitney's test. Meanwhile, the analysis of the Ki-67 relationship with bone destruction used the Spearman's rank correlation, Pearson's correlation and logistic regression with the relationship between the two variables was significant if the p value was <0.05.

RESULTS

Characteristics of Participants

Most participants were female (61.5%), with a mean age of 28.35 ± 11.5 years and a median of 26.5 (15 - 62) years. Participants experienced CSOM complaints for 10.58 ± 3.69 years, with a median of 10.5 (5-18) years. Most participants experienced complaints from CSOM within a span of 6-10 years as many as 53.8%. Most participants had a junior high school education as much as 35%, had complications of retroauricular fistula as much as 27%. Furthermore, most participants' audiogram results showed severe CHL as much as 54%, while the most tympanic membrane perforations in the participant were atic (27%; Table 1).

Table 1. Characteristics of Participant's Frequency Distribution

Characteristics	n (%)
Sex	
Male	10 (38.5)
Female	16 (61.5)
Symptoms duration	
<5 years	2 (7.7)
6-10 years	14 (53.8)
11-15 years	8 (30.7)
≥16 years	2 (7.7)
Education level	
Primary school	7 (27)
Junior high school	9 (35)
Senior high school	6 (23)
Diploma	1 (4)
Bachelor	3 (12)
Complication	
Retroauricular abscess	4 (15)
Facial nerve paralysis	4 (15)
Retroauricular fistula	7 (27)

Cerebral abscess	3 (12)
Audiogram	
Severe CHL	14 (54)
Very severe CHL	10 (38)
Very severe MHL	2 (8)
Perforation of Tympanic Membrane	
Marginal	5 (19)
Central	4 (15)
Total	5 (19)
Subtotal	5 (19)
Atic	7 (27)
Bone Destruction	
1st Degree	1 (3.8)
2nd Degree	7 (26.9)
3rd Degree	4 (15.3)
4th Degree	11 (42.3)
5th Degree	2 (7.7)
6th Degree	1 (3.8)

Bone Destruction

The most common bone destruction degree was the 4th degree by 11 people (42.3%), followed by the 2nd degree by 7 people (26.9%), then 3rd degree by 4 people (15.3), and the least was the 1st and the 6th degree by 1 person each (3.8%; Table 1). The average degree of bone destruction was 3.46 ± 1.21, with a median value of 4 (1-6; $p = 0.813$; table 2).

Table 2. Participant's Characteristics Based on Data Distribution

Variable	Sex		*p*
	Female	Male	
Age	30.5 (16-62)	22.5 (15-43)	0.141
Symptom Duration	10.62 ± 3.67	10.5 ± 3,923	0.935
Ki-67 Level	0.54 ± 0.16	0.51 ± 0.19	0.649
Bone Destruction Degree	4 (2-5)	4 (1-6)	0.813

Ki-67 Level

The average Ki-67 level was 0.53 ± 0.17, with a median value of 0.51 (0.30 - 0.90). The average Ki-67 level for female and male participants was 0.54 ± 0.16 and 0.51 ± 0.19, respectively ($p = 0.649$; Table 2).

Table 3. Correlation between Ki-67 Level, Bone Destruction Degree, Symptom Duration, and Age

Variable	*r*	*p*
Ki-67 vs Age	0.020	0.921
Ki-67 vs Bone Destruction Degree	0.906	0.001*
Symptom Duration vs Bone Destruction Degree	0.913	0.001*
Symptom Duration vs Age	0.152	0.460
Symptoms Duration vs Ki-67	0.887	0.000**

Note: *significant $p < 0.05$; **significant $p < 0.001$

Correlation between Ki-67 Level and Bone Destruction

The value of Ki-67 level at each degree of destruction was 0.301 (degree 1), 0.36 ± 0.34 (degree 2), 0.41 ± 0.07 (degree 3), 0.60 ± 0.85 (degree 4), 0.74 ± 0.12 (degree 5), and 0.90 (degree 6; $r = 0.906$; $p = 0.001$). In addition, there was a significant relationship between symptoms duration on bone destruction ($r = 0.913$; $p = 0.001$) and Ki-67 level ($r = 0.887$; $p < 0.001$; Table 3). The regression coefficient was 0.079 for independent variable, bone destruction with $p = 0.009$, and the regression coefficient 0.024 for symptoms duration with $p = 0.014$ (Table 4). In the regression analysis above, the first inserted variable was bone destruction ($r = 0.906$), and symptoms duration followed afterward with $r = 0.886$.

Table 4. Regression Analysis Result of Ki-67 on Bone Destruction and Symptoms Duration

Variable	*β*	St Error	*t*	*p*
Bone destruction	0.079	0.028	28.34	0.009*
Symptoms durations	0.024	0.009	26.25	0.014*

Note: *significant $p < 0.05$

Therefore, the regression formula was as follows KI-67 P = (0.0787 × Bone destruction) + (0.0239 × Symptom duration (years)) with a total correlation coefficient r = 0.99 (Figure 1). From the previous table, it was also seen that the Beta column showed the forming variables of the regression equation, namely bone destruction

contributed by 0.5161 (51.61 %) and symptoms duration contributed by 0.4781 (47.81%). It turned out that the contributions were in accordance with each variable's correlation coefficient. The higher the variable correlation coefficient with Ki-67, the higher the contribution. This formula could be used to predict Ki-67 from bone destruction and symptoms duration without Ki-67 testing in the laboratory.

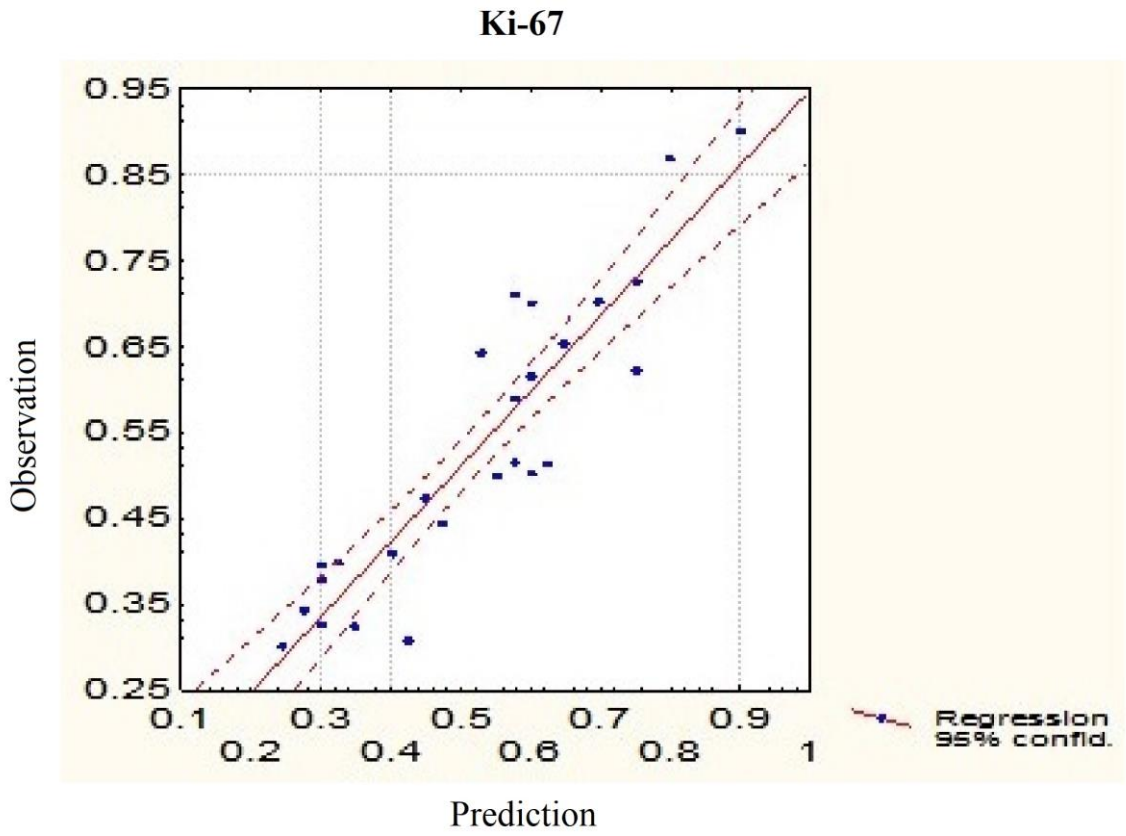

Figure 1. Prediction vs observation regression graph

DISCUSSION

Regarding the symptom duration, Aquino et al. stated that this issue is due to a low level of knowledge of the disease that makes the patients delay getting themselves checked [11]. According to Sade et al., there are two factors correlated to delayed management in diagnosis, namely the patient does not consider the symptoms as important unless it is painful, causes dizziness, and/or ear bleeding. Furthermore, the knowledge factor of cholesteatoma which only be familiar to ORL-HNS specialists, but less understood by general practitioners [3,12].

The effect of tympanic membrane perforation is mostly found in the atic area, pars flaxide, because there is a cavity called the prussax space in the posterior part of the pars flaxide. This space is a very important area because it is the area where cholesteatoma most often occurs. Cholesteatoma that grows in the Prussak space will spread to the posterior area along the side of the incus body which then enters the antrum and mastoid cavity [13]. Cholesteatoma is formed after a perforation of the tympanic membrane as a result of the entry of the skin epithelium from the ear canal or from the edge of the tympanic membrane perforation to the middle ear (migration theory) or occurs as a result of metaplation of the tympanic mucosa due to prolonged irritation of infection (metaplation theory) [14].

Moreover, the average Ki-67 level was least found in the 1st degree bone destruction with 0.301pg/ml, and the highest was in the 6th degree with 0.899 pg/ml. The overall average Ki-67 level in cholesteatoma was 0.532 ± 0.171 pg/ml (p < 0.001). This indicates a significant association between Ki-67 level and bone destruction. This finding corresponds to a study conducted by Aquino et al. that reported that the most common complication of cholesteatoma is bone destruction, as the bone destruction degree depends on the location and the expansion of the cholesteatoma [11]. Chae et al. stated that in cholesteatoma, Ki-67 expression is increased [15]. Sikka et al. stated that of a total of 67 samples used, 10 adult samples and 9 children samples, there was significant bone destruction [16]. Michael et al. described that cholesteatoma affects bone resorption by releasing OPGL from activated T-cells thereby triggering osteoclastogenesis [17]. This is in accordance with the theory which states that the mechanism of bone destruction in cholesteatoma starts from the pressure due to accumulated keratin and the influence of mechanical stress. This will affect the MIF (macrophage migration inhibitory factor) and the MMPs (matrix metalloproteinase) production. MIF will increase the production of the pro-inflammatory cytokines and chemokines thereby affecting the production of IL-1, IL-6, and TNF-α, receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [2,6,7,18,19]. This results in activated osteoblasts and osteoclasts. MMPs will work as angiogenesis and cell proliferation to stimulate the production of Ki-67. Increased Ki-67 and osteoclast activation affect the

occurrence of bone destruction process in cholesteatoma [8,9,15,20].

A study found that if Ki-67 has a significant relationship with bone destruction, the higher the value of Ki-67, the more severe bone destruction experienced by the participant [10]. In addition, the Ki-67 value in CSOM with cholesteatoma is higher than that in CSOM without cholesteatoma [21]. Ki-67 can be used as a proliferation marker to evaluate middle-ear cholesteatoma [22]. Thus, it can be concluded that Ki-67 can be used as an effective marker to evaluate bone destruction of CSOM patients with cholesteatoma [10,21-23].

CONCLUSION

This study finds a correlation between Ki-67 and bone destruction degree. The higher the Ki-67 level, the higher the bone destruction degree on chronic suppurative otitis media patients with cholesteatoma. Furthermore, this study obtains a formula to predict the value of Ki-67 on CSOM patients based on bone destruction degree and symptom duration before surgery.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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