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Research Report

Analysis of Ki-67 expression as clinicopathological parameters in predicting the prognosis of adenoid cystic carcinoma

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ABSTRACT

Background: *Adenoid cystic carcinoma is a malignant salivary gland tumor located in the head and neck region. Although complete surgical resection and complementary radiotherapy have been shown to improve long-term survival rates, the prognosis of adenoid cystic carcinoma remains poor. Ki-67 expression is considered a marker for the cellular proliferation rate, the detection of its expression usually being related to the aggressiveness and unfavorable prognosis of adenoid cystic carcinoma in the salivary gland.* ***Purpose:*** *This study was conducted to quantify the expression of Ki-67 in adenoid cystic carcinoma and to correlate the result with clinical parameters and histopathological grading in determining the prognosis.* ***Methods:*** *Twenty three cases of salivary gland adenoid cystic carcinoma were identified at the Department of Anatomical Pathology, Dr. Hasan Sadikin Hospital between 2013 and 2015. Clinical data such as age, gender, location of tumor and histopathological grading was also collected. The expression of Ki-67 was assessed by immunohistochemical means to determine the correlation of Ki-67 with clinical parameters and histopathological grading.* ***Results:*** *There were no significant differences between the expression of Ki-67 and clinical parameters, although a very strong correlation existed between the expression of Ki-67 and histopathological grading ($p < 0.01$).* ***Conclusion:*** *There were no correlation between the expression of Ki-67 and clinical parameters, although a correlation existed between the expression of Ki-67 and histopatological grading in salivary gland adenoid cystic carcinoma. Thus, clinical parameters were unusable in determining the prognosis of adenoid cystic carcinoma, although Ki-67 expression could be used for this purpose..*

Keywords: *adenoid cystic carcinoma; histopathological grading; clinical parameters; immunohistochemistry; Ki-67*

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INTRODUCTION

Adenoid cystic carcinoma is a malignant tumor of the secretory glands often occurring in the major and minor salivary glands situated in the head and neck regions. Adenoid cystic carcinoma presents certain clinical and histopathological properties, such as slow growth, perineural invasion and metastasis in isolated locations often leading to recurrence.[1] Nevertheless, the etiology and pathogenesis of adenoid cystic carcinoma remain unknown. Up to the present, no research results exist confirming that environmental and genetic factors cause this tumor. However, they do indicate abnormalities in chromosomes 6q, 9p, and 17p of regions 12-13. There are also frequent genetic deletions in 12q, 13q, and 19q.[2–4]

Clinically, adenoid cystic carcinoma develops slowly rendering detection of the tumor during examination difficult, while reducing patient survival rates. Adenoid cystic carcinoma is also known to affect all age groups with its peak occurring in middle or old age. However, there is no specific higher incidence rate with a particular gender.[2]

Histopathologically, adenoid cystic carcinoma demonstrates three different growth patterns, namely: 1) cribriform/glandular (classic); in the form of an epithelial cell nest with a cylindromatous cyst, 2) tubular; in the form of tumor cells forming ductal structures coating epithelial

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cells and 3) solid (basaloid); in the form of uniform, small, basofillic cells with hyperchromatic nuclei.[2,3] Although the solid type represents the most aggressive forms, the three types are often found to be present in a tumor, resulting in frequently unpredictable prognosis.[5]

Adenoid cystic carcinoma can afflict either the parotid gland or the submandibularis gland. However, it is most commonly found in the minor salivary gland with the highest frequency being in the area of the durum palate, followed by the tongue, buccal mucosa, lips and mouth floor.[2]

In general, the prognosis of adenoid cystic carcinoma is influenced by several factors including: growth type, stage, anatomical location, tumor size and metastasis. Although additional surgical therapy and radiotherapy are frequently administered, the survival rate of and prognosis for patients with adenoid cystic carcinoma remains poor.[6] In other words, the prognosis of adenoid cystic carcinoma is clinically unpredictable.[7]

Furthermore, a considerable body of research has linked Ki-67 expression with the aggressiveness and prognosis of adenoid cystic carcinoma, although results remain inconclusive.[8] Consequently, it is important to predict the prognosis of the tumor. Accurate identification of tumors by means of immunohistochemical examination is critical to reducing instances of misdiagnosis and establishing more appropriate therapies in order to avoid recurrence and metastasis and improve prognosis.[9]

Ki-67, a protein located in chromosome 10q26.2 on the 16th exon, is a double band of polypeptide with a molecular weight of 345 and 395 kD. This protein lies within the cell nucleus and is expressed by cells undergoing proliferation with expression levels changing throughout the cell cycle. Ki-67 will also be expressed in phase G1, phase S, phase G2 and then continually during phase M, but not the resting phase (phase G0).[2,10,11]

The occurrence of a malignancy fundamentally involves gene activity stimulating growth, in addition to gene mutation, which regulates apoptosis. In other words, a tumor can occur as the result of a higher rate of cell proliferation than cell death, resulting in the progression of the tumor.[12,13] Thus, in order to predict the aggressiveness of a tumor, any immunohistochemical examination conducted should employ Ki-67 as an antibody marker. By utilising Ki-67, cell proliferation will be detectable because this antibody will only be expressed under such conditions. The working principle of Ki-67 antibodies is based on the form of antigen and antibody reaction. The Ki-67 antibody will react positively to the tumor cell nucleus antigen, a process referred to as an immunoreactive condition.[2] The proliferation rate of a tumor is closely related to its biological behavior. Consequently, the higher and more rapid the rate of tumor proliferation, the greater its aggressiveness and the more serious the prognosis.[14] Therefore, this research aimed to investigate the relationship between Ki-67 expression and the clinical parameters and histopathological grading of salivary adenoid cystic carcinoma. Thus, Ki-67 expression can be confidently utilised as an indicator underpinning a medical prognosis, leading to the implementation of an appropriate therapy to avoid recurrence.

MATERIALS AND METHODS

This research constituted a retrospective study conducted between 2013 and 2015. The samples used consisted of twenty three paraffin blocks of adenoid cystic carcinoma derived from salivary glands examined at the Department of Anatomical Pathology, Dr. Hasan Sadikin Hospital, Bandung. Data relating to these paraffin blocks in terms of the age, sex and tumor locations of patients was then recorded.

With the help of two anatomical pathologists from FKUP/RSHS Bandung the paraffin blocks were re-stained with eosin hematoxylin enabling diagnoses and histopathological types to be established. Histopathological grading consisted of the following categories: grade I; if there was a tubular and cribriform pattern without a solid component, grade II; when only cribriform pattern was present or mixed with a solid component comprising less than 30% and grade III; when there was a tumor with a predominantly solid component.[9]

Immunohistochemical preparations were made using a Ki-67 antibody (Clone SP6, Biocare, USA) 1:50 dilution withjn a streptavidin-biotin peroxidase method (LSAB kit K0492, Dako, Carpinteria, CA). Such preparations were considered to be positive if a brownish immunoreactive tumor cell nucleus was present. The results were compared with both positive controls using lymphoid tissues and negative controls (performed on the same adenoid cystic carcinoma preparations using secondary/"non-immune serum" antibodies). Thereafter, Ki-67 immuno-expression was assessed according to its percentage and intensity. The percentage of Ki-67 expression was calculated by means of a "hand tally counter" (VWR, no catalog 23609-102) on 1000 tumor cells in ten representative fields using a CX-21 light microscope (Olympus America Inc. Melville, NY 11747) at a magnification of 400X.

In order to determine the relationship between Ki-67 expression and clinical parameters (age, sex and location), Ki-67 expression was categorized by the percentage of tumor cells falling within two categories, namely; one with a high proliferation index (PI↑) where the positive cell percentage was greater than 40%, and another with a low proliferation index (PI↓) with a positive cell percentage less than 40%.[15] In addition, to determine the relationship between Ki-67 immuno-expression and histopathological types, the percentage of Ki-67 expressed by tumor cells was categorized as score 1 if positive cells accounted for less than 20%, as score 2 if positive cells represented between 21-50%, as score 3 if positive cells amounted to between 51-80% as and score 4 if positive cells totally over 80%. The intensity of Ki-67 expression was also categorized into score 1 for weak intensity (light brown approaching

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negative control), score 2 for medium intensity (brown between score 1 and score 3) and score 3 for strong intensity (dark brown resembling positive control).[16,17]

Based on the percentage and intensity values, the Ki-67 expression score was calculated by multiplying the percentage value (score 1, 2, 3 or 4) by the intensity value (score 1, 2 or 3). The results obtained ranged from 1 to 12 and were then associated with histopathological grading of salivary adenoid cystic carcinoma.[9] Thereafter, the data obtained were recorded for further statistical analysis using a chi-square test.

RESULTS

Of the 23 cases of adenoid cystic carcinoma, 13 afflicted males and 10 females. The median of the average age was 48.44 years (with a range of 31-68 years). The ratio of male to female was 1: 0.77. There were four cases of tumors located in major salivary glands, three of which were located in the submandibular gland, with one being located in the parotid gland. On the other hand, the remaining nineteen cases were found in the minor salivary glands, thirteen of which were in the palate, two in the buccal mucosa, two in the tongue and two in the mouth (Table 1).

Based on the examination results of Ki-67 expression in the 23 cases of adenoid cystic carcinoma, four men had PI↓, while nine had PI↑. On the other hand, six women had PI↓, whereas four had PI↑. Moreover, the results also showed that four people at the age of < 48 years had PI↓, while five at the age of < 48 years had PI↑. In contrast, six people aged > 48 had PI↓, whereas eight aged > 48 had PI↑. In addition, one case located in the major salivary glands had PI↓, while the other three had PI↑. Then again, nine cases located in minor salivary glands had PI↓, whereas the other ten had PI↑.

Moreover, based on the statistical test results, there was no significant difference between the Ki-67 expression percentage and the clinical parameters, such as sex ($p = 0.16$), age ($p = 0.94$), and location ($p = 0.41$) in the cases of adenoid cystic carcinoma (Table 2, Figure 1).

Based on the contents of Table 3, of the 23 cases of adenoid cystic carcinoma studied, there were ten were of grade I, eight cases of grade II and five of grade III. The table also illustrated that the number of tumor cells expressed with Ki-67 varied from < 20% to> 80%, i.e. three cases (< 20%), ten cases (21% -50%), six cases (51% -80%) and four cases (> 80%). Furthermore, it was also known that the intensity of Ki-67 indicated nine cases of weak intensity, eleven cases of moderate intensity, and three cases of strong intensity (Table 3).

Based on the percentage value and intensity of Ki-67, the Ki-67 expression score was calculated by multiplying the percentage of Ki-67 by its intensity, resulting in a score of 1-12. The results confirmed two cases with score 1, five cases with score 2, two cases with score 3, six cases with score 4, three cases with score 6, two cases with score 8, one case with score 9, and two cases with score 12. The data was then related to the histopathological grading of adenoid cystic carcinoma (Table 4 and Figure 2).

In addition, based on the statistical test results, there was a significant difference between Ki-67 expression and histopathological grading of adenoid cystic carcinoma ($p < 0.01$). It means that the higher the value of Ki-67 expression, the higher the histopathological grading.

Table 1. Characteristics of patients with adenoid cystic carcinoma

Variables	n (%)
Median of Age (years)	
< 48	9 (39)
≥ 48	14 (61)
Sex	
Male	13 (56.5)
Female	10 (43.5)
Location	
Major salivary glands, 4 cases (17%):	
Submandibular gland	3 (13)
Parotid gland	1 (4)
Minor salivary glands, 19 cases (83%):	
Palate	13 (56)
Buccal mucosa	2 (9)
Tongue	2 (9)
Mouth base	2 (9)

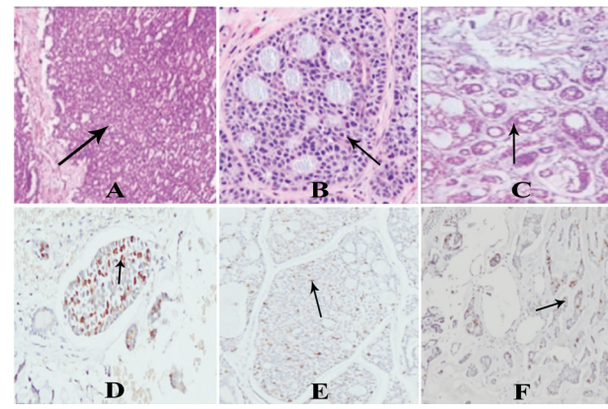

Figure 1. The results of hematoxylin eosin staining in adenoid cystic carcinoma: (A) Solid type, (B) Cribriform type, and (C) Tubular type. Ki-67 immunoxpression in adenoid cystic carcinoma: (D) Solid type, PI (> 40%), (E) Cribriform type, and (F) Tubular type, PI↓ (< 40%). (100× magnification)

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Table 2. The relation of the Ki-67 expression percentage and the clinical parameters in ACC

Characteristics	Ki-67 (%)		P
	PI↓(< 40%)	PI↑(≥40%)	
Sex			
Male(13)	4	9	0.16 NS
Female(10)	6	4	
Age (31–68 years old, mean 48,44 years old)			
< 48(9)	4	5	0.94 NS
≥48(14)	6	8	
Location:			
Major salivary glands (4)	1	3	0.41 NS
Minor salivary glands (19)	9	10	

NS = Non Significant

Table 3. The percentage and intensity of Ki-67 in the histopathological grading of adenoid cystic carcinoma

Histopathological Grading	Percentage (%)				Intensity			Total (n)
	< 20	21–50	51–80	> 80	Weak	Moderate	Strong	
I	3	7	–	–	7	3	–	10
II	–	3	4	1	2	6	–	8
III	–	–	2	3	–	2	3	5

Table 4. The relation of Ki-67 expression and histopathological grading of adenoid cystic carcinoma

Histopathological Grading	Ki-67 expression score								Total (n)	Sig P
	1	2	3	4	6	8	9	12		
I	2	5	–	3	–	–	–	–	10	0.009*
II	–	–	2	3	2	1	–	–	8	
III	–	–	–	–	1	1	1	2	5	

*) Significant

DISCUSSION

Adenoid cystic carcinoma is a rare tumor, 2% to 4% of such malignancies being found in head and neck.[5] In general, adenoid cystic carcinoma is mostly found in the minor salivary gland and palate.[2] Similarly, the results of this research showed that 56% of adenoid cystic carcinoma cases occur in the palate. The results also confirmed that the ratio of men to women was 1: 0.77. The mean age of the patients was 48.44 years. As with the results of this research, a number of previous investigations found that adenoid cystic carcinoma may affect all age groups with its highest incidence at both middle and old age, but with no specific predominance of a particular sex.[2,18]

In recent years, molecular biology has been widely developed leading to invaluable results. Various molecular markers have been identified and their association with oral cavity tumor development widely discussed.[19] Nowadays, considerable research has been focussed on Ki-67 expression and malignant tumor prognosis.[20] In mammary tumors, Ki-67 expression has an independent and useful prognostic value in predicting recurrence, survival rate and therapeutic response.[10] For example, since Ki-67 expression increases in malignant oral tumors, it may be used as an indicator in predicting the prognosis of squamous cell carcinoma within the oral cavity.[21] However, only limited research into Ki-67 expression in salivary gland tumors exists.

The basis of the occurrence of a malignancy actually lies in the fact that there is gene activation stimulating growth in addition to the gene mutation regulating apoptosis. As a result, when excessive cell proliferation and lack of apoptosis occurs, the tumor will develop.[12,13] In tumors experiencing aggressive growth, there can also be an imbalance between cell production and cell death, in which the former exceeds the latter. The proliferation rate of a tumor is closely related to its biological behavior. Thus, the faster and the higher the proliferation, the more aggressive the tumor will be and the greater the potential for a more serious prognosis. Similarly, the higher the level of anti-apoptosis, the more progressive the tumor and the worse the prognosis might be.[10,16]

In this research, the Ki-67 antibody marker was used to observe cell proliferation within a special

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immunohistochemical examination. Ki-67 is a specific cell proliferation marker. By using the Ki-67 antibody, cell proliferation will be detectable because the antibody will only be expressed in proliferative cells. The Ki-67 antibody will be expressed in the cell nucleus during some cell proliferation phases, namely: phase G1, phase S, phase G2 and phase M, but not during the resting phase (phase G0) of the cell cycle. The more actively a cell divides, the more rapidly Ki-67 antibodies will be expressed until phase M. Then, it will decrease and disappear during the resting phase where there is no cell proliferation, so the Ki-67 antibody cannot be expressed.[2,10,11] This condition can also distinguish the types of Ki-67 intensity, namely: weak, medium, and strong during the immunohistochemical staining process. Therefore, the more actively tumor cells proliferate, the stronger the intensity of the color. Meanwhile, the less actively tumor cells proliferate, the weaker the intensity of the color.

Moreover, many researchers have linked Ki-67 expression as a marker with malignancy and a prognosis of adenoid cystic carcinoma, but the results are still confusing.[8] Consequently, this research linked Ki-67 expression with clinical parameters (sex, age and location). However, the results of this research did not indicate any relation between Ki-67 and clinical parameters. This suggests that adenoid cystic carcinoma can occur in both men and women, either in the major or minor salivary glands. This also suggests that both the < 48 years age group and that of ≥ 48 years have the same chance of suffering adenoid cystic carcinoma, so it is ineffective in determining its aggressiveness and prognosis. There is a reference which suggests that the prognosis of adenoid cystic carcinoma is affected by growth type, stage, anatomical location, tumor size and metastasis. However, some references suggest that although additional surgical and radiotherapy therapies have been administered, the survival and prognosis rates of patients with adenoid cystic carcinoma remains poor. Consequently, the prognosis of adenoid cystic carcinoma remains clinically unpredictable.[6,7]

Furthermore, considerable previous research has linked Ki-67 expression with the aggressiveness and prognosis of adenoid cystic carcinoma, although the results are still perplexing.[8] For these reasons, the research reported here used previously unstudied samples of adenoid cystic carcinoma supplied by the Department of Anatomical Pathology, Dr. Hasan Sadikin Hospital, Bandung. Similar to the results of this research, those of two previous investigations conducted by Carlinfante *et al*. and Amoueian *et al*. also showed there to be no relationship between Ki-67 and the clinical parameters of adenoid cystic carcinoma.[18] Similarly, research conducted by Jiang *et al.* found no relationship between Bcl-2 expression and the clinical parameters in adenoid cystic carcinoma.[9] Since there was no relation between Ki-67 expression and clinical parameters within this research, the clinical parameters cannot be used to determine the aggressiveness and prognosis of adenoid cystic carcinoma.

In addition, this research linked Ki-67 expression with the histopathological grading of adenoid cystic carcinoma which, according to Szanto et al., is divided into three grades, namely: grade I, if there was a tubular and cribriform pattern without a solid component, grade II, with the presence of a sole cribriform pattern or one mixed with a solid component of less than 30% and grade III, when the presence of a tumor with solid component was the dominant one.[18] In this research, 43.5% of tumors were classified as grade I, 34.8% as grade II and 21.7% as grade III. In general, the Ki-67 expression in the 23 cases of salivary adenoid cystic carcinoma significantly augmented with the increase in histopathological grading. This result suggests that the higher the value of Ki-67 expression, the higher the histopathological grading. Similarly, research conducted by Norberg *et al.* showed that the number of tumor cells positively expressing Ki-67 correlates significantly with their tumor gradation. The same result was found in research conducted by Triantafillidou *et al*. and Suzzi *et al.*[18] Another previous piece of research conducted by Faur *et al*.,[10] also suggests that immunohistochemical examination can detect significant proliferation of tumor cells indicating an increasingly aggressive tumor.

Finally, it can be concluded that there is no relationship between Ki-67 expression and clinical parameters, although there is correlation between Ki-67 expression and the histopathological grading I, II and III of adenoid cystic carcinoma. As a result, clinical parameters are unusable in determining the prognosis of adenoid cystic carcinoma, although Ki-67 expression can be used for this purpose. Consequently, a more appropriate therapy can be implemented and recurrence avoided.

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