Immunoexpression of E-Cadherin and VEGF-A Proteins in Various Degrees of Histologic Malignancies of Adenoid Cystic Carcinoma of Salivary Glands

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Abstract

Fifty-one patients with adenoid cystic carcinoma (ACC) arising in salivary glands were studied retrospectively. This carcinoma is the most common salivary gland malignancy, it grows slowly, but is aggressively invasive, and easily metastasis. Three histologic grades were established; although these histologic growth patterns provide some indicators of clinical outcome additional predictors of biological activity were proven to be helpful for clinical management of the patient. The purpose of this study was to analyze the alteration of immune expression of E-cadherin adhesion protein and vascular endothelial growth factor-A (VEGF-A) protein in salivary gland ACC which was correlated with the degree of histopathological signs of malignancy based on the growth pattern type of ACC. A cross-sectional study was performed on 51 paraffin blocks from patients with ACC salivary gland, retrospectively. The examination began with a histopathological examination slide to diagnose the case of salivary gland ACC and to get data of the degree of histopathological signs of malignancy based on the growth pattern type of ACC. Subsequently, an immunohistochemistry examination of E-cadherin and VEGF-A was undertaken. The decrease or total absence of E-cadherin expression followed by the increase of VEGF-A expression had a very significant correlation with the increasing degree of histological malignancy ($p<0.001$) and had a probability of 81% of becoming a high malignancy of ACC. In conclusion, E-cadherin and VEGF-A were able to be used as markers for tumor to predict the prognosis of malignancy in adenoid cystic carcinoma of the salivary gland.

Experimental article (J Int Dent Med Res 2020; 13(3): 873-879)

Keywords: Adenoid cystic carcinoma, Salivary gland, E-cadherin, VEGF-A, Immunohistochemistry.

Received date: 23 May 2020 **Accept date:** 04 July 2020

Introduction

Adenoid Cystic Carcinoma (ACC) is an epithelial malignant tumor and the most common malignant tumor of the parotid gland and submandibular gland in the oral cavity.[1,2] ACC, formerly called cylindroma, is an infiltrative malignant tumor, generally grows slowly and tend to be aggressively invasive, and 40% - 60% of ACC easily metastasizes hematogenously (blood borne).[3,4] After surgery, more than 40% of ACC tends to recur, 25% of which spread to other parts of the body such as lungs, bones, liver, and brain. This spread usually does not cause any symptoms, and only by radiological examination can it often be found in the form of multiple nodules. In the early stages, if the boundary of the tumor tissue is still positive or has invaded into the perineural area, the lesion can be treated with radiation or excision and for postoperative reevaluation, radiotherapy will be used. In the advanced stage, a radical surgery will be performed combined with postoperative radiotherapy. For cases involving bone, palliative radiation will be a treatment option. However, many case reports found that after the removal of ACC tumor tissue, 50% or more can recur and spread to the lungs, bones, liver, or brain.[5,6]

The prognosis of ACC is based on clinical properties often used as prognostic prediction such as the location and size of the tumor, presence or absence of metastases at the time of diagnosis, and the expansion of adequate

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excision. About 50% of the patients with ACC tumor with a measurement of 2 cm or smaller were able to survive well for at least 10 years.[7] In the case of ACC with distant metastases, only 20% can be healed (within 5 -14 years), but 33% of the cases died in less than two years.[8]

Based on the histologic examination, ACC is divided into three types of growth patterns: tubular type, cribriform (glandular) type, and solid type. The most dominant type is the solid type which is often associated with poor prognosis, therefore researchers often classify the degree of histopathology malignancy based on the combination of three types of growth pattern and the presence or absence of the solid type in three levels in degrees of 1, 2, and 3.[8,9] Several researchers have suspected that the type of growth pattern was not related to survival rates, but the degree of histopathology malignancy played an important role in the prognosis of ACC.[9,Hata! Başvuru kaynağı bulunamadı.] Therefore, the existence of various differences in the biological behavior of tumors leads to the need for a combination of clinical examination, routine histopathology, and more accurate use of other diagnostic support factors.

E-cadherin is a cell-adhesion molecule-controlling gene that acts as an intermediate adhesion between epithelial cells and plays an important role in histogenesis and organogenesis. Mutation of the adhesion molecule control gene will affect its function as a contact control of cell inhibition up to intercellular communication, which is indispensable for normal proliferation and differentiation. In addition, these gene mutations can also lead to the release of tumor cells from primary tumor tissue, which is the initial stage of the process of invasion and metastasis of tumor tissue. Based on studies on carcinoma,a reduction or absence of E-cadherin expression may implicate the loss of the differentiation process, accompanied by high mobility and the invasion of metastasized epithelial cells.[7,Hata! Başvuru kaynağı bulunamadı.-13]

Invasion and metastasis are certain signs of malignancy in carcinoma, and the mechanism is determined by four important factors: oncogenes, proteolytic enzymes, adhesion molecules, and angiogenesis.[14] The process is very complex and begins with the release of cells from the primary tumor tissue, through the basement membrane, degrading the extracellular matrix, then entering the blood vessels or lymph, until finally metastasis occurs and grows in new places as secondary tumor tissue. At the time of the formation of primary and secondary tumor tissue, a new blood flow also formed through the angiogenesis process that serves as a nutrient and oxygen supplier that supports tumor tissue to survive and grow.[15] The occurrence of the angiogenesis process begins with the movement and migration of endothelial cells needed for the formation of new blood vessels. Therefore, vascular endothelial growth factor (VEGF) is an important factor in the regulation of tumor angiogenesis.[16] Most tumor cells are able to secrete VEGF, to induce angiogenesis in tumor tissue. Various studies have shown that there was a close relationship between high expression of VEGF with poor metastasis, recurrence, and prognosis in malignant tumor tissue. VEGF is grouped into VEGF-A, - B, - C, and – D.[17,18] The Mohammed R.A et al., study suggested that the elevation of VEGF expression was associated with hematogenous invasion of ACC invasion.[18] The description above shows that there are biological heterogeneities and various signal pathways in neoplasm tissue. Therefore, knowledge of the ACC progression mechanisms from various possibilities are needed because it is the modulation factor in the carcinoma progression pathways.

A better understanding of potential molecular mechanisms in carcinoma may be helpful in determining a more accurate prognostic marker to obtain a better understanding of the clinical properties and stratification of patients for the need of different treatment strategies.

Materials and methods

The population of this study was obtained from tissue biopsy of ACC of salivary gland patients which had been embedded in the paraffin block, completed with the patient's medical record and hematoxylin-eosin (HE) histology preparation. This study was designed using a non-random observational cross-sectional study to obtain a predictive model of E-cadherin and VEGF-A immunoexpression against the degree of histopathologic malignancy parameters of salivary gland ACC.

The subjects of the study were tumor tissues with histopathologic diagnosis of the ACC of salivary glands on 51 paraffin blocks, retrospectively. All tumor samples were re-

diagnosed to determine the diagnosis of ACC and to obtain the histopathological malignancy data from histologic examination using HE staining (modification Meyer's method). This was hen followed by an immunohistochemical examination (streptavidin-biotin method) towards E-cadherin and VEGF-A.

The degree of histopathological malignancy was determined by the growth pattern of ACC salivary glands (Tubular, Cribriform, and Solid). Assessment criteria were modifications of the methods of Szantos et al and Batsakis et al.[19,20] The criteria were: degree 1, a combination of tubular types with cribriform without solid type, degree 2, cribriform only or mixed with solid type (<30%), and degree 3, a mixed type with solid types (>30%).

The criteria of E-cadherin and VEGF-A immunoexpression based on the intensity of immunohistochemical staining were as follows: negative, weak, moderate, and strong. Meanwhile, the criteria based on the pattern distribution of immunoexpression protein on the entire cancer tissue present in the slide, were as follows: 0 if negative; if ACC tumor tissue cells were immunoreactive positively then the value was +1 (<25%); +2 (≥25% - 50%); +3 (>50% - >75%); and +4 (>75%).[19,21]

The assessments were modified semi-quantitatively from the combined scoring of the staining intensity and the distribution pattern as seen in Table 1.

Distribution	INTENSITY			
	- (Negative)	Weak	Moderate	Strong
0	0	-	-	-
+1	-	1	5	9
+2	-	2	6	10
+3	-	3	7	11
+4	-	4	8	12

Table 1. Scoring value for immunoexpression of E-cadherin and VEGF-A based on the assessment of intensity staining and distribution pattern.

The minimum score was 0, for the weak intensity of immunoexpression with distribution value was +1 = 1, whilst the score for strong intensity with the highest distribution value was +4 = 12 as a maximum score.

Data on the degree of histopathological malignancy were obtained from performing an histopathologic evaluation based on solid type percentage from the three growth pattern types for all cases of salivary glands ACC. Data for E-cadherin and VEGF-A immunoexpression were obtained from a semiquantitative assessment based on the percentage of intensity and distribution patterns of immunohistochemical staining. Bivariate analysis was carried out to determine the relationship and the magnitude of risk between each independent variable (E-cadherin and VEGF-A) with the dependent variable (histopathological malignancy degree based on ACC growth pattern type). While multivariate analysis was done simultaneously between independent variables with dependent variables to determine which independent variables were influencing the outcome the most. Data processing was done using SPSS statistical application and Med Clacd® software. For statistical analysis, the test type, statistical value, and significance of statistical test based on p-value < 0.05 (in 95% confidence level) were included.

Results

The immunohistochemical staining of E-cadherin displayed weak intensity in Figure 1. In Figure 2, the immunohistochemical staining of VEGF-A expressed weak and moderate intensity. In addition, immunohistochemical staining of VEGF-A with histopathological signs of malignancy degree 3 exhibited a strong immunoexpression intensity (Figure 3).

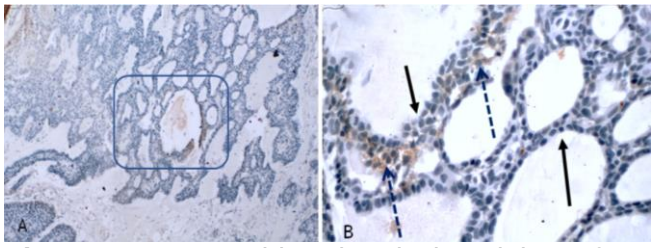

Figure 1. Immunohistochemical staining of E-cadherin with histopathological signs of malignancy degree 1: (A) Weak intensity (↑) distribution value of +1 (100 x); (B) Positive immunoexpression of E-cadherin with weak intensity (dotted arrow) and negative immunoexpression (full arrow) (400 x).

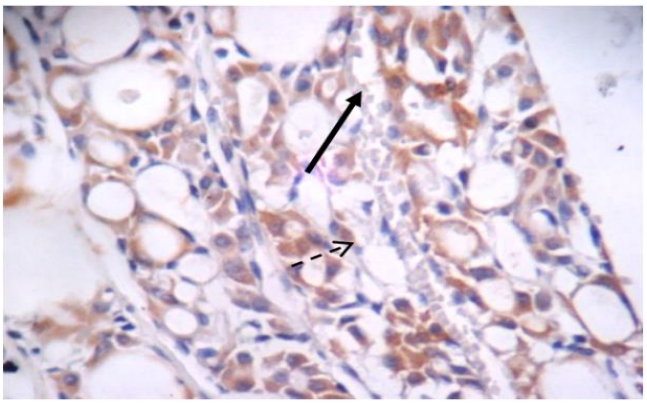

Figure 2. Immunohistochemical staining of VEGF-A with moderate intensity (400 x).

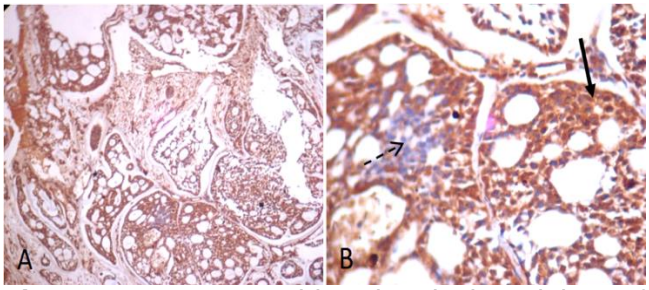

Figure 3. Immunohistochemical staining of VEGF-A with histopathological signs of malignancy degree of 3: (A) Strong intensity distribution value of +4 (100 x); (B) Strong intensity staining (full arrow), negative cells staining (dotted arrow) (400 x).

Protein	Expression	Frequency N=51 (100%)	Histopathological Signs of Malignancy Degree 1	2	3	Significance Value
E-cadherin	Negative	42 (82.4)	2 (4.8)	17 (40.5)	23 (54.8)	Zm-w = 3.364
	Positive					p < 0.001
	Intensity:					
	- Weak	9 (17.6)	3	6	-	
	- Moderate	-	-	-	-	
	- Strong	-	-	-	-	
	Distribution:					
	+ 1	7 (13.7)	2	5	-	r_s = - 0.4777
	+ 2	2 (3.9)	1	1	-	p < 0.001
	+ 3	-	-	-	-	
	+ 4	-	-	-	-	
VEGF-A	Negative		-	2	-	r_s = 0.782
	Positive				p < 0,001	
	Intensity:					
	- Weak	18 (35.3)	4	13	1	
	- Moderate	13 (25.5)	1	8	4	
	- Strong	18 (35.3)	-	-	18	
	Distribution:					
	+ 1	2 (2.0)	-	1	1	r_s = 0.322
	+ 2	12 (23.5)	2	6	4	p < 0.021
	+ 3	22 (43.1)	2	11	9	
	+ 4	14 (27.5)	1	3	10	

Table 2. The frequency of immunoexpression of E-cadherin, VEGF-A and the correlation towards histopathological signs of malignancy degree of ACC of salivary gland.

Notes : r_s = Spearman's rank correlation coefficient
Z_{m-w} = Mann-Whitney U test

The majority of E-cadherin expression of 51 cases of ACC of salivary gland tissues were on the negative value (82.4%) (Table 2). In 51 cases of VEGF-A, the negative expression was only 2 cases, whereas the positive expressions with strong intensity there were 35.5% (18 cases) and for the staining distribution of +3 and +4, were 43, 1% (22 cases) and 27.5% cases (14 cases) respectively.

The negative correlation between E-cadherin expression and histopathological malignancy was significant. (Z_{M-W} = 3.364; p < 0.001). E-cadherin negative expression of 42 cases was found to be highest in malignancy degree 3 (23 cases, or 54.8%). Positive E-cadherin expression with the weak intensity only existed in the histopathological malignancy degree 1 and 2, consecutively (3 and 6 cases, respectively). Similarly, the lower the staining distribution, the higher the value of histopathological malignancy degree (rs = - 0.477; p< 0.001), it is seen on the distribution of +1 that there were 5 cases of malignancy in degree 2. On the contrary, the VEGF- A expression evidenced a positive relationship with malignancy, which was the higher the intensity and distribution, the higher the histopathological malignancy degree (intensity: rs = 0.782, p < 0.001; distribution rs = 0.322, p < 0.001). VEGF-A expression with strong intensity was found to be dominant in histopathological signs of malignancy degree 3 (18 cases), whereas for the staining distribution of +4 = 10 cases were found. Negative expression of VEGF-A was only found in 2 cases, which was on the histopathological malignancy degree 2. Positive correlation was observed between the intensity and distribution of VEGF-A, in which the higher the VEGF-A distribution, the stronger the intensity (rs = 0.376, p = 0.007).

VEGF-A score	E-Cadherin score	Malignancy probability
<6	0	0.07
≥6	0	0.81
<6	≥1	0.00
≥6	≥1	0.00

Table 3. The probabilities of maligancy occurence from the combination of VEGF-A and E-cadherin.

Based on Table 3 above, it can be calculated the probability of malignancy from various scores of E-cadherin and VEGF-A immunoexpression. The higher the VEGF-A score (≥ 6) and the lower the E-cadherin score

(0), the higher the probabitlity change of a malignancy ACC at degree 3 (81%).

Discussion

Some researchers used various criteria of the ACC of salivary gland histopathological signs of malignancy degree, but in principle, the classification was based on the presence of any solid type. In this study, histopathological examination of all ACC tumor tissues showed mixture of the three types of growth patterns. Therefore, the criteria of degrees 1, 2, and 3 were the modification of Batsakis, in order to be adjusted to the cases; however, the percentage of solid type was a determining condition of the high malignancy degree.[19,20]

The degree of histopathological malignancy 1 in this study was found the least in only 9.8% of cases, whereas the degree 1 was the least aggressive ACC tumor tissue compared to degrees 2 and 3. This was in accordance with the condition of patients with carcinoma in Indonesia that generally came for treatment to the doctor at the advanced stage.[20,22] This may also be explained by the poor level of health education in developing countries, including Indonesia.

It is known that the sign of malignancy progressivity in carcinoma was the occurrence of invasion and metastasis. The mechanism of metastasis is determined by several important factors including adhesion molecules, and angiogenesis.[23,24] The process of invasion is the beginning of metastasis, starting when the carcinoma cell is released from the tumor due to the adhesion factor that fails to function perfectly in maintaining network integrity. The most important adhesion molecule in epithelial tissue is E-cadherin which functions as catenin complex formation. Cadherin is a family of glycoproteins trans-membrane involved in intercellular adhesion that depends on the calcium ion, and if other adhesion systems are inactive it will have a little effect on cellular adhesion as long as cadherin is still functioning normally. Several studies have linked E-cadherin expression, which acts as an invasive suppressor gene, with tumor growth in several carcinomas.[24,25]

The classification of histopathological malignancy degree in this study was based on solid type percentage of tumor tissue, where the degree of 3 ACC tissue were mostly of the solid type (more than 30%). The majority of E-cadherin immunoexpression from 51 cases of the salivary gland tissue ACC, were found to be negative (82.4%) and mostly found in the malignancy degree 3 as much as 54.8% (23 cases), while on the degree 1 was only found in 4.8% (2 cases).

The correlation between E-cadherin immunoexpression and histopathological malignancy of the salivary gland ACC also was very significant ($Z_{M\text{-}W} = 3.364$, $p < 0.001$), in other words, the negative intensity toward the degree of 3, and the lower the distribution pattern of immunoexpression, the higher the degree of histopathological malignancy. Using multiple logistic regression analysis, the results showed that E-cadherin was strongly related with histopathological malignancy ($p = 0.002$), in other words, the higher the E-cadherin immunoexpression score, the lower the risk of malignancy (OR < 0.001). This condition showed that the absence or reduction of E-cadherin expression in ACC of salivary glands was closely related to the high degree of histopathological signs of malignancy. Therefore, as in the ACC of the breast gland, E-cadherin immunoexpression on ACC of the salivary gland can be used as a marker of malignancy. Decreasing E-cadherin immunoexpression will increase the degree of histopathological malignancy, so that the progression of malignancy in the salivary gland increases. This is because when the cell adhesion becomes weaker it causes cell differentiation to disappear and makes it easier for cells to invade.[26,27]

Angiogenesis is also an important factor in malignancy because it helps maintain the life of tumor tissue and facilitates metastasis. The stages of metastasis as a sign of malignancy is preceded by the activity of proto-oncogene changes into oncogenes, then through the angiogenesis process, the tumor forms a new blood flow (neovascular) to enable the tumor to continue growing and subsequently the ability of tumor cells to avoid immunosurveillance. The role of VEGF-A in terms of angiogenesis and bone marrow metastases has been widely known. It was stated that members of the VEGF family were involved in lymph node metastasis, and the role of VEGF-A helped the growth of metastatic tumors through angiogenesis. VEGF-A is one member of the VEGF family that plays an important role in vasculogenesis and angiogenesis.[18] Study of the correlation between

VEGF and micro-vessel density (MVD) of salivary gland ACC tissue showed that excessive VEGF expression contributed to the increased value of MVD.[28,29]

The results of this study showed that from 51 cases of ACC of the salivary gland, VEGF-A expressions with positive value were the majority with as much as 48 cases, which for strong intensity was 35.5% (18 cases) and for distribution of +3 and +4 were 43.1% (22 cases) and 27.5% cases, respectively. Whereas for immunoexpression, negative VEGF-A was only found in 2 cases (3.9%). There was a significant relation between the intensity and distribution pattern of the VEGF-A immunoexpression, in other words, the higher the distribution of VEGF-A expression, the stronger the staining intensity.

The study by Zhang et al., regarding the correlation between VEGF with nuclear factor of kB and Nitric oxide synthase in ACC of the salivary gland tissue, stated that the VEGF relation with two factors above was found to be higher in the solid type compared to the other two growth pattern types (glandular and tubular types).[28] In this study, it was found that the higher the VEGF-A immunoxpression (score ≥6), the higher the chance of the risk of increased malignancy, which was as much as 58.8 times compared to the low degree of immunoexpression (score <6) and very significant ($p < 0.001$). This showed that the increased expression of VEGF-A of the salivary glands ACC was closely related to the high degree of histopathological malignancy.

When the primary tumor tissue was formed, the tumor will form a new bloodstream (neo-vascular) in order for continuing tumor growth (through the angiogenesis process). Neovascularization is necessary not just for activating nutritional supplements but for being the most frequent place for tumor cells to reach the circulation. It is also known that hypoxia will increase VEGF expression. This occurred because solid tumor tissue often led to hypoxic conditions so that VEGF production will increase, and will encourage the occurrence of tumor angiogenesis. The tumor cells have the ability to avoid immune surveillance that results in tumor cells being released from the primary tumor and entering the local of blood and the lymph nodes (circulation) to form a new tumor mass. By the time the tumor cells are released from the tumor mass, the most important factor is the adhesion factor of E-cadherin. The loss of E-cadherin expression or the failure of its function in forming a complex with catenin will lead to a potentially autonomous tumor cell.[7,**Hata! Başvuru kaynağı bulunamadı.**] The theory above was supported by the results of this study which showed a significant relation ($p < 0.01$) between immunoexpression of E-cadherin and VEGF-A. As shown in Table 3, the higher the VEGF-A score (≥ 6) and the lower the E-cadherin score (0), the higher the chance of malignancy (degree 3) with a value of 0.81 (81%).

Conclusions

This study concluded that the probability of the increasing degree of histopathological signs of malignancy of ACC of the salivary glands was influenced by the absence or decrease of E-cadherin expression and increased of VEGF-A expression. The absence or low immunoexpression of E-cadherin, along with the increased immunoexpression of VEGF-A, was a correlation model that can be used as a tumor marker to predict the prognosis of malignant progressivity of ACC of the salivary gland.

Acknowledgements

We would like to thank all co-examiners, staff, and respondents willingly involved in this research.

Declaration of Interest

The author declared no conflicts of interest.

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