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Correlation of Immunoexpression of E-Cadherin and Vascular Endothelial Growth Factor to Histopathology Gradation and Enlargement Regional Lymph Node in Oral Squamous Cell Carcinoma

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Abstract: ***Introduction: Oral Squamous Cell Carcinoma (OSCC)is the most common factor of secondary infection in the head and neck. Changes in the expression function of both E-Cadherin and Vascular Endothelial Growth Factor (VEGF) genes are known to provide an increased chance of invasion, metastasis and tumor growth. Immunohistochemistry immune techniques are known to examine the expression of tumor-linked gene proteins (immunoexpression) with a high degree of cellular accuracy. Method: A total of 30 body tissue subjects were selected from patients of Anatomical Pathology Faculty of Medicine Universitas Padjadjaran-Hospital Dr. Hasan Sadikin Bandung. Subsequently prepared using immunohistochemistry principles to test the correlation of histopathology gradation and immunoexpression of both E-Cadherin and VEGF. This cross sectional study with secondary data was retrospective and then evaluated correlation ofboth immunoexpressions of E-Cadherin and VEGF. Correlation using Correlation Rank Spearman with significance p <0,05. Results: There was a negative correlation (r_s=-0.522) and significant (p <0.005) between E-Cadherin and histopathology gradations of OSCC. Results also showed no significant correlation between immunoexpression of both E-Cadherin and VEGF to enlargement of lymph nodes regional of OSCC. Conclusion: Oral Squamous Cell Carcinoma . Either E-Cadherin or VEGF is unrelated in enlargement of regional lymph nodes of Oral Squamous Cell Carcinoma.***

Keywords: Oral Squamous Cell Carcinoma, E-Cadherin, VEGF, histopathology gradations,immunoexpression.

1. Introduction

Tumors occur as a result of accumulation of genetic changes including activation of proto-oncogenes and inactivation of tumor suppressor genes.[1]Tumors can be prevented by early examination as investigations marker known as marker for prognosisbiology of tumor.Immunohistochemistryknown as the potentialtechnique to prognosis of tumor growth which is still need evaluate in the future.[2-5]Immunohistochemistry staining technique can examine the protein expression of the gene known as immunoexpression which is related to tumor cell level and it the accuracy is quite high and can be performed in conjunction with routine pathological examination of paraffin tissue blocks or fresh tissue with Fast and at a relatively more affordable cost.[3, 4-6]

One of the oral cavity tumors of concern to date is Oral Squamous Cell Carcinoma caused by extrinsic factors including cigarettes, tobacco, alcohols, viruses, and other chemical agents. Intrinsic factors such as malnutrition or deficiency anemia, and immunological factors.[7,8,9]

E-Cadherin is an adhesion molecule that is expressed by normal epithelial tissues that contribute to the formation of the gland, stratification and polarization epitel.[10,11] E-Cadherin gene is located on chromosome 16q22.1 associated with invasive karsinoma.[12,13,14] History Research Reported that E-Cadherin is a tumor suppressor gene.[15,16.] Specifically, E-Cadherin is associated with intercellular adhesion activity in tumor tissue in the early stages of invasion and metastasis.[17,18]

Vascular Endothelial Growth Factor (VEGF) contributes to the growth of endothelial cells to support blood vessel formation. This protein has an effect on the mechanism of angiogenesis. Tumor growth is always correlated with the development of VEGF function, by pressing the signal angiogenesis.[19.20]Addition, the ability to grow and survival of cancer cells is dependent on the intake of oxygen and nutrients, one of them the role of hypoxia inducible factor / HIF-1 regulation governing the response to hypoxia on tumor growth .[17.21]

E-Cadherin which serves as the adhesion between cells, the loss of expression of this gene is associated with increased power of invasion and metastasis, VEGF [21] and role in the process of angiogenesis growth tumor.[22] Both of these proteins can be used as one indicator to assess neovascularization on squamous cell carcinoma.[17, 21]This study also evaluate immunoexpressi of E-Cadherin and Vascular Endothelial Growth Factor (VEGF) with gradation and enlargement of regional lymph nodes in squamous cell carcinoma of the oral cavity

2. Methods

This study was conducted cross-sectionalally with secondary data taken from Immunohistochemistry immuno-expression against E-Cadherin and VEGF immunoexpression based on gradation and metastatic and non-metastatic on Oral Squamous Cell Carcinoma. The sample population is 30 people taken from the Anatomical Pathology of Faculty of Medicine Universitas Padjadjaran-Hospital Dr. Hasan Sadikin Bandung.Sample based on Hosmer.[23]

Hematoxylin Eosin

The preparations were placed into the xylol solution for 2 minutes, then dried between two sheets of filter paper and then dipped successively into 96%, 80% and 50% alcohols for 10 times respectively, rinsed in running water for 2 minutes, then inserted into Hematoxylin Eosinsolution for 10-15 minute. After let it dried, immersed into 2% ammonia solution for 5 times, rinsed in water for 1 minute, dried it and then put into eosin solution for 1-2 minutes. The preparation is subsequently dipped in 96% alcohol at three different places in succession 10 times. After it was inserted in a solution of carboxyl for 1 minute then put into xylol solution for 1 minute in a row on two places then spilled with an entrainment and covered with cover glass then incubatedovernight at 37°C.[24]

Immunohistochemistry

Avidin-Biotynilated horseradish peroxidase Complex staining system is first performed by mixing in a special bottle, which is readily available by: Serum block: mixed in bottle 1 consisting of 75 µl of normal blocking serum with 5 ml PBS. Biotinylated secondary antibody: mixed in bottle 2 consisting of 75 µl normal blocking serum, 5 ml PBS and 25 µl biotinylated secondary antibody. AB enzyme reagent: mixed with bottle AB consisting of 50 ul reagent A (avidin), 5 µl reagent B (biotinylated HRP) and 2.5 ml PBS, mixed and allowed for 30 minutes. Peroxidase substrate: mixed in a substrate bottle consisting of 1.6 ml of aquadest, 5 drops of diluted substrate buffer 10 times, 1 drop of diluted 50 DAB chromogene and 1 drop of dilute peroxidase substrate 50 times. This mixture is for 15 - 20s.[25]

Tissue samples were fixed in 10% neutral buffered formalin for 24 hours. Tissue samples were processed in an auto processer and embedded in paraffin wax on an embedding station. The tissue blocks were sectioned by microtome into 4µM sections that were dried overnight at 37°C. Prior to antibody staining, the slides were pre-treated with microwave irradiation to unmask binding epitopes. After blocking endogenous peroxide activity with a 3% solution of hydrogen peroxide in methanol for 30 minutes, slides were immersed in 200 mL of 10 mM citric acid (pH 6.0) for 5 minutes at 100 Watt power in a microwave oven, followed by 4 cycles of 5 minute each on 50 Watt power. After topping up the buffer with distilled water, these steps were repeated. The slides were then left to stand for 10 minutes in buffer at room temperature before being washed thoroughly in tap water.

After three washes in tris-buffered saline (TBS), the slides were incubated with a 1:50 dilution of mouse anti-E-cadherin monoclonal primary antibody (DakoCytomation, Denmark) in TBS for 1 hour at room temperature. After three more washes in TBS, the secondary antibody, biotinylated goat antibody antibodi (Lab Vision)at a dilution of 1:100 in TBS was applied for 1 hour at room temperature. After an additional three washes, a streptavidin-biotin-horseradish peroxidase (HRP) complex was formed. After an additional three washes, the staining was visualized by adding diaminobenzidine (DAB) for 5 minutes at room temperature. The slides were washed well in tap water and counterstained with meyerhematoxylinfor 2minute and then dehydrated, cleared, and mounted in Xylene. The entire stained slide was evaluated for immunostaining by light microscopy. The slides were first observed under a 10X objective to confirm that the cells were still attached to the slide. Final evaluation was performed under 400X objective magnification. All images were taken under 400X objective magnification without oil immersion lens. [26]

Analysis of results

Histopathology gradation of squamous cell carcinoma of the oral cavity evaluate based on: gradation I (differentiated / well differentiated), gradation II (moderately differentiated / Moderate differentiated) and gradation III (poorly differentiated/ Poorly differentiated).[27] Immunoexpression level of E-Cadherin is evaluate based on group deployment pattern Immunohistochemistry staining according to the modification of Hiraki's method [28]: (-) = no staining, (+) = +1 staining, but less than 25%, (++) = staining +2, between 25% - 75%, and (+++) = staining +3 extends more than 75%. Rate VEGF staining similar to the E-Cadherin evaluated by Immunohistochemistry staining pattern as follows: (-) = no staining, (+) = +1 staining, but less than 25%, (++) = +2 staining, between 25-50%, (+++) = +3 widespread staining, between 50% -75%, and (++++) = staining +4, more than 75%.[29] Data analysed by Correlation Rank Spearman with significance p <0,05.

3. Results

In well-differentiated Oral Squamous Cell Carcinoma (OSCC) body tissues, arrows show E-Cadherin Immunohistochemistry immune in the cell membrane and cytoplasm of brownish color. In moderately differentiated OSCC tissues, arrows show E-Cadherin immunexpression in the cell membrane and cytoplasm of brownish color. Results of histopathological images of OSCC E-Cadherin and VEGF networks can be seen in Figure 1 below.

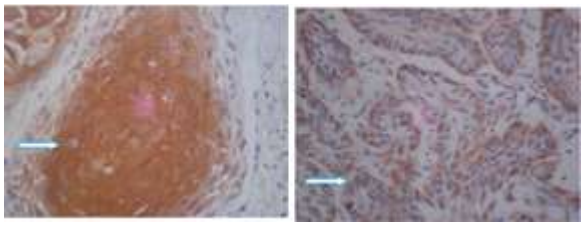

Figure 1: ImunoekspresiE-Cadherin (left) dan VEGF (right) Magnification 400x

Clinical characteristics of 30 cases of Oral Squamous Cell Carcinoma (OSCC)by age were: the average patient aged 54.5 years with age ranges from 40 years to 80 years and by gender the most were men than women, ie men as many as 17 cases (57%) and female patients as many as 13 cases

(43%). The most common sites of OSCC are the tongue area of 10 cases (33%) and the buccal mucosa of 9 cases (30%), followed by the lower lip of 3 cases (10%), 3 mandibular gingivae (10%), 2 cases of palate (7%), maxillary gingiva 2 cases (7%) and retromolar trigonum 1 case (3%). Clinical characteristics of OSCCcan be seen in Table 1.

Table 1.Clinical characteristics of OSCC

Description	*Amount*	*Percent (%)*
SEX		
Male	17	57
Female	13	43
AGE		
$\bar{x}$(SD)	56,2 (12,7)	
Median	54,5	
Range	40-80	
LOCATION		
Lingual	10	33
MucosaBuccal	9	30
Lip	3	10
Gingiva Mandibule	3	10
Palatum	2	7
Gingiva Maxilla	2	7
Trigonumretromolar	1	3

Based on histopathologic gradation, gradation I was found there were 20 cases (67%) and the smallest in Grade III category were 3 cases (10%). Category of histopathologic gradations of Oral Squamous Cell Carcinoma (OSCC)can be seen in Table 2.

Table 2: Category of histopathologic gradations of OSCC

Gradation of Histopatologic	Amount	%
Gradation I *(Well Differentiated)*	20	67
Gradation II *(Moderately Differentiated)*	7	23
Gradation III *(Poorly Differentiated)*	3	10
Total	**30**	**100**

Based on the results obtained a significant relationship (p <0.005) between E-Cadherin with histopathologic gradation of Oral Squamous Cell Carcinoma (OSCC). The association between E-Cadherin and the histopathologic gradation of OSCCrevealed a moderate relationship. (r_s = -0.522). In contrast, no significant association was observed between VEGF and histopathologic gradation in patients with OSCC. The two relationships can be seen in Table 3 below: this:

Table 3: Relationshipsof E-Cadherin, VEGF and histopathologic gradation of OSCC

Variabel	Gradasi histopatologis			r_s	p
	I	II	III		
E-cadherin					
0	0	1	1	-0,522	0,0031
1	0	0	0		
2	0	0	2		
3	3	3	2		
4	1	5	0		
5	6	1	0		
6	3	2	0		
VEGF					
0	1	3	1	-0,029	0,8771
1	0	0	0		
2	1	2	0		
3	5	1	0		
4	1	0	1		
5	2	3	3		
6	2	3	0		
7	1	0	0		

Table 4: Correlation ofE-cadherin, VEGFandregional enlargement of lymph nodes on OSCC

Variable	enlargement of lymph nodes		r_s	p
	(+)	(-)		
E-cadherin			0,012	0.9506
0	1	1		
1	0	0		
2	1	1		
3	4	3		
4	4	3		
5	1	6		
6	4	1		
VEGF				
0	2	3	0,018	0.5356
1	0	0		
2	2	1		
3	4	2		
4	2	1		
5	3	4		
6	2	3		
7	0	1		

4. Discussion

Oral squamous cell carcinoma occurs mostly in old age. This is consistent with studies suggesting that the incidence of oral squamous cell carcinoma will increase with the age of a person whose majority of the cases occur in people over 40 years of age.[8]The tumor is known to be an age-related disease. Initially, in the United States nearly 50% of cases of this tumor have occurred in women aged over 65 years. However, at this time, cases in women have passed the number of cases that hit men over the age of 65 years.Most cases of oral cavity tumors are diagnosed in the sixth and seventh decades of a person's life, with the highest prevalence seen in patients over 65 years. However, other studies reported an alarming increase in the incidence of oral tumors, especially tongue tumors, in young white men under 40 years of age.[17,26]

Based on its location, the most common sites occur on the tongue and buccal mucosa. This corresponds to most of the literature that has reported the most frequent site of infection is the tongue. Several other studies suggest that of the three most common sites as sites of oral squamous cell carcinoma are the tongue, lips, and oral floor.[29] This is because the lymphatic flow in the tongue is related to the early spread of carcinoma of the tongue.[28]As we know, oral squamous cell carcinoma may caused by a multifactor, which includes extrinsic and intrinsic factors. The extrinsic factors such as cigarettes, tobacco, and alcohol for a long time then is very intense with the tongue and buccal mucosa.

In this study, the number of male patients more than female, with a ratio of 1.33: 1. This is not matches with epidemiological studies of oral tumors that the ratio between male and female is 2: 1. However, the ratio of oral tumors still shows that it is higher in males. Oral tumors have shown

some significant epidemiological changes. The ratio of men to women has changed steadily from 6:1 in 1950 to 2:1 in 1997.This changing ratio may be the result of an increase in the number of female smokers and those exposed to carcinogenic agents especially those from food. [9,29]

The absence of a link between E-Cadherin and VEGF with KGB explains that, in determining the prognosis and management of cancer sufferers more to the role of angiogenesis intact in tumor or cancer growth.[22,31] In subsequent developments, much of the research evidence suggests that growth and Tumor metastasis is heavily dependent on angiogenesis.[22] A complex angiogenesis process explains that many factors affect the ability to grow and survive cancer cells other than the vascular endothelial growth factor.[19,20] Some research also suggests that oxygen and nutrient intake, one of which is the role of hypoxia inducible factor / HIF-1 regulating the regulation of hypoxic response to tumor growth.[17,21]

Meanwhile, E-Cadherin showed his relation with early stages of metastatic growth. This is in line with the research report that E-Cadherin has a role during the observation period of metastatic spread to regional KGB and distant metastases.[17,33] As is known, almost all malignant tumors go through a four-stage process of transformation, differentiation, invasion, and metastasis. At the stage of cell differentiation, there are variations in cell size and shape, abnormal nucleus morphology, many mitoses resulting in high proliferation activity.

Proliferation of tumor cells is known to have association with cell adhesion. One transmembrane glycoprotein on the cell surface mediating homophilic interactions between cells and the surrounding environment is E-Cadherin.10,11 In relation to metastasis it is known that the loss or occurrence of E-Cadherin dysfunction causes the tumor to be more invasive and potentially increase metastasis.3,17 This Explains the existence of E-Cadherin relationship with histopathologic grading of squamous cell carcinoma of the oral cavity at an early stage of growth and development. However, the variation in size and shape of the cells in the differentiation stages that have been described makes this relationship so meaningless. More samples are needed to clarify this relationship.

Meanwhile, the absence of an association between E-Cadherin and enlargement of regional lymph nodesmay also be due to normal cell mutations or transformation as a result of damage to growth regulatory genes and cell differentiation in the final stages. This statement is supported by other research report that mentions squamous cell carcinoma is often the final stage of a series of changes in epithelial cells that begin as epithelial dysplasia and continues until these epithelial epithelial cells penetrate the basement membrane and invade into the connective tissue.[8,34]

5. Conclusion

Oral Squamous Cell Carcinoma . Either E-Cadherin or VEGF is unrelated in enlargement of regional lymph nodes of Oral Squamous Cell Carcinoma.

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