RESEARCH ARTICLE

Expression of Ki67 and CD105 as Proliferation and Angiogenesis Markers in Salivary Gland Tumors

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Abstract

<u>Objective</u>: To investigate the association between CD105 and tumor cell proliferation in salivary gland tumors. <u>Methods</u>: In this study, 59 samples of salivary tumors from Khalili Hospital archive, including 20 cases of pleomorphic adenoma (PA), 20 cases of mucoepidermoid carcinoma (MEC) and 19 cases of adenoid cystic carcinoma, as well as 10 cases of normal salivary gland tissue, were reviewed by immunohistochemistry (IHC) for CD105 and Ki67 staining. <u>Results</u>: CD105 positive vessels were absent in normal salivary gland tissue in the vicinity of tumors (51.6% of all tumors were positive). There was a statistically significant difference in frequency of CD105 staining between PA and malignant tumors and between four groups of different lesions (p<0.000) being highest in MEC. Intratumoral microvessel density was also elevated in malignant neoplasms (2.61±3.1) as compared to PA (0.46 ± 0.6). Normal salivary glands did not express Ki67. There was a statistically significant difference in frequency and percentage of Ki67 immunoreactivity in malignant neoplasms (86.5% and 10.7±10.8 respectively) compared to PA (50% and 0.78 ± 0.2) and among the four groups values were highest in MEC (p<0.000). <u>Conclusion</u>: n this study, it was observed a higher rate of angiogenesis and cellular proliferation was noted in malignant tumors compared to benign tumors, but no correlation was observed between these two markers.

Keywords: Ki67 - CD105 - salivary gland tumor - proliferation - angiogenesis

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Introduction

Angiogenesis, development of new blood vessels from previous blood vessels, is necessary for tumor growth and metastasis. During recent years, a lot of effort has been put into identifying markers of angiogenesis (Meng et al., 2012). CD105 is not only a marker that has an important role in angiogenesis (Nassiri et al., 2011) but also it is essential for proliferation of endothelial cells and stimulates active phase of angiogenesis (Goumans et al., 2003). CD105 is a membranous homodimer protein with a molecular weight of 180 KDa, and the gene for this protein is located on chromosome 9 which is expressed on the cell surface (Dallas et al., 2008). There are two isoforms of protein with the capacity of binding to Transforming Growth Factor β (TGF- β) that differ in the cytoplasmic amino acid composition. Most of CD105 functions are likely to be associated with TGF- β signaling , however in some processes it is independent of TGF- β signaling (Levi et al., 2011).

Endothelial cells of blood vessels are the main source of CD105 (Duff et al., 2003). Other cells including vascular smooth muscle cells, fibroblasts, macrophages, can also express CD105 to a lesser extent (Zijlmans et al., 2009). In addition, expression of CD105 is a prominent feature of newly formed blood vessels, and minimally expressed in preexisting ones (Fonsatti et al., 2003). Expression of CD105 in blood vessels around the tumor has been shown previously (Fonsatti et al., 2003), suggesting that the role of CD105 in tumor angiogenesis.

Diagnostic use of CD105 has an important role in controlling the clinical signs of the disease. Moreover, it may be used in the field of diagnostic follow-up, determining the response to treatment and prognosis of the disease (Ali et al., 2011).

Microvessel density (MVD), is an independent factor to determine prognosis in many human malignancies. With increased MVD, survival decreases. Several studies have shown that compared with other endothelial markers such as von Will brand and CD34, CD105 antibodies are more specific to tumor vessels and therefore, are more suitable for determining the MVD (Brewer et al., 2000).

Ki67 is a nuclear protein that is encoded by the gene MKI67 (Bullwinkel et al., 2006). This protein is associated with cell proliferation and is associated with transcription of ribosomal RNA (Bullwinkel et al., 2006).

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Inactivation of Ki-67 leads to inhibition of rRNA synthesis (Rahmanzadeh et al., 2007).

The Ki-67 is used as a marker for cell proliferation. During inter-phase, the Ki-67 protein is found specifically in the cell nucleus, whereas in mitosis most of the protein is transported to the surface of the chromosomes. This protein is present in all active phases of the cell cycle (G2, S, G1 and mitosis) but is absent in resting cells (G0) (Scholzen et al., 2000).

Ki-67 is very important for determining prognosis of tumors and determine the recurrence rate after radiotherapy in patients with adenocarcinoma (Scalzo et al., 1998).

In various studies the relationship between MVD (by using CD105 marker) and factors such as metastasis, Stage of tumor, proliferation rate of tumor cells and survival has been investigated (Liu et al., 2012), but in salivary gland tumors, the association between this factor and tumor cell proliferation rate has not been studied, therefore this study was based on investigating the relationship between these two factors.

Materials and Methods

In this study, 59 samples of salivary tumors from Khalili Hospital archive included 20 cases of pleomorphic adenoma, 20 cases of mucoepi dermoid carcinoma and 19 cases of Adenoid systetic carcinoma were reviewed. The control group was consisted of 10 cases of normal salivary gland tissue.

Firstly, H and E slides of available blocks were reviewed and then cases with definite diagnosis and adequate cellular tissue were selected for immunohistochemical staining (IHC). IHC staining was performed by using EnvsionLabled Peroxides System (DAKO, Carpentaria, CA, USA). All the samples have been fixed in 10% buffered formalin and have been embedded in paraffin. Sections with 4 μ thickness were prepared, deparaffinized in xylene ,rehydrated in graded alcohol and were washed with distilled water. Antigen retrieval was performed by using DAKO cytomation target retrieral solution with P=9, for 20 minutes. Internal peroxidase activity was inhibited by 3% H₂O₂.

Tissue sections were then incubated for 30 minutes with the anti-CD105monoclonal antibody(mouse ,Dako Corporation, Denmark) at a 1/10 dilution and 1hour with the anti-Ki67 monoclonal antibody (mouse anti-human Ki60, Clone Mib-1, Dako, Denmark) at a 1/50 dilution.

Normal samples were stained with the same amount of antibody used for staining tumor tissues.Omission of primary antibody was employed as negative control,while tonsil tissue and liver were used as positive control for Ki67 and CD105 respectively.

Brown nuclear staining for Ki-67 and brown cytoplasmic staining for CD105 was considered as positive. Immunohistochemical results were interpreted by two pathologists. Immunoreactivity was expressed by determining the percentage or positive tumor cells. Briefly, of least 1000 neoplastic nuclei in the area with the highest Ki-67 positivity of scaning magnification (X40) were counted at X400 magnification intensity of staining

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Figure 1. Frequency of CD105 Staining in Different Lesions.



Figure 2. Intratumoral Microvessel Density in Different Groups



Figure 3. Frequency and Percentage of Ki-67 Staining in Different Groups



Figure 4. ki67 (Left) and CD105 (Right) Immunoreactivity : In Plemorphic Adenoma (a, b), in Adenoid Cystic Carcinoma (c, d), and in Mucoepidermoid Carcinoma (e, f). In mucoepidermoid carcinoma, numerous ki67 positive cells and CD105 positive capillaries are evident

was not considered for evaluation.

Intratumoral microvessel density (IMD) was quantified according to a recent consensus statement (Cardoso et al., 2009). Briefly, in an optical microscope hot-spot areas for CD105 expression in discrete blood vessles were in itially identified by scaning the entire tumor of low power (X40). The number of CD105 high ligted vessles in 10 of these areas was then counted in high power magnification (X400)

Mann-Withney, Chi squre and Kruskalwalis tests were used to compare test results between the two markers and 00.0 Spearmanscorrelatia was used to assess the relationship between two markers.

Results

Gender of patients with salivary gland tumors included age of 49.1 years.

CD105 positive vessels were absent in normal salivary tumors were positive). Frequency of CD105 staining in each group is shown in Figure 1. There was a statistically significant difference in frequency of CD105 staining between PA and malignant tumors and between four groups of different lesions(p<0.000). Among malignant neoplasms, positivity for CD105 being the highest in MEC (83%).

IMD was also elevated in malignant neoplasm (2.61 ± 3.1) compared to PA (0.46 ± 0.6) (P=0.01). IMD was similar in PA and normal salivary gland and also in MEC and ACC (respectively P=0.08 and P=0.2) as shown in Figure 2. Normal salivary gland didn't express Ki67.There was a statistically significant difference in frequency and percentage of Ki67 immunoreactivity in malignant neoplasm (86.5% and 10.74±10.8 respectively) compared to PA (50% and 0.78 ± 0.2) and between four groups (p<0.000) (Figure 3).

The relation between the two markers, CD105 and Ki-67 was evaluated by Mann-Whitney test and Spearman's correlation coefficient and no correlation was found between two markers (P=0.07).

Discussion

Tumor growth is limited by the balance between the need for oxygen and nutrients and diffusion of these substances from vessels around tumors and therefore the formation of new blood vessels (angiogenesis) is a necessary step in tumor progression (Kubota, 2012). Counting blood vessels in tumors by IHC is a common method for assessing angiogenesis. However, due to the use of endothelial markers, which may not be able to determine the angiogenic activity of endothelial cells, different results have been reported in tumors (Sharma et al., 2005).

CD105 is not only a marker that has an important role in angiogenesis (Nassiri et al., 2011) but also it is essential for proliferation of endothelial cells and stimulates active phase of angiogenesis (Goumans et al., 2003). In addition, expression of CD105 is a prominent feature of

newly formed blood vessels, and minimally expressed in preexisting ones. (Fonsatti et al., 2003). Expression of this marker can be also observed in non-neoplastic tissue with increased angiogenic activity such as embryonic development and wound healing (Hillen and Griffioen, 2007).

In previous studies, the higher accuracy of these markers in showing new blood vessels in comparison with other factors such as CD34 and factor VIII has been shown (Minhajat et al., 2006).

In this study, expression of these markers was ndt00.0 found in<u>619</u>e bloof vessels of normal salivary glands. Moreover, not being expressionin approximately half of 75.0^{tumors} shows the specificity of this marker in detecting75,80.0 new blood vessels. This is also indicative of this fact that development of near stic angiogenesis may not always require a clear and noticeable angiogenesis and tumor 34 females (57.6%) and 25 males (42.4%) with a mean 50.0 metabolic activity may be carried out the non 50.0 **30.0** oxygen-dependent process

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None

In this study, in comparison with benign tumors, a gland tissue in the vicinity of the tumors (51.6% of all_{25.6} ignificant increase of blood vessels in malignant tumors_{25.0} has been observe**s son** indicates that development 30.0 of blood vessels reflects invasiveness of salivary gland tumors n

0 These findings are consistent with reports of multiplestep tumor genesis model tumors. It has been reported in this study that angiggenesis was found to be activated in midstage lesions pefore the appearance of full-blown tumors suggesting that the formation of new blood vessels is ssential for Clonal Expansion and formation of macroscopic tumers (Hanakan and Weinberg, 2000). But in the macrosco ic tumor the main factor affecting the density of blood vessels is the metabolic needs of tumor cells which us fally increase with tumor progression (Sharma 豪 al., 200岁) Thus, in malignant tumors with severe invasiveness and progression, metabolic needs and consequently the development of blood vessels increases (Romani et al., 2006).

In the study conducted by Cardoso and others, which was similar to our study, it was detected that there is a higher increase in development of blood vessels within the malignant tumors compared to benign tumors (Cardoso et al., 2009).

In this study, a statistically significant was found in the frequency of CD105 expression between ACC and MEC, so that a higher percentage of MEC express this marker. However, there was not a significant difference in IMD; among MEC and ACC.

Numerous studies have shown that tumors with a phenotype of myoepithelial cells, have specific nature (Barsky and Karlin,2005). Myoepithelial cells, produce high levels of proteinase inhibitors and angiogenesis inhibitors and low amounts of proteinase angiogenic factors (Nguyen et al., 2000). With respect to this evidence, Barsky and Karlin stated that myoepithelial cells are angiogenesis inhibitors (Barsky and Karlin, 2005).

MEC is the most common malignant salivary tumor which lacks myoepithelial cells. Due to lack of these cells, a higher percentage of these tumors show this marker.

In the same study conducted by Costa and Cardoso, it was evidenced that there is an increased incidence of

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blood vessel in MEC compared to ACC (Costa et al., 2008; Cardoso et al., 2009).

In this study, in adenoid cystic carcinoma lesions, neoplastic cells usually formed large hypo vascularized aggregates that were surrounded by large vessels. In contrast, in MEC cell aggregates were smaller and had some small blood vessels around it. The difference in shape , size and distribution of vessels in two type of carcinoma may be explained by the characteristics of myoepithelial cells. These cells are able to produce large amounts of extracellular matrix devoid of vesseles that contains angiogenenic inhibitors (Nguyen et al., 2000). It seems that large blood vesselesin ACC are needed to compensate decreased angiogenesis.

One of the most important biological mechanisms in oncogenesis is cell proliferation. Ki-67 is essential for cell proliferation (Liu et al., 2012). Ki-67 is required for the synthesis of ribosomes during the cell cycle. Therefore it is relevant to the rate of protein synthesis (MacCallum and Hall, 2000). In this study, expression of Ki-67 in three groups consist of normal tissues, pleomorphic adenoma, malignant tumors, (ACC and MEC) was studied. Frequency and the percentage of staining of this marker in normal tissues in comparison with PA and in PA in comparison with malignant tumors was significantly lower, which indicates higher rate of proliferation in tumor lesions in comparison with normal tissues and also indicates higher rate of proliferation in malignant tumor cells in comparison with benign tumors.

In this study, the frequency of staining of Ki-67 in mucoepi dermoid carcinoma was significantly higher than that of adenoid cystic carcinoma. In the study conducted by Alves frequency of Ki-67 expression in 53.3% mucoepi dermoid carcinoma and 40% of adenoid cystic carcinoma were reported as negative (Alves et al., 2004). In our study 11% and 16%, of muco epidermoid carcinoma and adenoid cystic carcinoma were respectively negative for this marker. In the study reported by Cleveland expression of Ki67 was observed in 88.2% of adenoid cystic carcinoma (Lazzaro and Cleveland, 2000) but Fonseka et al reported a low incidence of this marker in adenoid cystic carcinoma (Fonseca et al., 1997).

The difference in the expression of this marker in different studies may be related to the type of antibody used (monoclonal and polyclonal), and the differences in how the cells are counted. However in accordance with our study ,in all of these studies the frequency of Ki67 expression was lower in benign tumor in comparison with malignant tumors and lower in ACC than MEC (Hollstein et al., 1991; Nordgard et al., 1997).

Several studies have shown the relationship between cell proliferation, and aggressive behavior or prognosis of tumors. Nordgard et al has studied the relationship between Ki-67 and the prognosis in 44 adenoid cystic carcinoma and showed that this marker has a role in determining the short-term prognosis (Nordgard et al., 1997).

In this study, due to unavailability of information associated with prognosis, survival and follow up of the patients, it was impossible to assess the relationship between Ki-67 marker and factors associated with prognosis. Despite the increase of both markers in malignant tumors in comparison with benign tumors, and in MEC in comparison with ACC, there was not a significant relationship between them, indicating that CD105 expression levels are not affected by the rate of cellular proliferation. This finding may suggest that these tumors maintain their metabolic activities predominantly through oxygen-independent pathways such as glycolysis.

In conclusion, in this study, it was observed that there is a higher rate of angiogenesis and cellular proliferation in malignant tumors compared to benign tumors, indicating their role in the malignancy and aggressive behavior of these tumors, but no correlation was observed between these two markers. Moreover, the role of myo-pithelial cells was detected in the control of angiogenesis.

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