Immunohistochemical analysis of CD34 and PCNA expression in salivary and laryngeal adenoid cystic carcinoma

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Abstract---Background/purpose: Adenoid cystic carcinomas (ACC) are malignant tumors that affect major and minor salivary glands. LACC is an extremely rare location for this type of tumour. This study describes the immunohistochemical analysis of CD34 and PCNA expression in salivary and laryngeal ACC. Materials and methods: The study included 40 formalin-fixed paraffin-embedded blocks were used, 20 of them were previously diagnosed as SACC, and 20 as LACC. All sections were stained with hematoxylin and eosin (H&E) and immunohistochemical marker to detect CD34 and PCNA protein. Results: Regarding the expression of CD34, SACC had recorded the highest mean area % (31.58), while LACC recorded the lowest mean area % (14.25) and the comparison revealed that there was a highly significant difference between these lesions where P value was (<0.001) concerning the expression of PCNA, SACC had recorded the highest mean area % (36.28), while LACC recorded the lowest mean area % (20.7) and the comparison revealed that there was a highly significant difference between these lesions where P value was (<0.001). Conclusions: immunohistochemical analyses presented here indicated that LACC tends to nonaggressive oncological behavior and angiogenesis when compared to SACC.

Keywords---adenoid cystic carcinoma, laryngeal adenoid cystic carcinoma, salivary adenoid cystic carcinoma, SACC, LACC, CD34, PCNA.

Introduction

Salivary gland neoplasms account for approximately 1–4% of all human tumors. The most common malignancies of the salivary glands include pleomorphic adenoma, mucoepidermoid carcinoma, and ACC. [1] ACC is one of the most frequent malignant neoplasms that affect either the major or minor salivary glands of the oral cavity with significant risk of recurrence and metastasis. [2] Around 50% of ACC develops on the hard palate, the rest occurring in other

intraoral locations such as the lower lip, the region of the retromolar/tonsillar pillar, the sublingual gland and the buccal mucosa. [3] In addition to salivary glands, ACC may infrequently occur in the esophageal, bronchial, and mammary glands. [4] Due to the relatively low frequency of minor glands in the larynx (23-57 glands / cm2) compared to the oral cavity (600-1000 glands / cm2), LACC is sporadic (1% of all laryngeal cancer). [5] LACC is clinically characterized by dyspnea and hoarseness. The most prevalent site is in the subglottic region, although the gender distribution remains debatable. [6]

LACCs are characterized by a high rate of distant late metastasis and local recurrence, despite their slow growth rate. ACC is formed of epithelial and myoepithelial cells and is classified into three subgroups histologically: The most prevalent subtype is cribriform, followed by tubular with a favorable prognosis, and finally solid with a poor prognosis. [7] Meanwhile, malignant transformation of the salivary glands is dependent on a range of distinct factors, the most critical of which are considered to be cell cycle regulators such as cyclins, tumour suppressors, and transcription factors, as well as histo-type-specific oncogenes such as mucoepidermoid carcinoma translocated-1 (MECT1) for mucoepidermoid carcinoma and v-myb avian myeloblastosis viral oncogene homolog (MYB) for ACC. Additionally, cancers require blood to grow, invade, spread, and provide oxygen and nutrition to cancer cells. If the tumour exceeds 2-3 mm in diameter, angiogenesis, a complex process that occurs in both normal and malignant situations, is unavoidable. [8, 9]

Angiogenesis is the process through which new blood vessels are formed from the structure of the primary blood vessel of the host. Due to the heterozygous nature of tumors, the density of blood vessels in different tumour locations varies. [10] The counting of tumor blood vessels using immunohistochemistry is a commonly used technique to determine the angiogenic activity of a tumor. [11] CD34 is a pan endothelial marker that stains both newly generated and normal blood vessels trapped within tumor tissues. [12] Tumors with a high vascular density have a more significant metastatic potential and shorter survival. [13] CD34 is a membrane glycoprotein that belongs to the Sialomucin protein family. Its molecular weight ranges between 105 and 120 CDS. The anti-CD34 antibody is linked to the Sialomucin membrane protein. Then, endothelial cells in their precursor or mature state might be detected. [14]

It would be difficult to evaluate angiogenesis and detect endothelial cells, as well as to distinguish vascular channels from lymphatic channels; however, these issues can be resolved using immunohistochemical staining and simply reported as microvessel density (MVD). Angiogenesis not only plays a critical role in tumour progression, but also angiogenesis can predict the clinical outcomes and prognosis of tumors. [14, 15] Evaluation of epithelial proliferative activity is a valuable method to examine biological distinctions between tumors and cysts. [16] Furthermore, Proliferative activity is an important predictor of biologic behavior of pathologic condition and a potential guide for therapy. [17] PCNA is required for the replication and repair of DNA. Through symmetrical connection of three identical monomers, PCNA forms a toroidal, ring-shaped structure of 90 kDa. The ring surrounds DNA, serves as a binding site for polymerases and other proteins involved in various DNA metabolic activities, and functions as a cofactor for the

DNA polymerase delta. [18] In malignancy, immunohistochemical detection of this protein is a valuable indicator of a tumor's aggressiveness and is connected with prognosis and survival rate. [19, 20]

PCNA expression is also found in salivary gland tumors, and as a tumour becomes more aggressive, its expression increases and the prognosis deteriorates. PCNA expression is recognized as a sign of cellular proliferation in neoplasia, and its detection is typically associated with a high risk of metastasis and an unfavorable prognosis. [21] As a result, the current research was conducted in order to examine the immunohistochemical evaluation of CD34 and PCNA expression as angiogenesis and proliferation markers, respectively, and to understand the clinicopathological and biological characteristics in salivary and laryngeal ACC.

Materials and Methods

Samples Selection

40 formalin-fixed paraffin-embedded blocks, 20 of which were previously diagnosed with SACC and 20 of which were previously diagnosed with LACC, were obtained from the archives of the Pathology Department, National Cancer Institute (Cairo University), Department of Oral and Dental Pathology, Faculty of Dental Medicine, (Boys), Al-Azhar University, Assiut, and Oral Pathology Department, Al-Azhar University, Faculty of Dental Medicine (Cairo - Boys). Each section was stained with hematoxylin and eosin (H&E) and an immunohistochemical marker for CD34 and PCNA protein detection.

Immunohistochemistry and Staining Interpretations

The EnVision immunohistochemistry method (Dako) was used to detect the presence of CD34, which was conducted in accordance with the manufacturer's specifications. To summarize, tissue sections were deparaffinized and rehydrated routinely, and then treated for 10 minutes at 100°C in a pressure cooker with 1 mM EDTA for antigen retrieval. After 5 minutes of incubation with 3 percent hydrogen peroxide to remove endogenous peroxides, the sections were washed with phosphate-buffered saline and incubated with rabbit monoclonal CD34 (clone EP373Y; dilution, 1:100, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (dilution, 1:100, USA). The 3,3′-diaminobenzidine substrate was used to detect the signal, and subsequently the sections were counterstained with hematoxylin to show the nuclei. CD34 expression in vascular endothelial cells using the Weidner method. [22] Individual microvessels were defined as groups of endothelial cells expressing CD34 and creating a lumen or vessel. In contrast, luminal areas larger than the total diameter of eight erythrocytes, a blood vessel with a thick muscle layer or a single positive cell were excluded from the microvessel count. Light microscopy was used to scan tumour sections at low magnification (x40) to identify dense areas of vascular (hot spots). Individual tumour microvessels were then counted in five fields at high magnification (x100), and the mean vessel count in three hot spots was used to calculate the MVD. Unlike Keyhani's methods [23], the present study

defined a low expression level of CD34 as an MVD value <15 / HPF and a high expression level of CD34 as an MVD value of >15/HPF.

Concerning the PCNA protein, sections were washed in phosphate-buffered saline and incubated with normal serum (1:10, 10 min) before being treated with primary anti-PCNA antibody (PC-10, diluted 1:80, USA) for 18 hours; incubation in secondary serum; incubation in streptoavidin-biotin complex for thirty minutes at room temperature; and the reaction with diaminobenzidine is developed. Between phases, the material was immersed in a trisphosphate buffer solution with a pH of 7.4 (trisphosphate buffer solution). Following the reaction's development, sections were washed and counterstained with Mayers hematoxylin. To determine the prevalence of immunopositivity PCNA in the analyzed sections, immunostaining sections were examined using an ordinary light microscope. Positive cells for PCNA were counted within at least 1000 tumor cells, and the positive index (PI) was calculated using an image analyzer computer system to determine the percentage of positive cells in the area. Image analysis was performed using a computer system (Leica Quin 500). This research was conducted at the Department of Oral and Dental Pathology, Faculty of Dental Medicine, Boys, Cairo, Al- Azhar University.

Statistical Analysis

All results derived from computer image analysis were statistically analyzed. SPSS V20 was used to present and analyze the data in this study. Mean, standard deviation, student t-test unpaired, and analysis of variance (ANOVA) tests were used. Significant level: non-Significant >0.05 Significant <0.05* High Significant <0.001*.

Results

SACC CD34 and PCNA Expression Results

The expression of CD34 for the 20 cases of SACC (cribriform, tubular, and solid pattern) tissue sections showed high widely distributed expression located in the nuclei of endothelial cells of blood vessels around tumor nests that form the cribriform pattern and negative staining in cystic spaces. Note, high positive expression in solid pattern compared to the cribriform and tubular pattern, with an MVD of >15/HPV. The expression of PCNA for the 20 cases of SACC (cribriform, tubular, and solid pattern) tissue sections showed high widely distributed expression in the nucleus of basal epithelial cells that form the cribriform pattern and negative staining in cystic spaces. Note that there is a high positive expression in solid pattern than cribriform and tubular pattern (Fig. 1A-I).
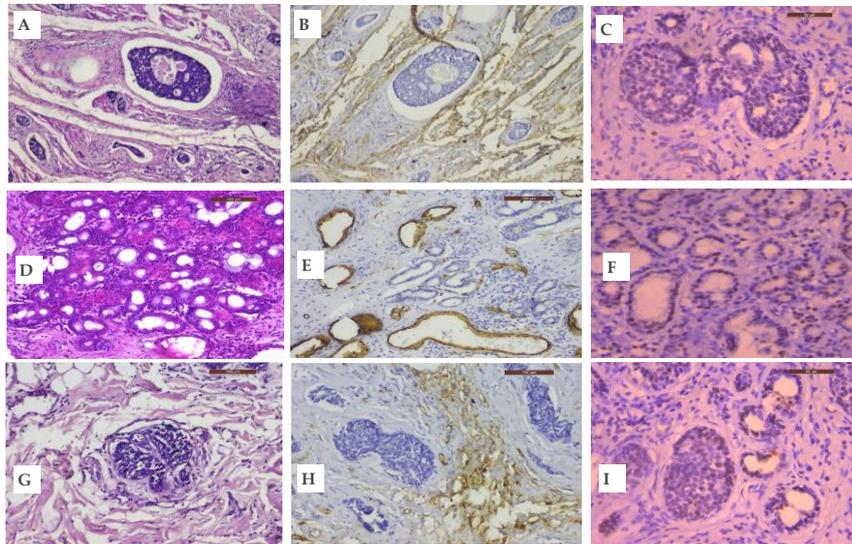
Figure 1. (A) H&E stain of SACC (cribriform pattern) showing small cuboidal cells similar to basal cells that form multiple cylindrical cyst-like patterns resembling honeycomb pattern within the hyalinized connective tissue stroma; (B) CD34 stain of SACC (cribriform pattern) showing high expression in vascular endothelial cells; (C) PCNA stain of SACC (cribriform pattern) showing high expression in neoplastic epithelial cells; (D) H&E stain of SACC (tubular pattern) showing tubular structures within the hyalinized connective tissue stroma; (E) CD34 stain of SACC (tubular pattern) showing high expression in vascular endothelial cells; (F) PCNA stain of SACC (tubular pattern) showing high expression in neoplastic epithelial cells; (G) H&E stain of SACC (solid pattern) showing cuboidal cells with little tendency towards duct or cystic formation within hyalinized connective tissue stroma; (H) CD34 stain of SACC (solid pattern) showing high expression in vascular endothelial cells; (I) PCNA stain of SACC (solid pattern) showing high expression in neoplastic epithelial cells.

LACC CD34 and PCNA Expression Results

Unlike SACC, the expression of CD34 for the 20 cases of LACC expressed low levels of CD34, with an MVD of <15 / HPV located in the nuclei of endothelial cells of blood vessels around the tumor nests. Similarly, the expression of PCNA for the 20 cases of LACC expressed low levels of PCNA (Fig. 2A-I).
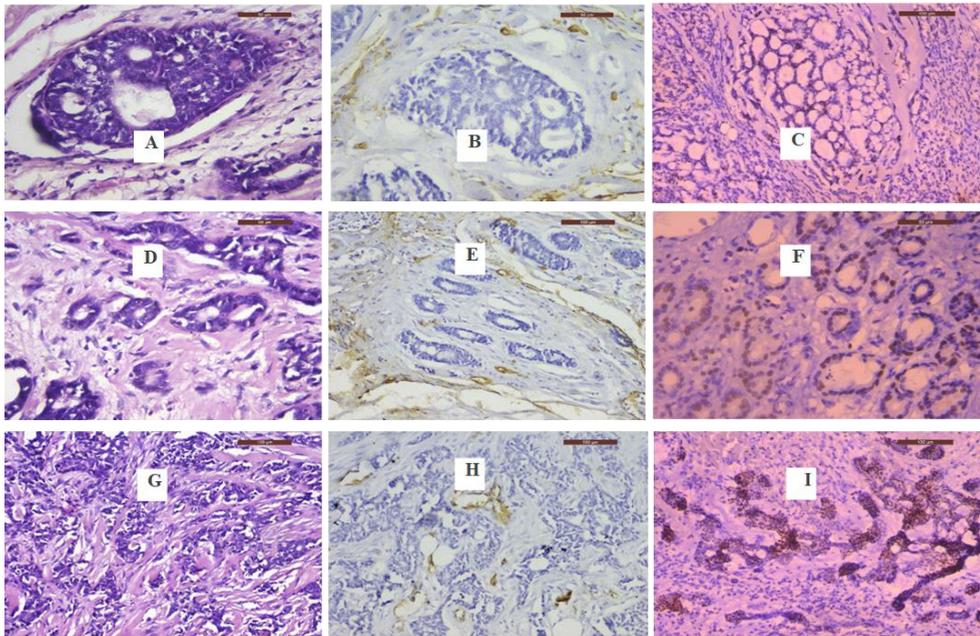
Figure 2. (A) H&E stain of LACC (cribriform pattern) showing a cell lined with ducts arranged in a cribriform pattern within the hyalinized connective tissue stroma; (B) CD34 stain of LACC (cribrifrm pattern) showing low expression in vascular endothelial cells; (C) PCNA stain of LACC (cribrifrm pattern) showing high expression in neoplastic epithelial cells; (D) H&E stain of LACC (tubular pattern) showing that epithelial cells take the form of tubules within the hyalinized connective tissue stroma; (E) CD34 stain of LACC (tubular pattern) showing low expression in vascular endothelial cells; (F) PCNA stain of LACC (tubular pattern) showing high expression in neoplastic epithelial cells; (G) H&E stain of LACC (solid pattern) showing cuboidal epithelial cells form solid masses with minimal cystification; (H) CD34 stain of LACC (solid pattern) showing low expression in vascular endothelial cells; (I) PCNA stain of LACC (solid pattern) showing high expression in neoplastic epithelial cells.

Statistical Analysis Results

The results of the statistical analysis of the expression of CD34 & PCNA were obtained by comparing the area % between SACC and LACC. The results of the statistical analysis revealed that, with respect to CD34 expression, SACC had recorded the highest mean area % (31.58), while LACC recorded the lowest mean area % (14.25), and the comparison revealed that there was a high significant difference between these lesions where P was (<0.001) (Table 1, Fig. 3)., with respect to PCNA expression, SACC had recorded the highest mean area % (36.28), while LACC recorded the lowest mean area % (20.7), and the comparison revealed that there was a high significant difference between these lesions where P value was (<0.001), (Table 2, Fig. 4).

Table 1

Mean, standard deviation (SD), P-values and results of comparison between
expression of CD34 in SACC and LACC

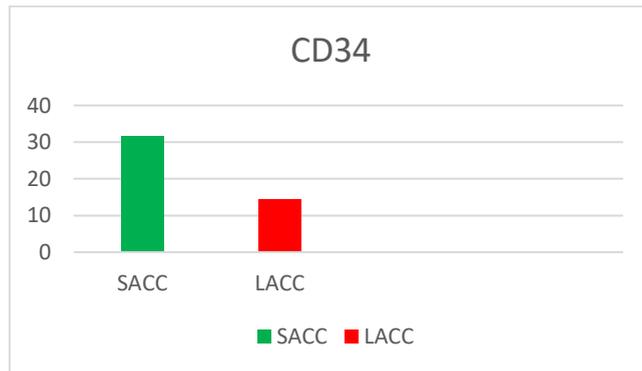| Tumor Type	CD34 Expression Results		
	Mean	SD	p = 0.002*
SACC		31.58	3.97
LACC		14.25	2.39

Figure 3. The bar chart representing mean area % results of CD34 expressions in
SACC and LACC

Table 2

Mean, standard deviation (SD), P-values and results of comparison between
expression of PCNA in SACC and LACC

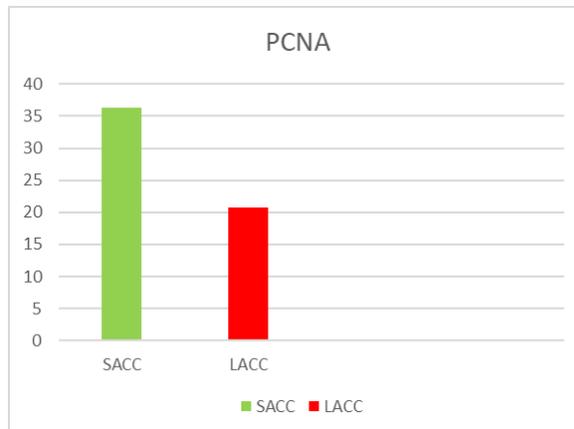| Tumor Type	PCNA Expression Results		
	Mean	SD	p = 0.020*
SACC		36.28	7.77
LACC		20.7	5.18

Figure 4. The bar chart representing mean area % results of PCNA expressions in
SACC and LACC

Discussion

While it is evident that there is a correlation between the expression of specific angiogenic factors, angiogenesis, and the development of salivary gland tumours, the precise nature of these correlations remains unknown. An increase in MVD is involved in numerous of physiological and pathological situations. Angiogenesis is a multistep process that involves the disintegration of the basement membrane, the sprouting and migration of endothelial cells into the interstitial space, the proliferation of endothelial cells, and the development of lumens. [9, 24] The primary element affecting vascular density in macroscopic tumours is the metabolic requirement, which usually increases with tumour growth. [25, 26] In the current study, salivary and laryngeal ACC vasculature generally formed a ring of capillaries surrounding tumour nests and stained positively in a solid pattern rather than in a cribriform or tubular pattern. The present study agrees with Moghadam, et al. (2015) [27], who found that SACC vasculature commonly formed a capillary ring surrounding tumour nests. Large blood vessels appear to be required in SACC to compensate for reduced angiogenesis.

According to Zhang et al , the microvessels surrounding the solid tumour nests of SACC were significantly denser than those surrounding the cribriform and tubular nests. [28] Additionally, MVD in SACC was associated with tumour size, clinical stage, vascular invasion, recurrence, perineural invasion, and distant metastasis. The ability of a tumour to generate vascularization ensuring that the tumour receives an appropriate quantity of blood and nutrients, as well as a mechanism for metastasis. Thus, tumor growth is required for intratumoral angiogenesis, which provides nutrition to invasive or metastatic cancers. Among the different angiogenic agents, vascular endothelial growth factor (VEGF) is well established as a major regulator of physiological and pathological angiogenesis. In human malignancies, it is related with increased MVD, advanced disease stage, and a bad prognosis. [29, 30] CD34 attaches to undifferentiated endothelial cell progenitors during angiogenesis. [31, 32] However, a study by Costa et al. [33] revealed no significant increase in MVD in the metastatic group.

In addition, Chen et al. [34] showed no association between CD34 expression and age, sex, or tumour size in esophageal SCC samples. Li et al. [35] reported that CD34 expression was found to be much higher in esophageal SCC samples than in normal mucosa. In the present work, we examined CD34 levels in salivary tissue levels to identify their potential utility as predictive and distinguishing markers for SACC and LACC. CD34 and PCNA were found to have significantly higher expression in SACC than in LACC. These findings could be explained by the microenvironment's role in tumour growth and invasion between the oral and laryngeal areas. Genetic and epigenetic alterations and anomalies in the cancer-associated signalling pathways lead to cancer-related phenotypes, which are then acquired by cancer cells, including those with head and neck squamous cell carcinoma. [36, 37] This includes cancers' infinite replication capacity, growth signals self-sufficiency, resistance to antigrowth signals, ability to dodge apoptosis, enhanced angiogenesis, invasion, and metastasis.

Cancers, on the other hand, are complex tissues. They contain tumour cells and the stroma that surrounds them, which is composed of numerous mesenchymal

cell types and the extracellular matrix. This tissue is collectively known as the microenvironment of the tumour. As a result, the tumour cell-centric perspective of cancer ignores the environment in which malignant cells exist. Indeed, when cancer advances, the surrounding microenvironment co-evolves into an active state due to ongoing tumor-stromal interactions. As a result, diverse stromal components contribute to the six distinguishing characteristics of cancer outlined by Pietras and Ostman. [38] In coclusion, compared to SACC, the immunohistochemical findings provided here demonstrate that LACC has a predisposition toward nonaggressive oncological behaviour and angiogenesis. These findings contribute to our present understanding of the clinicopathological and biological characteristics of SACC and LACC, and shed additional light on the oncological behaviour of this uncommon pathological variation of ACC.

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Data Availability Statement

The data sets used during current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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