Cyclin D1 Expression in Salivary Gland Tumors: An Immunohistochemical Analysis
Saede Atarbashi Moghadam, Neda Jahani, Sepideh Mokhtari

Abstract
Background: Gene amplification and over-expression of Cyclin D1 have been frequently demonstrated in many malignant tumors. The alterations in Cyclin D1 expression by immunohistochemical methods in salivary gland tumors have shown conflicting data concerning the molecular events involved in the salivary gland tumors. Hence the present study was performed to evaluate Cyclin D1 expression in salivary gland neoplasms. Method and Materials: Tissue specimens of 15 pleomorphic adenoma and 15 malignant salivary gland tumors including mucoepidermoid carcinoma, adenoid cystic carcinoma and salivary duct carcinoma were examined by immunohistochemistry for Cyclin D1 protein. Cyclin D1 expression was evaluated based on the percentage of nuclei expressing this protein in any intensity. Results: Twenty-five specimens (83.3%) were negative for this marker and only three pleomorphic adenomas and two adenoid cystic carcinomas were positive (16.6%). There was no significant difference between benign and malignant salivary gland tumors. Conclusion: Overexpression of Cyclin D1 probably does not play a role in pathogenesis of benign and malignant salivary gland tumors.

Keywords: Adenoid Cystic Carcinoma; Cyclin D1; Immunohistochemistry; Mucoepidermoid Carcinoma; Pleomorphic Adenoma; Salivary Gland Neoplasms.

Introduction
It is generally accepted that cancers develop through molecular events and genetic changes. Therefore, a better understanding of tumor pathogenesis will improve the management of cancer. CCND1 gene, located on chromosome 11q13, encodes the key cell cycle G1 regulatory protein CyclinD1 to make cell proliferation rapidly. Cyclin D1 is essential in cell cycle progression from G1 to S phase and is frequently overexpressed or genetically altered in breast, prostate and colon cancers and in squamous cell carcinomas of the head and neck. Gene amplification and over-expression of Cyclin D1 have also been frequently demonstrated in esophageal squamous cell carcinoma.

Salivary gland tumors (SGT) are a heterogeneous group of lesions. Mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (AdCC) are the most common salivary malignancies. Salivary duct carcinoma (SDC) is a high grade tumor that histologically resemble ductal carcinoma of breast. Pleomorphic adenoma (PA) although classified as benign, has tendency for recurrence and malignant transformation. There is conflicting data concerning the molecular events involved in these tumors and few studies have examined the alterations in Cyclin D1 expression by Immunohistochemical (IHC) methods in SGTs. The aim of this study was to evaluate the Cyclin D1 expression in a variety of benign and malignant salivary gland tumors in order to evaluate the implication of these molecular events in both subtypes of tumors.

Method and Materials
The 30 paraffin embedded blocks of salivary gland tumors including 15 benign pleomorphic adenomas and 15 malignant tumors including mucoepidermoid carcinoma, adenoid cystic carcinoma and salivary duct carcinoma (MEC: 6, AdCC: 5, SDC: 4) were selected from Imam Khomeini Hospital archive. The diagnostic criterion for AdCC was dual epithelial and myoepithelial differentiation as luminal and abluminal cells respectively. An admixture of epidermoid, mucous and intermediate cells helped us identify MEC. Morphologic features similar to breast ductal carcinoma served as diagnostic clues in SDC cases. Epithelial differentiation as ductal/non-ductal structures and mesenchymal differentiation (myxoid, hyaline, chondroid) were used to identify all PA cases. No carcinoma ex-PA was included in our study. All tumors were primary. Clinical information was also recorded. The specimens were fixed in 10% neutral formalin, processed routinely and embedded in paraffin. Five-micron sections were exposed to the anti-Cyclin D1 (DAKO),
using a streptavidin-biotin immune-staining method. After deparaffinization and dehydration in a graded ethanol series, the sections were treated with citric acid (10 mM, pH 6.0) in a water bath before exposure to Cyclin D1 for antigen retrieval. Diaminobenzidine (DAB) was used as a chromogen followed by counterstaining. Tonsillar tissue and omission of primary antibody were employed as positive and negative controls, respectively. Investigations show that Cyclin D1 may be present in the cytoplasm, nucleus or both of these cell compartments in salivary glands. To clarify the extent to which Cyclin D1 could be localized in the cytoplasm and in the nucleus, De Falco et al examined the subcellular localization of Cyclin D1 by electron microscopy. They observed both cytoplasmic and nuclear staining in many tissues. They also found that the protein was localized to the immediate vicinity of both sides of the nuclear pore. This indicated that protein could move between the two compartments. However, in this study according to Greer et al scoring, brown nuclei staining for Cyclin D1 was considered positive. Score +1 was assigned to tumors with cells containing 5–40% nuclear staining; +2 for tumors with cells containing 40–80% nuclear staining and +3 for tumors with >80% nuclear staining. Tumors with less than 5% of the cell nuclei stained were considered negative.

Statistical Analysis: Mann-whitney test was used to compare benign and malignant tumors. The results with p <0.05 were considered significant. All the statistical procedures were performed using SPSS, WIN program package 13.0 (SPSS Inc., Chicago, IL, USA).

Results
The mean age of patients in benign and malignant tumors was 34.86 and 52.06 respectively. In both benign and malignant tumors, 40% of neoplasms were occurred in females and 60% in males. With respect to anatomical location, 12 PA tumors were located in the parotid and 3 PA in minor salivary glands. In malignant tumors group, 9 cases were located in minor glands and 6 in the parotid.

Cyclin D1 expression was negative in twenty-five specimens (83.3%). Only three PA and two AdCC were positive (16.6%). Among positive cases, two specimens showed score 3 and three cases were scored as +2. In positive groups, both epithelial and myoepithelial cells showed immunoreactivity with Cyclin D1. There was no significant difference between benign and malignant salivary gland tumors (P=0.775). Figures illustrate Cyclin D1 expression in positive groups (Figure 1, & 2).

Discussion
Better understanding of salivary gland tumors biology provides important data to establish novel treatment strategies. Factors modulating cell progression from G0 through G1 phase, including Cyclins, are critical in determining growth rates and sensitivity of cells to cytotoxic agents and radiotherapy.

![Figure 1: Immunohistochemical expression of Cyclin D1 in AdCC (×100)](image1)

![Figure 2: Immunohistochemical expression of Cyclin D1 in PA (×400)](image2)

Overexpression of Cyclin D1 has been observed in many human tumors. Amplification of Cyclin D1 results in growth advantage for tumor cells and enhances tumorigenesis. Some studies indicate that overexpression of Cyclin D1 occurs at the beginning of tumor development and is an early marker of cell proliferation, whereas others have demonstrated late overexpression of this protein during the development of malignant tumors. Some investigators state that normal salivary
glands exhibit less Cyclin D1 expression than benign and malignant tumors but there is no difference between benign and malignant tumors.\textsuperscript{14} Liu W et al found that Cyclin D1 gene polymorphism, A870G, was associated with an increased risk of salivary gland tumors in the Chinese population.\textsuperscript{15} Yasumutso et al stated that there was no association between Cyclin D1 expression and proliferative activity of AdCC cells. They also concluded that Cyclin D1 overexpression was not linked to poor prognosis.\textsuperscript{1} In contrast, some investigations have shown Cyclin D1 overexpression in AdCC suggesting a relationship with elevated cell proliferation.\textsuperscript{16,17} Zhou et al detected Cyclin D1 immunoreactivity in most cases of AdCC.\textsuperscript{17} Ferrazzo et al showed specific nuclear staining in 10/15 cases of AdCC.\textsuperscript{16} As well, Greer et al found that Cyclin D1 overexpression was present in 90% of evaluated AdCC cases, but it was not related to tumor subtype or its location. They suggest that Cyclin D1 plays a role in overall AdCC tumor progression rather than contributing to the genesis or characteristics of a specific AdCC subtype.\textsuperscript{10}

Miguel et al stated that this protein does not participate in the etiopathogenesis of MEC and other genes are likely responsible. In fact, it seems that Cyclin D1 is not sufficient for malignant transformation in most cells and other nuclear transcription factors are involved in the pathogenesis of MEC.\textsuperscript{13} Some authors suggest that b-catenin, in cooperation with Cyclin D1, plays a critical role in the Wnt-signaling pathway and contribute to the adverse outcome and high-grade tumor staging of MEC.\textsuperscript{10}

Studies have also shown the weak expression of Cyclin D1 in polymorphous low-grade adenocarcinoma (PLGA). No Cyclin D1 immunoreactivity was detected in 14 cases of 15 PLGA in Ferrazzo et al study.\textsuperscript{16} Some evidence shows the important role of Cyclin D1 in the development of PA and in the progression of PA to carcinoma. In a series of cases including PA and carcinoma ex-pleomorphic adenoma, Cyclin D1 was more likely to be expressed in the malignant components of carcinoma ex-PA than in the benign components of PA (50% versus 31% and 31%, respectively), but the trend was not statistically significant.\textsuperscript{19} Cyclin D1 expression has not been investigated in SDC so far. In our research, all the SDCs were negative for this marker.

Overexpression of Cyclin D1 is rare in tonsillar carcinomas but frequent in carcinomas of the tongue, implying a relationship between Cyclin D1 overexpression and distinct tumor sites.\textsuperscript{20} In the present study all the positive cases were located in the parotid gland. Therefore, it seems that distinct molecular alterations occur at different tumor sites in head and neck cancers.

Acknowledgement
We would like to thank the staff members from Dental College for their support.

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Source of Support: Nil, Conflict of Interest: None Declared.