Immunolocalization of DNMT1 and DNMT3a in Salivary Gland Neoplasms

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Key Words

DNA methyltransferase • Methyltransferase • Salivary gland neoplasms • Methylation • Epigenetics

Abstract

Objective: Salivary gland neoplasms pathogenesis has not been well established. DNA methylation occurs when methyl groups are added to cytosine nucleotides in specific areas of the gene by the enzyme DNA methyltransferase (DNMT). This chemical modification can alter gene expression without altering DNA sequence. While DNMT3a is mostly involved in de novo methylation, DNMT1 acts as a maintenance methyltransferase. We aimed to investigate the immunoexpression of DNMT3a and DNMT1 in minor salivary gland neoplasms, comparing it with normal tissue. **Material:** Forty-four formalin-fixed and paraffin-embedded samples of pleomorphic adenoma, adenoid cystic carcinoma, mucoepidermoid carcinoma and polymorphous low-grade adenocarcinoma were included in the study. The DNMT1 and DNMT3a proteins were identified by using a highly sensitive polymer-based system. **Results:** Positive nuclear and cytoplasmic labeling for DNMT1 was observed in all samples, including the controls. Positive nuclear labeling for DNMT3a was found only in few neoplasms: 1 pleomorphic adenoma (9.0%), 2 adenoid cystic carcinoma (16.6%) and 1 mucoepidermoid (9.0%) cases. **Conclusion:** Our results were not able

to demonstrate a clear correlation between DNMT1 and DNMT3a immunoexpression and salivary gland neoplasms development.

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Introduction

Salivary gland neoplasms are relatively uncommon lesions representing 0.3–1.5% of all biopsies processed in oral pathology laboratories, predominantly affecting major salivary glands [1]. Minor glands account for 9–23% of all salivary gland neoplasms [1]. The intraoral site most commonly affected by benign and malignant neoplasms is the palate. Pleomorphic adenoma (PA) is the most common benign salivary gland neoplasm, while mucoepidermoid carcinoma (MEC) is the most common malignant one, followed by adenoid cystic carcinoma (ACC) and polymorphous low-grade adenocarcinoma (PLGA) [1].

Recent studies have demonstrated that the development and progression of human malignancies are associated with accumulation of alterations in proto-oncogenes and tumor suppressor genes [2, 3]. Epigenetic alterations such as DNA methylation are important for human carcinogenesis, but its role in salivary gland neoplasias has not been well established.

Table 1. Clinical data of the cases of the normal salivary glands, PA, PLGA, adenoid cystic carcinoma and MEC included in the study

	Mean age, years	Gender		Location					
		female	male	lower lip	buccal mucosa	mandible	tongue	floor	palate
NSG	19 (13–22)	3	4	7	–	–	–	–	–
PA (n = 11)	45 (16–86)	8	3	–	1	–	1	1	7
PLGA (n = 10)	54 (20–84)	5	4	–	1	1	–	–	6
ACC (n = 12)	38 (21–85)	10	2	–	3	2	–	–	7
MEC (n = 11)	45.5 (14–73)	7	3	–	–	5	–	–	5

Figures in parentheses are ranges. NSG = Normal salivary glands.

DNA methylation is a phenomenon that occurs when methyl groups are added to cytosine nucleotides in specific areas of the gene by the enzyme DNA methyltransferase (DNMT) [4]. This chemical modification of DNA and its associated proteins can alter gene expression without altering DNA sequence. A number of DNMTs have been identified in mammals. DNMT3a and DNMT3b are mostly involved in de novo methylation, whereas DNMT1 acts as a maintenance methyltransferase [5–7]. Epigenetic silencing of gene expression by promoter CpG island hypermethylation has been shown to be important in the formation of a variety of cancers including oral squamous cell carcinoma [8]. Indeed, several studies have demonstrated hypermethylation events in ACC [9–11], MEC [11–13], PA [11–14], malignant PA [10], salivary duct carcinoma [11] and acinic cell carcinoma [11].

DNMTs were initially found to be overexpressed in tumorigenic cell lines [15] and in a few types of human neoplasms [16–18]. There is scarce information about DNMT1 immunoexpression in MEC, and apparently no data about immunoexpression pattern of DNMT3a in salivary gland neoplasms [12]. In this study, the immunoexpression pattern of DNMT3a and DNMT1 was investigated in benign and malignant minor salivary gland neoplasms, as well as in minor normal salivary glands.

Materials and Methods

Tissue Samples

Forty-four formalin-fixed and paraffin-embedded tissue samples of PA (n = 11), ACC (n = 12), MEC (n = 11) and PLGA (n = 10) as well as the normal salivary glands (n = 7; control tissue) included in the present study were obtained from the files of the Oral Pathology Service, School of Dentistry. The age, gender and tumor location in each group are summarized in table 1. The nor-

mal salivary gland tissues used as control were associated with oral mucocoele biopsies. Tissue sections were stained with DNMT1 (diluted 1:150, clone 60B1220.1; IMGENEX, San Diego, Calif., USA) and DNMT3a antiserum (diluted 1:100, clone 64B1446; IMGENEX). Heat-induced epitope retrieval was performed with 10 mM citrate buffer pH 6.0 for 30 min in a steamer at 96°C. Primary antiserum incubation was performed diluted in BSA 0.5% for 18 h at 4°C. Immunohistochemical stains were performed using the high-sensitive polymer-based system (EnVision; Dako Corporation, Carpinteria, Calif., USA) with diaminobenzidine substrate solution as chromogen (Sigma, St. Louis, Mo., USA). The sections were counterstained with hematoxylin, dehydrated and mounted. A negative control, in which the primary antibody was omitted, was used in each reaction.

Only sections containing sufficient tumor cells to assess the antibody reactivity were included in our investigation. Two experienced pathologists examined multiple fields and made an independent analysis of each case, regardless of staining intensity. All samples were individually scored as grade 1 (less than 25% of positive cells) or grade 2 (more than 25% of positive cells). The score of the nuclear staining was performed separately from the cytoplasm.

Results

All lesions investigated showed broad positive nuclear and cytoplasmic labeling for DNMT1 (grade 2; fig. 1). In the normal salivary glands, positive nuclear staining for DNMT1 was observed in the ductal and acinar cells (grade 2). The immunoexpression pattern of DNMT3a in normal as well as in tumor cells is shown in figure 2. Ductal and acinar cells in normal salivary glands presented only cytoplasmic staining (grade 2). One PA (9.0%) presented nuclear (grade 1) and cytoplasmic labeling (grade 2). Nuclear staining was also observed in 2 ACC (grade 2, 16.6%) and 1 MEC (grade 1, 9.0%; fig. 2). Therefore, only 4 (9.0%) salivary gland neoplasms investigated presented DNMT3a nuclear staining.

Color version available online

Fig. 1. DNMT1 immunohistochemical staining in a representative case of normal salivary gland (a), PA (b), ACC (c), MEC (d) and PLGA (e). All lesions (b–e) exhibited nuclear and cytoplasmic immunostaining in all cell layers, showing the same immunohistochemical staining pattern of normal salivary glands (a).

Color version available online

Fig. 2. DNMT3a immunohistochemical staining in a representative case of normal salivary gland (a), PA (b), ACC (c) and MEC (d). Ductal and acinar cells in normal salivary glands presented positive cytoplasmic labeling (a). Nuclear and cytoplasmic staining was observed in 1 PA (b). Two ACC (c) and 1 case of MEC (d) showed positive nuclear labeling.

Discussion

In the present investigation we examined the immunoreexpression pattern of two catalytically active DNMT in minor salivary gland neoplasms. Overexpression of both DNMT1 and DNMT3a has previously been reported in human neoplasias, including hepatocarcinomas, prostate and breast neoplasms, as well as gastric carcinomas [19–21]. Apparently, no such study has been performed in salivary gland neoplasms.

Hypermethylation of tumor suppressor genes that are normally unmethylated correlates with loss of expression in cancer cells. Hypermethylation of the tumor suppressor genes p16INK4a, p14ARF, p27Kip1, E-cadherin, 14-3-3 σ , RASSF1A, DAPK, MGMT, RAR β 2 and RB1 has been demonstrated in some cases of salivary gland neoplasms [9–14, 22]. Also, overexpression of various DNMTs in lung cancer may result in promoter hypermethylation of multiple tumor suppressor genes which then leads to poor prognosis [18].

While some studies revealed significant overexpression of DNMT1 in some human neoplastic tissues [15, 18, 23–25], others have found no significant difference of this protein expression in neoplasms when compared to matched normal tissues [26, 27]. Although DNMT1 is considered a maintenance form of DNMT that copies methylation patterns after DNA replication, in human cancers unknown factors could target this enzyme to unmethylated substrate DNA. So some authors believe that DNMT1 protein overexpression could result in de novo DNA hypermethylation during carcinogenesis [25]. Regarding DNMT3a, its expression was shown to be slightly increased in neoplasms from bladder, colon, kidney and pancreas when compared to matched normal tissue [26, 27], and it is suggested that its overexpression may take part in gastric carcinogenesis [28]. In hepatocarcinogenesis, a sequential decrease in cytoplasmic immunoreactivity for DNMT3a and a concurrent increase in nuclear DNMT3a were observed in high-grade dysplasia and carcinomas when compared to nonneoplastic and low-grade dysplasia [29].

All cases included in our study showed a broad nuclear positive immunostaining for DNMT1 in all cell layers, and so did the fragments of normal salivary glands, suggesting that this enzyme may not be relevant to salivary gland tumorigenesis. In addition, only 9% of the salivary gland neoplasms analyzed presented nuclear expression of DNMT3a. Although hypermethylation of tumor suppressor genes is reported in salivary gland tumors [9–14, 22], our data suggests that these epigenetic changes might not be associated with an increased expression of DNMT3a.

In conclusion, our results were not able to demonstrate a clear correlation between DNMT1 and DNMT3a immunoreexpression and salivary gland neoplasms development. The present findings, however, need to be confirmed by studies using other assays.

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