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Overexpression of cyclooxygenase-2 correlates with cytoplasmic HuR expression in salivary mucoepidermoid carcinoma but not in pleomorphic adenoma

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BACKGROUND: The overexpression of cyclooxygenase (COX)-2 in several human carcinomas suggests that COX-2 is related to carcinogenesis. Although COX-2 expression has been shown to be up-regulated in carcinomas of the salivary gland, its mechanisms are not completely understood. HuR is an mRNA-binding protein that controls the stability of certain transcripts including COX-2.

METHODS: The expression of COX-2 and HuR was determined by immunohistochemistry in 28 cases of salivary pleomorphic adenoma and 18 cases of salivary mucoepidermoid carcinoma.

RESULTS: 28.6% and 72.2% of the pleomorphic adenomas and mucoepidermoid carcinomas showed high COX-2 expression respectively. 35.7% of pleomorphic adenomas and 72.2% of mucoepidermoid carcinomas were tested positive for HuR in the cytoplasm of tumor cells. There was a correlation between a high COX-2 immunoreactivity and cytoplasmic HuR expression in mucoepidermoid carcinomas but not in pleomorphic adenomas.

CONCLUSION: This study suggests that cytoplasmic HuR is correlated with COX-2 expression in salivary mucoepidermoid carcinomas. In addition, the immunoreactivity of COX-2 and cytoplasmic HuR might be used to evaluate the nature of a borderline malignancy in the salivary glands.

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Keywords: mucoepidermoid carcinoma; pleomorphic adenoma; COX-2; HuR

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Introduction

A pleomorphic adenoma and mucoepidermoid carcinoma are the most common benign and malignant neoplasia of the salivary glands (1). Salivary gland tumors are characterized by a variety of morphologies and clinical behaviors and this heterogeneity of tumors makes it difficult to predict the clinical course (2). Although irradiation, heavy smoking, and alcohol consumption are associated with the development of tumor, the precise pathogenesis is unclear (3). Some reports have shown that a mutation in p53 and Rb and the activation of c-myc and ras are associated with benign and malignant salivary gland tumors (4–6).

The cyclooxygenase (COX) enzymes, COX-1 and COX-2, perform the rate-limiting step in the synthesis of eicosanoids from arachidonic acid. Increased COX-2 gene expression and elevated eicosanoids levels are often observed in chronically inflamed tissues and solid tumors, which possibly reflects the existence of mechanisms common to both inflammation and tumorigenesis (7, 8). COX-2 overexpression is associated with the progression, proliferation, and metastasis as well as inhibition of apoptosis in various tumors. Moreover, COX-2 is known to induce the expression of several growth factors through the mediation of prostaglandins (9–12). Therefore, COX-2 has a variety of activities in various tumors. However, there are few reports on COX-2 expression in salivary gland tumors (13–15).

The COX-2 gene is tightly regulated at both the transcriptional and post-transcriptional levels, and its 3′-untranslated region (UTR) is the main factor determining the inherent instability of COX-2. The 3′-UTR contains several copies of the mRNA-destabilizing motif, AUUUA. These AU-rich elements (AREs) regulate the mRNA stability through interactions with the sequence-specific RNA-binding protein, which influences either of two critical steps in eukaryotic mRNA decay: deadenylation and/or the subsequent 3′ to 5′

degradation of the mRNA body (16). HuR is an mRNA-binding protein that can stabilize COX-2 mRNA leading to an increase in COX-2 expression (17).

This study measured the expression of COX-2 and HuR in a pleomorphic adenoma and mucoepidermoid carcinoma using immunohistochemistry. The relationships between the expression of COX-2 and HuR and between their expression and the clinicopathological parameters were also investigated.

Materials and methods

Materials

The Ethics Committee of Chonbuk National University School of Dentistry approved this study. Twenty-eight cases of pleomorphic adenoma and 18 cases of mucoepidermoid carcinoma were collected from the 1992–2004 pathological files of the Chonbuk National University Hospital and H&E stained sections were examined by light microscopy. All available clinical data were obtained from a review of the patients' medical records. The histopathological diagnosis was verified by an independent evaluation of all H&E slides by two pathologists. The mucoepidermoid carcinomas were graded histopathologically based on the criteria reported by Goode *et al*. (18) as follows: cystic component ≤20%, four or more mitotic figures per 10 high-power fields, neural involvement, necrosis, and anaplasia. Of the 18 mucoepidermoid carcinomas, 11 cases were low grade and the remaining seven were high grade. The tumor size and nodal status were classified according to the American Joint Committee on Cancer (AJCC) staging system. Twelve cases were T1 and the remaining six cases were T2. There was no case of lymph node or distant metastasis. Follow-up data were obtained from 10 mucoepidermoid carcinoma cases. The mean follow-up period was 5.2 years and there was no recurrence or cancer-related death during this period.

Immunohistochemistry

Immunohistochemical staining was carried out using an EnVision™ Detection Kit System (Dako, Glostrup, Denmark), which is based on the peroxidase-catalyzed deposition of biotinylated tyramide. Briefly, 4-μm sections were mounted onto silanized slides, dewaxed in xylene, and rehydrated with a graded series of ethanol. The sections were immersed in a 0.1 M citrate buffer solution at pH 6.0. After boiling twice in a microwave oven for 5 min, the slides were cooled to room temperature and rinsed in phosphate-buffered saline (pH 7.4) for 10 min. The endogenous peroxidase activity was quenched by treating the specimens with 3% hydrogen peroxide for 5 min at room temperature. The sections were incubated with a COX-2 specific monoclonal antibody (Cayman Chemical, Ann Arbor, MI, USA; diluted 1:100), or with a mouse HuR monoclonal antibody (Zymed, South San Francisco, CA, USA; diluted 1:100) for 1 h at 37°C. The sections were then incubated with the secondary antibody for 30 min at room temperature, visualized using diaminobenzidine and counterstained with Mayer's hematoxylin. The negative control was performed with the antibody diluent as a substitute for the primary antibody.

Scoring

Two investigators scored the intensity of COX-2 and HuR staining in a blinded manner. All specimens with discordant scores were re-evaluated and the consensus score was used for statistical analysis. COX-2 expression was scored using the following scale: 0 = no staining, 1 = weak diffuse cytoplasmic staining (may contain a stronger intensity in less than 10% of the cells), 2 = moderate granular cytoplasmic staining in more than 10% of the cells, and 3 = strong granular cytoplasmic staining in more than 50% of cells. In HuR scoring, the nuclear and cytoplasmic staining were scored separately on the following scale: 0 = no staining, 1 = weak and/or focal (≤5% of the cells) staining, 2 = moderate or strong staining (≤50% of the cells), and 3 = moderate or strong staining (≥50% of the cells).

Statistical analysis

The COX-2 scores 0 and 1 were combined to represent low COX-2 expression, and scores 2 and 3 were combined to represent high COX expression. HuR scores 2 and 3 were combined and were considered strong staining. The correlation between the COX-2 and HuR staining intensity and clinicopathological parameters were assessed using a chi-squared test. A value of $P < 0.05$ was considered statistically significant.

Results

Expression of COX-2 and its correlation with clinical parameters

Of the 28 pleomorphic adenoma cases, there were 15 males and 13 females with a mean age of 37 ranging from 15 to 66 years. Of the 18 cases of mucoepidermoid carcinoma, there were eight males and 10 females with a mean age of 14 ranging from 21 to 63 years. The sites of the pleomorphic adenoma were the parotid gland ($n = 18$), the palate ($n = 6$), and submandibular gland ($n = 4$) (Table 1). The sites of the mucoepidermoid carcinoma were the parotid gland ($n = 12$), the palate ($n = 4$), and submandibular gland ($n = 2$) (Table 2).

High COX-2 (score 2–3) expression was observed in 13 (72.2%) of the 18 mucoepidermoid carcinoma cases (Fig. 1a–c). High COX-2 expression was observed in eight (28.6%) of the 28 pleomorphic adenoma casas (Fig. 1d and e). There was no correlation with age, gender, and origin sites. The level of COX-2 expression in the luminal epithelial cells was similar to that in the myoepithelial cells. There was a higher percentage of high COX-2 expression in the high-grade mucoepidermoid carcinomas (85.7%) than in the low grade (63.6%), but the difference was not statistically significant ($P = 0.308$). There was no correlation between a high COX-2 expression and age and gender in the mucoepidermoid carcinoma. The pattern of COX-2 expression was relatively similar among the specific cell types in the mucoepidermoid carcinoma. The frequency of a high COX-2 expression was 13/18 (72.2%) in the

Table 1 Association between the clinical parameters and high COX-2 (scores 2–3), strong nuclear and positive cytoplasmic HuR protein expression in the pleomorphic adenomas

Clinical parameters	*High COX-2/ n (%)*	*P-value (chi square test)*	*Strong nuclear HuR/n (%)*	*P-value (chi square test)*	*Positive cytoplasmic HuR/n (%)*	*P-value (chi square test)*
All patients	8/28 (28.6)	NA	15/28 (53.6)	NA	10/28 (35.7)	NA
Age						
<37	5/16 (31.3)	0.717	9/16 (56.3)	0.743	6/16 (37.5)	0.820
≥37	3/12 (25.0)		6/12 (50.0)		4/12 (33.3)	
Origin site						
Parotid gland	7/18 (38.9)	0.228	10/18 (55.6)	0.416	6/18 (33.3)	0.678
Submandibular gland	0/4 (0)		1/4 (25.0)		1/4 (25.0)	
Palate	1/6 (16.7)		4/6 (66.7)		3/6 (50.0)	
Submandibular gland + palate	1/10 (10.0)	0.105[a]	5/10 (50.0)	0.778[a]	4/10 (40.0)	0.724[a]

NA, not applicable.
[a]Vs. parotid gland.

Table 2 Association between clinicopathological parameters and high COX-2 (scores 2–3), strong nuclear and positive cytoplasmic HuR protein expression in mucoepidermoid carcinomas

Clinicopathological parameters	*High COX-2/ n (%)*	*P-value (chi square test)*	*Strong nuclear HuR/n (%)*	*P-value (chi square test)*	*Positive cytoplasmic HuR/n (%)*	*P-value (chi square test)*
All patients	13/18 (72.2)	NA	14/18 (77.8)	NA	13/18 (72.2)	NA
Age						
<45	6/8 (75.0)	0.814	8/8 (100)	**0.043**	7/8 (87.5)	0.196
≥45	7/10 (70.0)		6/10 (60.0)		6/10 (60.0)	
Origin site						
Parotid	10/12 (83.3)	0.051	9/12 (75.0)	0.725	9/12 (75.0)	0.758
Submandibular gland	2/2 (100)		2/2 (100)		1/2 (50.0)	
Palate	1/4 (25.0)		3/4 (75.0)		3/4 (75.0)	
Submandibular gland + palate	3/6 (50.0)	0.137[a]	5/6 (83.3)	0.688[a]	4/6 (66.7)	0.710[a]
Histologic grade						
Low	7/11(63.6)	0.308	8/11 (72.7)	0.518	7/11 (63.6)	0.308
high	6/7 (85.7)		6/7 (85.7)		6/7 (85.7)	
Staging						
I (T1N0M0)	9/12 (75.0)	0.710	9/12 (75.0)	0.688	8/12 (66.7)	0.457
II (T2N0M0)	4/6 (66.7)		5/6 (83.3)		5/6 (83.3)	

NA, not applicable.
[a]Vs. parotid gland.
The value in bold was considered statistically significant.

mucoepidermoid carcinoma and 8/28 (28.6%) in the pleomorphic adenoma and was statistically different ($P = 0.004$; Table 3). In this study, COX-2 was expressed in the cytoplasm of the ductal epithelium of the normal salivary glands, particularly in the striated ducts (Fig. 1f).

Nuclear and cytoplasmic expression of HuR and its correlation with clinical parameters

In the pleomorphic adenoma, the tumor cells expressed HuR in the nucleus only in 64.3% of cases (18/28) (Fig. 2a) and in both the cytoplasm and nucleus in 35.7% of cases (10/28) (Fig. 2b). Among the 10 pleomorphic adenomas, nine cases showed weak or focal staining, and only one case showed moderate cytoplasmic expression (Table 1). In the mucoepidermoid carcinoma, the tumor cells expressed HuR in the nucleus only in 27.8% of cases (5/18) (Fig. 2c) and in both the cytoplasm and nucleus in 72.2% of cases (13/18) (Fig. 2d and e). Among the 13 mucoepidermoid carcinomas, seven cases showed weak or focal staining, and six cases showed moderate or strong cytoplasmic expression (Table 2). The level of HuR expression in the nucleus was similar among the specific cell types of pleomorphic adenoma and mucoepidermoid carcinoma. However, the level of HuR expression in the cytoplasm was higher in the epidermoid cells than in the mucous cells of mucoepidermoid carcinoma. The frequency of cytoplasmic positivity for HuR was higher in the mucoepidermoid carcinomas than in the pleomorphic adenomas ($P = 0.009$; Table 3). There was no correlation between the cytoplasmic HuR expression and age, gender, and origin sites in the pleomorphic adenomas and mucoepidermoid carcinomas. The frequency of cytoplasmic HuR expression was higher in the high-grade mucoepidermoid carcinomas than in the low-grade mucoepidermoid carcinomas (63.6%), but the difference was not statistically significant ($P = 0.308$).

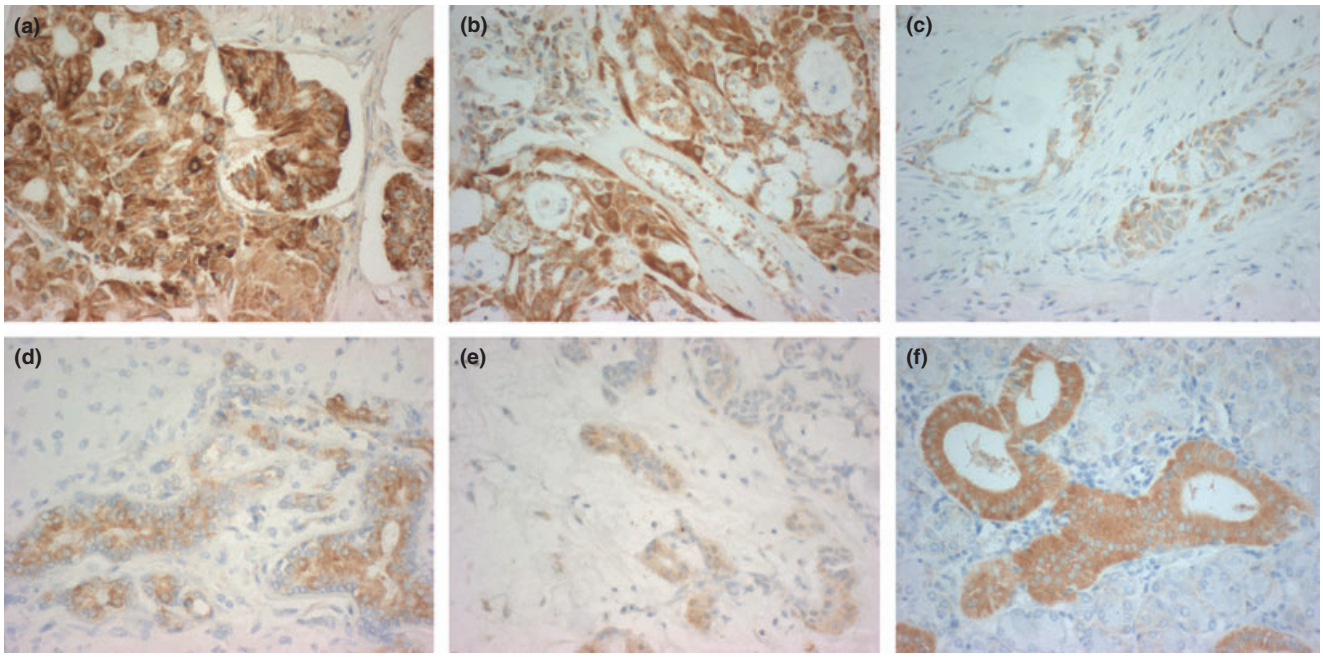

Figure 1 Immunohistochemical staining for cyclooxygenase-2 (COX-2). (a) Strong COX-2 expression in the high grade mucoepidermoid carcinoma. (b) Moderate and (c) weak COX-2 expression in the low-grade mucoepidermoid carcinomas. The pattern of COX-2 expression was relatively similar among the specific cell types in the mucoepidermoid carcinoma. (d) Moderate and (e) weak COX-2 expression in the pleomorphic adenomas. The level of COX-2 expression in the luminal epithelial cells was similar to that in the myoepithelial cells. (f) COX-2 expression in the normal salivary gland. The intralobular striated ductal epithelial cells show moderate intensity.

Table 3 Comparison of the pleomorphic adenomas and mucoepidermoid carcinomas according to HuR and COX-2 expression

Immunohistochemical reactivity	*Pleomorphic adenoma (n = 28)*	*Mucoepidermoid carcinoma (n = 18)*	*P-value (chi square test)*
COX-2			
Weak	20	5	**0.004**
Moderate	7	7	
Strong	1	6	
Nuclear HuR			
Negative or weakly	13	4	0.097
Strong	15	14	
Cytoplasmic HuR			
Negative	18	5	**0.009**
Weakly	9	7	
Strong	1	6	
Cytoplasmic HuR/High COX-2	($n = 8$)	($n = 13$)	
Negative	4	3	0.116
Weakly	4	5	
Strong	0	5	

The values in bold were considered statistically significant.

Nuclear and cytoplasmic expression of HuR and its correlation with COX-2 expression

High COX-2 immunoreactivity correlated with the cytoplasmic HuR expression in the mucoepidermoid carcinoma ($P = 0.027$) but not the nuclear HuR expression ($P = 0.528$) (Table 4). In the pleomorphic adenoma, there was no correlation between a high COX-2 immunoreactivity and either cytoplasmic or nuclear HuR expression. Among the eight cases of pleomorphic adenoma showing high COX-2 immunoreactivity, four cases were negative for cytoplasmic HuR expression and the remaining four cases showed only weakly positive cytoplasmic HuR expression. In the mucoepidermoid carcinomas, 13 cases showed high COX-2 expression. Among them, 10 cases showed weak or strong (five cases, weak; five cases, strong) cytoplasmic HuR immunoreactivity and only three cases were negative. Among the 13 cases showing high COX-2 expression, the staining intensity was homogeneous in five cases and heterogeneous in the remaining eight. Among these eight cases, the areas showing high COX-2 intensity within a sample showed

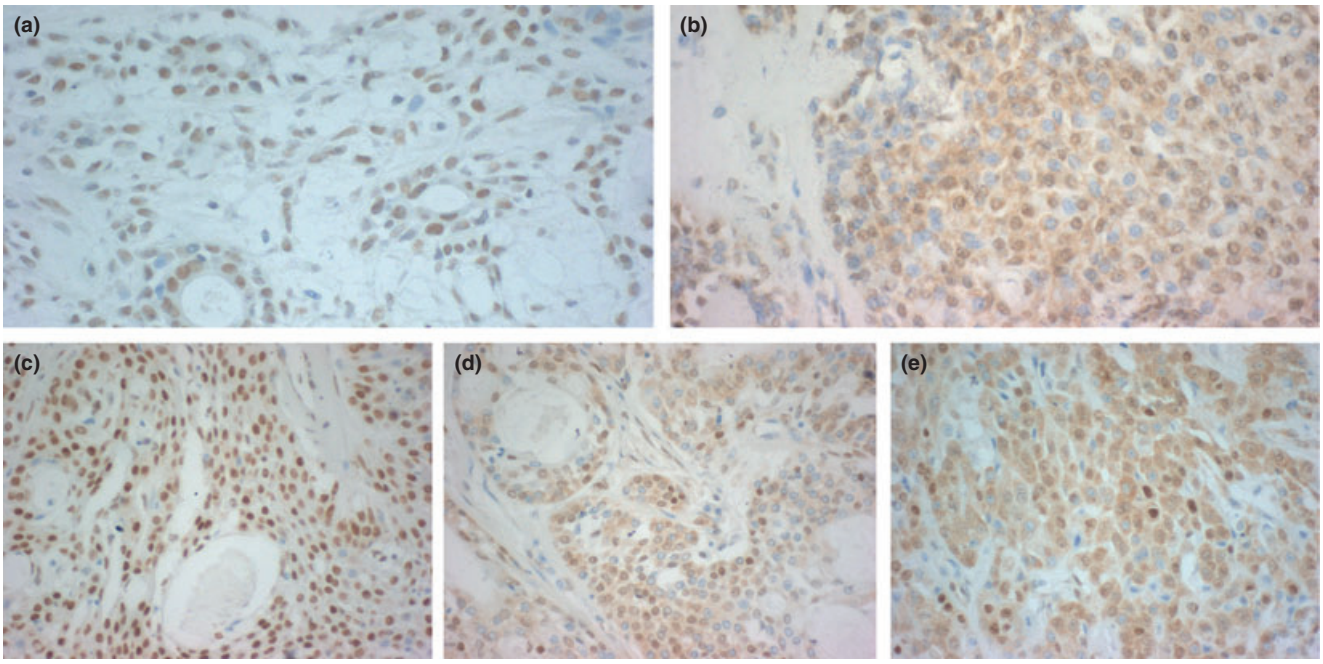

Figure 2 Immunohistochemical staining for HuR. (a) Nuclear but not cytoplasmic immunoreactivity for HuR in the pleomorphic adenoma. (b) Both nuclear and cytoplasmic staining for HuR in the pleomorphic adenoma. (c) Only nuclear, (d) both nuclear and weak cytoplasmic, and (e) nuclear and strong cytoplasmic immunoreactivity for HuR in the mucoepidermoid carcinoma. The level of HuR expression in the nucleus was similar among the specific cell types of pleomorphic adenoma and mucoepidermoid carcinoma. However, the level of HuR expression in the cytoplasm was higher in the epidermoid cells than in the mucous cells of mucoepidermoid carcinoma.

Table 4 Association between the nuclear and cytoplasmic HuR immunoreactivity and COX-2 expression in the pleomorphic adenomas and mucoepidermoid carcinomas

	Pleomorphic adenoma (n = 28), COX-2 positive			*P-value (chi square test)*	*Mucoepidermoid carcinoma (n = 18), COX-2 positive*			*P-value (chi square test)*
HuR immunoreactivity	*Weak*	*Moderate*	*Strong*		*Weak*	*Moderate*	*Strong*	
Nuclear								
Negative or weakly positive	12	1	0	0.072	2	1	1	0.528
Strong positive	8	6	1		3	6	5	
Cytoplasmic								
Negative	14	3	1	0.525	2	3	0	**0.027**
Weakly positive	5	4	0		2	4	1	
Strong positive	1	0	0		1	0	5	

The value in bold was considered statistically significant.

a tendency for higher HuR expression in the cytoplasm than in those with a low COX-2 intensity. However, there was no significant difference between these two tumors in terms of the relationship between a high COX-2 expression and cytoplasmic HuR immunoreactivity ($P = 0.116$; Table 3).

Discussion

The overexpression of the COX-2 protein in several human carcinomas indicates its association with carcinogenesis. Several studies have reported the expression and distribution of the COX-2 protein in carcinomas of the stomach (19), colon (20, 21), esophagus (22), lung (23, 24), pancreas (25), ovary (26), prostate (27), skin (28), and salivary gland tumors (9, 13–15). Normally, COX-2 expression is tightly regulated, and the altered expression of the mRNA stability factor, HuR, promotes COX-2 expression. There have been some papers showing that the constitutive overexpression of COX-2 is the result of HuR overexpression in ovarian and colon cancers, and that this process plays a key role in the carcinogenesis of several cancers (17, 26). This is the first study to report the expression of HuR and the correlation of HuR and COX-2 expression in salivary gland tumors. Dixon *et al.* reported that mRNAs of COX-2, vascular endothelial growth factor (VEGF), and IL-8 were transcribed constitutively and turned over slowly in colon cancer cells showing enhanced growth and tumourigenecity (17). They believed that the observed mRNA stabilization is the result of the defective recognition of class II-type AREs present within the

COX-2, VEGF, and IL-8, as well as the dysregulatory overexpression of HuR, which has been detected in tumors. Several studies have reported that the overexpression of HuR in gastric, ovarian, and breast carcinomas is associated with a higher grade, poor prognosis, and COX-2 overexpression (29–31). Other studies have recently reported that in ovarian carcinoma, cytoplasmic HuR expression is associated with COX-2 expression and a poor outcome. HuR binds to certain mRNAs in the nucleus, and this complex is then transported to the cytoplasm (19). Owing to the nucleocytoplasmic translocation of HuR being necessary for its activity and cytoplasmic presence of HuR found in several types of carcinomas, it was hypothesized that cytoplasmic HuR expression could be a prognostic marker in cancer patients (8–10, 26, 30, 32). In this study, there was a higher level of COX-2 and cytoplasmic HuR expression found in the high-grade mucoepidermoid carcinomas than in the low-grade carcinomas but the difference was not significant. Moreover, there was a correlation between a high COX-2 immunoreactivity and cytoplasmic HuR expression in the mucoepidermoid carcinoma but not in the pleomorphic adenoma. Therefore, it is believed that cytoplasmic HuR expression is essential for COX-2 overexpression, which might be partly associated with the progression of the mucoepidermoid carcinoma. In addition, it was assumed that the prognostic implication of cytoplasmic HuR and COX-2 overexpressions on a mucoepidermoid carcinoma is less definite than in other neoplasms, such as ovarian cancer.

Sakurai *et al*. (13) reported increased COX-2 expression in human salivary gland tumors. The COX-2 protein was detected in 27 of 30 salivary gland adenomas and in all cases of salivary gland carcinomas (13). In this study, the COX-2 protein was detected in 18 of the 28 pleomorphic adenomas (64.3%). Among them, high COX-2 expression was detected in only eight cases (28.6%). On the other hand, 15 of the 18 mucoepidermoid carcinomas (83.3%) showed COX-2 immunoreactivity and high COX-2 expression was detected in 13 cases (72.2%). In contrast to Sakurai *et al*. (13), there was a considerable difference in the incidence of COX-2 overexpression between the pleomorphic adenomas and mucoepidermoid carcinoma ($P = 0.004$). The cytoplasmic HuR expression was lower in the pleomorphic adenomas than in the mucoepidermoid carcinomas ($P = 0.009$). This suggests that the immunoreactivity of COX-2 and cytoplasmic HuR may be used to determine the degree of malignant behavior in the salivary gland tumors, particularly in those of a borderline nature. Sully *et al*. (33) attempted to perform a structural and functional dissection of a conserved destabilizing element of COX-2 mRNA. They reported that HuR does not play a prominent role in the conserved region 1 (CR1)-mediated regulation of mRNA stability and concluded that at least one critical regulator of COX-2 mRNA stability that is similar to HuR in its RNA-binding specificity but is not readily detected is likely to remain unidentified. In this study, there was no correlation found between COX-2 overexpression and the cytoplasmic or nuclear HuR immunoreactivity in pleomorphic adenomas. It is possible that the COX-2 mRNA stability is regulated by more complex mechanisms.

In this study, the COX-2 protein was expressed in the cytoplasm of the ductal epithelium of the circumferential normal salivary glands, particularly in the striated ducts. These results are similar to those reported by Sakurai *et al*. (13). Therefore, it is likely that COX-2 plays some role in maintaining the physiological state and function through an unknown mechanism.

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