

## Originals

Immunohistochemical Localization of REG I $\alpha$  Protein in Salivary Gland Tumors

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## SUMMARY

The *regenerating gene* (Reg) I $\alpha$  protein has a trophic effect on gastric epithelial cells, and its overexpression is reported in gastrointestinal cancers. The salivary gland is a component of the digestive system, and therefore, REG I $\alpha$  protein may play some role in the pathophysiology of salivary gland tumors. In the present study, we determined the immunohistochemical localization of REG I $\alpha$  protein in salivary gland tumors and moreover investigated its relationship to clinicopathological features. Twenty-eight patients with salivary gland tumor were enrolled. The specimens resected by surgery from those patients were examined using immunohistochemistry for REG I $\alpha$  protein and Ki67. Five of the 16 pleomorphic adenomas (31.3%) were positive for REG I $\alpha$  protein. Regarding salivary gland carcinomas, four of five mucoepidermoid carcinomas (80%), three of five adenoid cystic carcinomas (60%), one of two polymorphous low-grade adenocarcinomas (50%) were also positive for REG I $\alpha$  protein. However, no relationships were found between REG I $\alpha$  protein expression and clinicopathological features. Regarding the Ki67 expression, strong signal was observed in the tumor cells of patients with salivary gland adenoma as well as carcinoma. REG I $\alpha$  protein is expressed not only in adenocarcinoma but also precancerous adenoma cells proliferating actively, suggesting that REG I $\alpha$  protein may play a role at least in part in the development of salivary gland tumors.

**Key Words** : REG I $\alpha$  protein, proliferation, salivary.

## INTRODUCTION

The *regenerating gene* (Reg) was originally isolated from a complementary DNA (cDNA) library derived from regenerating rat pancreatic islets, and its human homologue was named REG I $\alpha$ <sup>1)</sup>. REG I $\alpha$  protein is expressed not only in the pancreas but also the gastro-

intestinal tract<sup>2~4)</sup>. Recently, REG I $\alpha$  protein is reportedly overexpressed in various malignancies including pancreatic<sup>5)</sup>, gastric<sup>3,6,7)</sup> or colorectal cancers<sup>8~10)</sup>, suggesting that REG I $\alpha$  protein play a role in the carcinogenesis of digestive system. The salivary gland is a component of the digestive system and its tissue structure, comprising acinar and ductal cells, is similar to that of the exocrine pancreas. Therefore, it is tempting to speculate that REG I $\alpha$  protein may play some role in the development of salivary gland tumors. In the present study, we initially determined the immunohistochemical localization of REG I $\alpha$  protein in salivary gland tumors and moreover investigated its relation-

Received November 25, 2008 ; accepted December 10, 2008  
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ship to clinicopathological features.

## MATERIALS AND METHODS

### *Patients, tissue samples, and histology*

Twenty-eight patients with salivary gland tumor (10 males, 18 females; mean age 49.3 y, range 13–85 y) who were diagnosed and treated at Dokkyo University Hospital between 1994 and 2005 were enrolled. Samples of salivary gland tissue were obtained by surgery from patients with salivary gland tumor, and also from 5 controls (2 males, 3 females; mean age 40.6 y, range 34–50 y) who were treated for mucocele. Tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Multiple hematoxylin-eosin-stained sections of each sample were examined histologically. The tumor stage was according to the International Union Against Cancer TNM staging system<sup>11)</sup>.

### *Immunohistochemistry*

Immunohistochemical staining was performed with a LSAB-2 kit (DAKO, Marseille, France) as described previously<sup>3)</sup>. Ki67 was used as markers for measures of cell proliferation<sup>12)</sup>. Anti-human REG I $\alpha$  antibody was provided by Dr. Okamoto et al. as previously described<sup>2)</sup>. In brief, 4- $\mu$ m-thick sections were placed on slides, deparaffinized, and dehydrated. They were then placed in 0.01 mol/L citrate buffer (pH 6.0) and treated by microwave heating (MI-77, Azumaya, Tokyo, Japan; 400 W, 95°C) for 10 and 40 min to facilitate antigen retrieval for REG I $\alpha$  and Ki 67, respectively. Then, the sections were followed by pretreatment with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min at room temperature to quench endogenous peroxidase activity. The sections were incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, and then with anti-REG I $\alpha$  (dilution 1:100) and anti-Ki 67 (DAKO Japan, Kyoto, Japan; dilution 1:50) for 1 hour. Thereafter, the sections were incubated with biotinylated secondary antibody for 15 min, washed with PBS, and treated with peroxidase-conjugated streptavidin for 20 min. Finally, the sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H<sub>2</sub>O<sub>2</sub> for 3 min and then counterstained with Mayer's hematoxylin.

### *Evaluation of REG I $\alpha$ expression*

We assessed the immunoreactivity of REG I $\alpha$  in the tissue sections including the invasive front of salivary gland tumors. To evaluate the immunoreactivity of REG I $\alpha$ , at least 500 tumor cells were counted in five different visual fields for each tumor tissue sample. A specimen was considered positive for REG I $\alpha$  protein, if  $\geq 20\%$  of the tumor cells were positively stained; otherwise, the specimens were considered negative.

### *Statistical analysis*

Statview 5.0J statistical software (Abacus Concepts Inc., Berkeley, CA) was used for all analyses. Chi-squared analyses were performed to investigate the relationship between REG I $\alpha$  expression and clinicopathological features. All values were expressed as the mean  $\pm$  SEM, and the significance of differences between two groups was assessed using Mann-Whitney U-test. Differences at  $P < 0.05$  were considered to be significant.

## RESULTS

### *Expression of REG I $\alpha$ in salivary gland tumors*

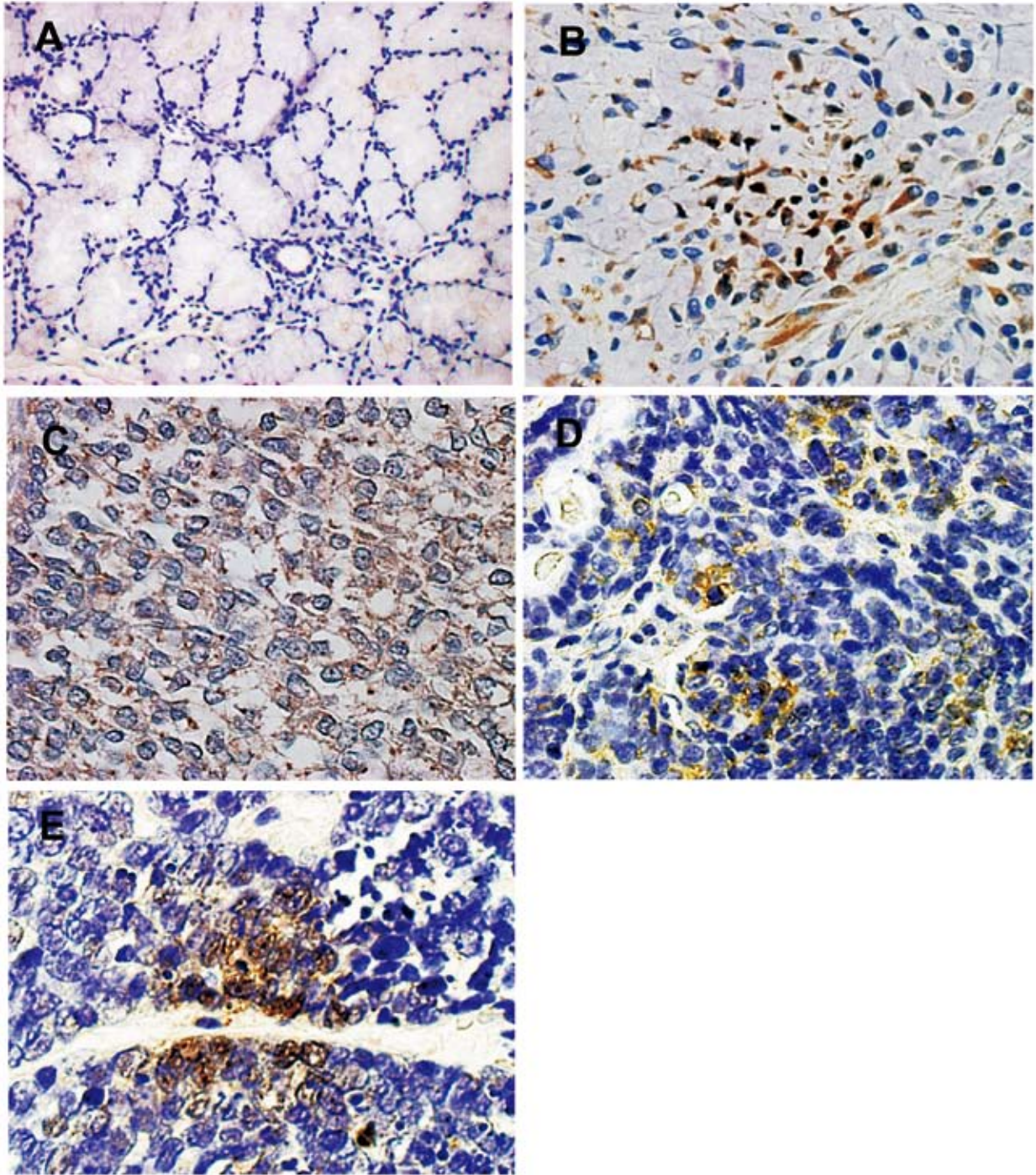
The immunoreactivity for REG I $\alpha$  protein was hardly observed in the ductal epithelial cells of normal salivary glands. Additionally, no immunoreactivity for REG I $\alpha$  protein was observed in either acinar or interstitial cells in normal salivary glands (Fig. 1A).

On the other hand, in salivary gland tumors, REG I $\alpha$  protein immunoreactivity was detected in the cytoplasm of tumor cells (Fig. 1B–E). REG I $\alpha$  protein immunoreactivity was detected in various types as well as stages of salivary gland tumors.

Five of the 16 pleomorphic adenomas (31.3%) were positive for REG I $\alpha$  protein. Regarding salivary gland carcinomas, four of five mucoepidermoid carcinomas (80%), three of five adenoid cystic carcinomas (60%), one of two polymorphous low-grade adenocarcinomas (50%) were also positive for REG I $\alpha$  protein (Table 1). However, no relationships were found between REG I $\alpha$  protein expression and sex, age, histology or stage of carcinoma (Table 1).

### *Expression of Ki67 in salivary gland tumors*

Immunoreactivity for Ki67 was scattered in the normal salivary gland (Fig. 2A). In detail, a few epithelial



**Figure 1** Immunostaining of REG I $\alpha$  in normal and salivary gland tumor tissues. (A) REG I $\alpha$  expression is negative in any types of cell in normal salivary gland tumor tissues. However, REG I $\alpha$ -immunoreactivity is detectable in the cytoplasm of tumor cells in (B) pleomorphic adenoma, (C) polymorphous-low grade adenocarcinoma, (D) mucoepidermoid carcinoma, (E) adenoid cystic carcinoma.

**Table 1** Relationship between REG I $\alpha$  expression and clinicopathological factors

		REG I $\alpha$ expression		P value
		negative	positive	
Sex	Male/Female	3/6	7/12	NS
Age		54.7 $\pm$ 4.0	46.7 $\pm$ 5.2	NS
Histology				
	Pleomorphic adenoma	11	5	
	Mucoepidermoid carcinoma	1	4	NS
	Adenoid cystic carcinoma	2	3	
	Polymorphous low-grade Ad Ca	1	1	
	Adenoma	5	10	NS
	Carcinoma	4	9	
Stage	I	2	8	NS
	II, III, IV	7	11	

cells per duct showed Ki67 immunoreactivity. However, strong immunoreactivity of Ki67 was observed in the tumor cells of patients with salivary gland adenoma as well as carcinoma (Fig. 2B-E).

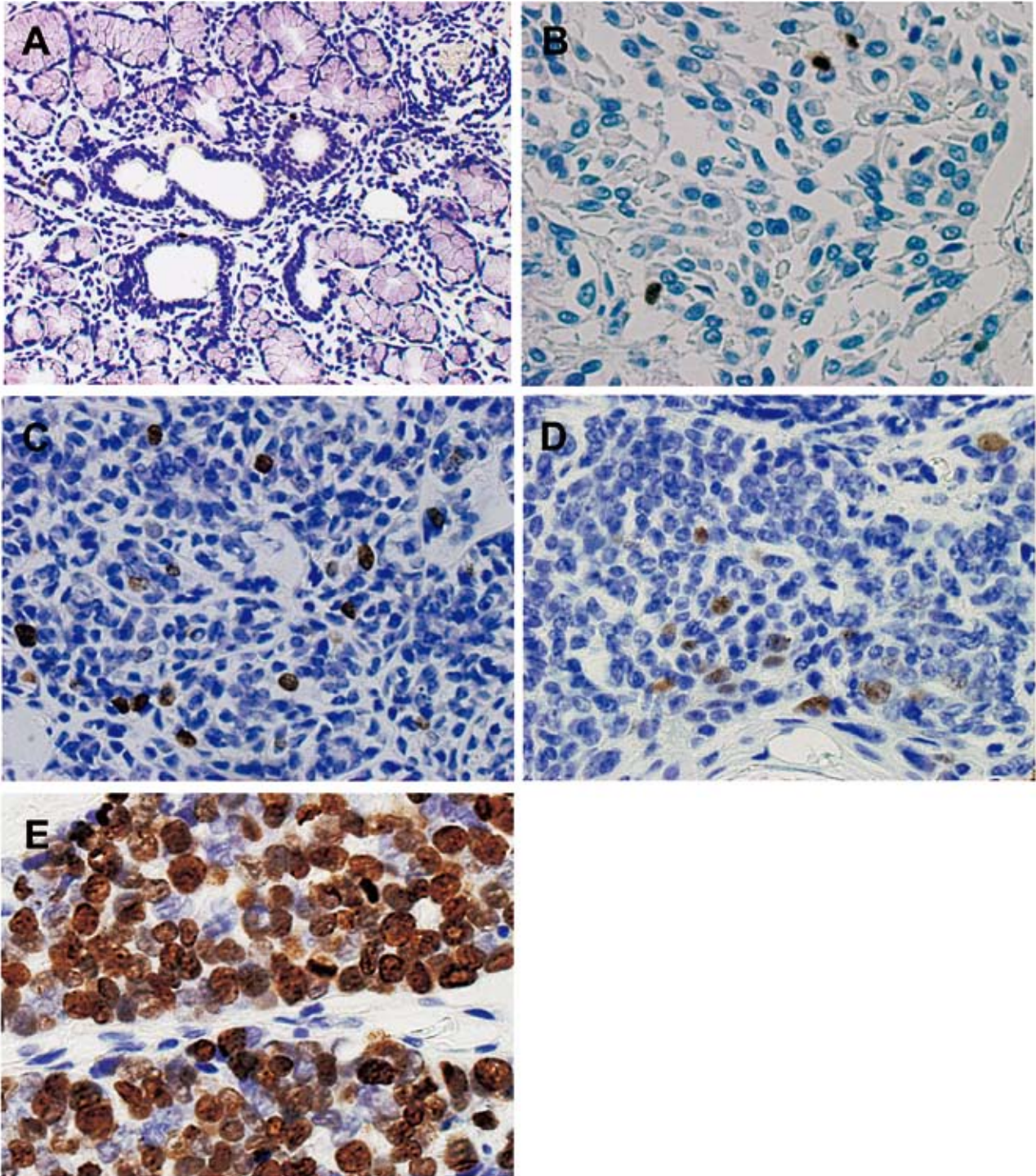
## DISCUSSION

We have previously shown that REG I $\alpha$  protein is expressed not only in the pancreas but also in the gastrointestinal tract<sup>3,4</sup>; however, its expression in salivary glands still remained unclear. Since the structure of salivary gland is similar to that of the exocrine pancreas, we expected that REG I $\alpha$  protein would be expressed in salivary gland as well. However, we observed that REG I $\alpha$  protein expression is not detectable in normal salivary glands although its expression is detectable at very high level in the pancreas<sup>13</sup>. We have no answer to explain this discrepancy but speculate that tissue-specific differences in the regulatory mechanism of gene expression may affect the level of REG I $\alpha$  protein expression. On the other hand, it is interesting that REG I $\alpha$  protein expression is detectable in the ductal epithelial cells in salivary glands under inflammatory condition<sup>14</sup>. This finding may suggest that the ductal epithelial cells in salivary glands have a potential to express REG I $\alpha$  protein but its expression is suppressed under normal condition.

The most important finding in this study was that a considerable number of salivary gland tumors expressed REG I $\alpha$  protein. In detail, REG I $\alpha$  expression

was detectable in the cytoplasm of tumor cells, being compatible with the character of REG I $\alpha$  as a secretory protein. Recent studies have reported that not only gastroenterological cancers but also lung cancers<sup>15</sup> and seminomas<sup>16</sup> express REG I $\alpha$  protein, suggesting that REG I $\alpha$  protein plays a role in the pathogenesis of various malignancies. In this context, although we investigated the relationship between REG I $\alpha$  expression and clinicopathological features in patients with salivary gland tumors, we found no relationship between them. However, since REG I $\alpha$  protein has a trophic effect on gastrointestinal epithelial cells<sup>17</sup>, we furthermore examined whether REG I $\alpha$  protein is involved in the cell proliferation in salivary gland tumors. Of note, the distribution of Ki67-positive cells was similar to that of REG I $\alpha$ -positive cells in salivary gland tumors, suggesting that REG I $\alpha$  protein may be associated with the proliferative behavior of tumor cells in salivary gland tumors. Moreover, it is noteworthy that REG I $\alpha$  overexpression was observed not only in adenocarcinoma but also adenoma cells, suggesting that REG I $\alpha$  protein play a role from the early stage of carcinogenesis in the salivary glands.

In summary, REG I $\alpha$  protein is expressed in various salivary gland tumors in histology. Moreover, REG I $\alpha$  protein is expressed not only in adenocarcinoma but also precancerous adenoma cells proliferating actively. These data suggest that REG I $\alpha$  protein may play a role at least in part in the development of salivary



**Figure 2** Immunostaining of Ki67 in normal and salivary gland tumor tissues. (A) Ki67 immunoreactivity is scattered in the normal salivary gland tissues. However, strong signal of Ki67 immunoreactivity is detectable in the nuclei of tumor cells in (B) pleomorphic adenoma, (C) polymorphous-low grade adenocarcinoma, (D) mucoepidermoid carcinoma, (E) adenoid cystic carcinoma.

gland tumors. REG I $\alpha$  expression has been recently suggested to be useful to predict the prognosis in patients with gastrointestinal cancers<sup>6-8</sup>). In this regard, we will need to investigate the prognostic significance of REG I $\alpha$  expression in patients with salivary gland tumors.

*Acknowledgements.* The authors thank Chiaki Matsuyama, Ayako Shimizu, Takako Ono, Midori Katayama, Nozomi Nagashima, Mikiko Ishikawa, Atsuko Kikuchi, and Sachiko Miyahara (Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, Tochigi, Japan) for their excellent technical and secretarial assistance. We are grateful to Dr. Hiroshi Okamoto in Tohoku University Graduate School of Medicine, Sendai, Japan, for providing anti-REG I $\alpha$  antibody.

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