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Original contribution

Human epididymis protein 4 is up-regulated in gastric and pancreatic adenocarcinomas☆,☆☆

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Summary Upper gastrointestinal neoplasia in the esophagus, stomach, and pancreas is associated with the formation of preneoplastic metaplasias. We have previously reported the up-regulation of human epididymis protein 4 (HE4) in all metaplasias in the stomach of humans and mice. We have now sought to evaluate the expression of HE4 in metaplasias/preneoplastic precursors and cancers of the human stomach, pancreas, and esophagus. Tissue microarrays for gastric cancers, pancreatic cancers, and esophageal adenocarcinoma were stained with antibodies against HE4. Immunostaining was quantified by digital imaging, and the results were evaluated to assess the expression in metaplasias, the expression in cancer pathological subtypes, and the effects of expression on survival in patients with cancer. In patients with gastric cancer from Korea, HE4 was detected in 74% of intestinal and 90% of diffuse

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cancers, whereas in a gastric cancer cohort from Johns Hopkins, HE4 was detected in 74% of intestinal-type and 92% of diffuse cancers. Nevertheless, in both cohorts, there was no impact of HE4 expression on overall survival. In the esophagus, we observed the expression of HE4 in scattered endocrine cells within Barrett esophagus samples, but Barrett columnar metaplasias and HE4 were detected in only 2% of esophageal adenocarcinomas. Finally, in the pancreas, HE4 expression was not observed in pancreatic intraepithelial neoplasia lesions, but 46.8% of pancreatic adenocarcinomas expressed HE4. Still, we did not observe any influence of HE4 expression on survival. The results suggest that HE4 is up-regulated during gastric and pancreatic carcinogenesis.

1. Introduction

Upper gastrointestinal adenocarcinomas carry an especially poor prognosis. Between 1999 and 2005, only 17% of patients with esophageal cancer, 26% of patients with stomach cancer, and 6% of patients with pancreatic cancer survived 5 years after identification of the disease [1]. Although many aspects of these cancers are likely distinct, all these adenocarcinomas display one common feature: association with preneoplastic metaplastic lineages. We have previously demonstrated that preneoplastic lineages in the human stomach demonstrate up-regulation of specific protein markers [2,3]. In particular, in both human stomach metaplasias and metaplasia models in mice, we have demonstrated a marked up-regulation of human epididymis protein 4 (HE4)/whey-acidic protein (WAP) 4-disulfide core domain protein 2 [3]. HE4/WAP 4-disulfide core domain protein 2 is a WAP 4-domain protein and is a member of a larger family of WAP domain–containing secreted putative extracellular protease inhibitor proteins [4,5]. Originally identified in human epididymis, HE4 has recently received a great deal of attention as a serum marker for ovarian cancer [4,5]. Patients with ovarian cancer show early up-regulation of detectable protein in the serum, and HE4 screening is under extensive evaluation as a serum screening test for ovarian cancer or a method for follow-up of resected patients [6-8].

We and others have found that HE4 is undetectable in the normal human stomach [3,4]. Thus, the up-regulation of HE4 in metaplasias makes HE4 an important candidate marker of the metaplastic process. Nevertheless, it is unclear whether HE4 is up-regulated in other upper gastrointestinal metaplasias and cancers. We have now evaluated the expression of HE4 in metaplasias/preneoplastic precursors and adenocarcinomas from the stomach, esophagus, and pancreas. Our studies have revealed that HE4 is up-regulated in gastric and pancreatic ductal adenocarcinomas, but not in esophageal adenocarcinoma. The results, therefore, indicate that up-regulation of HE4 expression is characteristic of carcinogenesis in the pancreas and stomach, but it is not a universal marker of metaplastic processes in the upper gastrointestinal tract.

2. Materials and methods

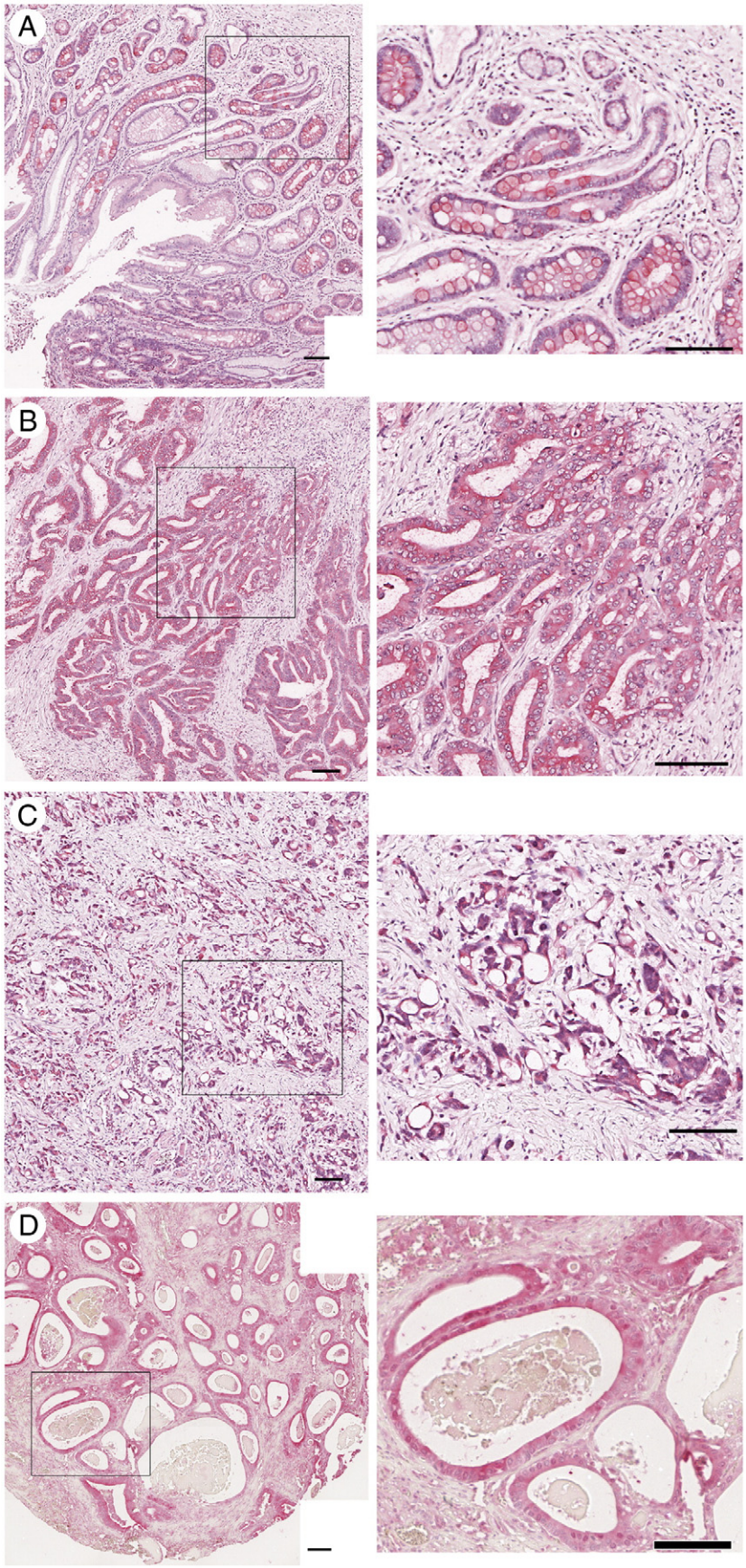
2.1. Tissue analysis

All esophageal tissue samples, including 12 archived cases of the Barrett epithelium, were obtained from the archives of the Department of Pathology at Vanderbilt University (Nashville, TN). The use of specimens from the archival tissue repository was approved by the institutional review board. A tissue microarray (TMA) that contained 75 tumors of lower esophageal or gastroesophageal junction–origin adenocarcinomas was constructed with each tumor sample on the TMA represented by 3 tissue cores (1-mm core size). Adjacent normal gastric and esophageal tissues were available for some cases for comparisons. Histopathologic diagnosis of the esophageal adenocarcinomas was verified according to the Vienna classification of gastrointestinal epithelial neoplasia [9]. The adenocarcinomas ranged from moderately differentiated to poorly differentiated, stages II to IV based on the American Joint Committee on Cancer (AJCC) seventh edition staging manual [10] and were all of the intestinal type.

For analysis of gastric cancer, 2 sets of TMAs were used. One set was obtained from Johns Hopkins Medical Institutions containing 481 tissue cores (1.5 mm) constructed from 131 patients with gastric carcinomas resected between 1985 and 1995 [11]. A second set of TMAs contained 450 gastric carcinomas (2.0-mm cores) resected at Seoul National University Hospital in 2004 (SNUH-2004-GC) [2].

For analysis of pancreatic lesions, 2 sets of tissue arrays were used from Johns Hopkins Medical Institutions. To determine the staining in pancreatic intraepithelial neoplasia (PanIN) lesions, we evaluated a tissue array constructed with

Fig. 1 Immunohistochemical staining of HE4 in intestinal metaplasia and gastric cancers. A, HE4 staining in intestinal metaplasia. B and C, HE4 staining in gastric cancer specimens from Korean patient arrays. D, HE4 staining in intestinal-type gastric cancer specimen from Johns Hopkins arrays. Right: higher magnification views. All scale bars are 100 μm.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samples from 55 PanIN lesions including 16 PanIN 1A, 18 PanIN 1B, 14 PanIN 2, and 7 PanIN 3 lesions [12]. To assess staining in pancreatic adenocarcinomas, we evaluated an array set containing samples from 673 tissue cores constructed from 172 patients who had pancreatic ductal adenocarcinomas.

2.2. Immunohistochemistry

Paraffin-embedded TMA sections were heated at 60°C for 30 minutes and cooled to room temperature. Deparaffinization and rehydration of tissue occurred in Histoclear (National Diagnostics, Atlanta, GA), graded concentrations of ethanol, and distilled water. Antigen retrieval was performed using Target Retrieval Solution (Dako North America Inc, Carpinteria, CA) at 120°C for 15 minutes and was cooled afterward at 4°C. Sections were blocked with 5% normal goat serum provided with the Vectastain kit (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Primary antibody application was performed overnight at 4°C. The primary antibody used was an antirabbit human HE4 at 1:1000 [5]. The next day, sections were incubated with biotinylated rabbit immunoglobulin G (IgG) (1:200; Vector Laboratories) for 30 minutes at room temperature, followed by incubation with avidin-biotin complex for 15 minutes at room temperature. Slides were developed with 3,3′-diaminobenzidine or alkaline phosphatase using a kit from Vector Laboratories and counterstained with Mayer hematoxylin (Sigma-Aldrich Corp, St Louis, MO) solution, dehydrated, and mounted.

2.3. Immunofluorescence

For double staining with anti-HE4 and anti–chromogranin A, deparaffinized sections were blocked with protein blocking (Dako) and incubated with rabbit IgG anti-HE4 (1:1000) and mouse IgG anti–chromogranin A (1:25; AbD Serotec, Raleigh, NC) at the same time overnight at 4°C, followed by incubation with Cy3-labeled goat antirabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Alexa488 goat antimouse IgG (Invitrogen, Grand Island, NY, USA). After washing with phosphate-buffered saline, sections were mounted using ProLong Gold Antifade Reagent with 4,6-diamino-2-phenylindole (Invitrogen) as a nuclear counterstain.

2.4. Quantitative immunohistochemistry analysis

Stained TMA slides were analyzed using the Ariol SL-50 automated slide scanner (Applied Imaging, San Jose, CA) to quantitate the amount of positive staining for each tissue core. TMA slides stained for HE4 were analyzed using a Multistain HighRes script trained to the desired intensity and color for the Vector Red staining. Before analysis, areas of nonepithelial cells were removed from the cores. After analysis, percent epithelium that was positive for HE4 was determined based on the area of Vector Red staining subtracted from the total area of epithelium.

2.5. Statistical analyses

For each set of data, 3 groups were analyzed based on percent HE4 staining. The first group encompassed those with no staining (for the pancreatic cancer set this was staining equal to zero, whereas for the other 2 sets, staining <0.1% was considered as zero). The nonzero staining cores were then stratified as low staining and high staining based on the median level of staining for all those with more than zero staining.

Estimated median survival times for each group were obtained using Kaplan-Meier estimates. The log-rank test was used to test for a difference between the groups for the Korean data set. The 2 Johns Hopkins data sets had multiple cores per subject, so a Wald test statistic was used to test differences in survival between the groups; these test statistics were obtained using the robust sandwich estimate of Lin and Wei [13] for the covariance matrix that describes the correlation between cores within subjects.

3. Results

3.1. Analysis of cohorts of gastric cancer resections

We evaluated the expression of HE4 in 2 large cohorts of gastric cancer specimens, one from Johns Hopkins Medical Institutions and the other from Seoul National University in Korea. As previously demonstrated [3], intestinal metaplasia showed strong HE4 staining (Fig. 1A). The Korean set contained 433 different patient resections that had complete data for both HE4 staining and survival. Most cases in this cohort were stages 1 or 2. The cancers were annotated according to their Lauren classification as intestinal-type, diffuse, or mixed cancers (Table 1). Twenty-five percent of intestinal-type gastric cancers showed high expression for HE4, compared with 46% with low levels of expression and 29% that displayed

Table 1 HE4 immunostaining in TMA from Korean patients

Lauren class	HE4 high (n = 181)	HE4 low (n = 167)	HE4 none (n = 85)	Total cases (N = 433)
Intestinal	40 (25%)	73 (46%)	46 (29%)	159
Diffuse	101 (61%)	47 (29%)	17 (10%)	165
Mixed	19 (28%)	32 (47%)	17 (25%)	68
Undetermined	0	2 (100%)	0	2
Unclassified/ Missing	21 (54%)	13 (33%)	5 (13%)	39

NOTE. The data displayed here are from only those cores with both staining and survival data (433/480 cores).

Table 2 HE4 immunostaining in Johns Hopkins gastric cancer tissue array

Lauren class	HE4 high (n = 97)	HE4 low (n = 64)	HE4 none (n = 39)	Total cases (n = 200)
Intestinal	44 (34%)	51 (40%)	33 (26%)	128
Diffuse	53 (74%)	13 (18%)	6 (8%)	72

NOTE. The data here reflect removal of 10 cores (from the 210 used in the survival analysis) that were missing Lauren grade. All analyzed cores were from cancers, so they do not include adjacent normal or other gastrointestinal tissue cores that were included on the tissue array.

no detectable expression (Fig. 1B). Sixty-one percent of diffuse-type gastric cancers showed high expression for HE4, compared with 29% with low levels of expression and 10% that displayed no detectable expression (Fig. 1C). In comparison, among mixed tumors, 28% showed high levels of HE4, 47% had low levels of expression, and 25% showed no HE4 expression. There was a statistically significant difference between staining pattern and the Lauren grades for intestinal, diffuse, and mixed (the χ^2 test statistic resulted in $P < .001$); these differences were found predominately in the high staining for diffuse type and low

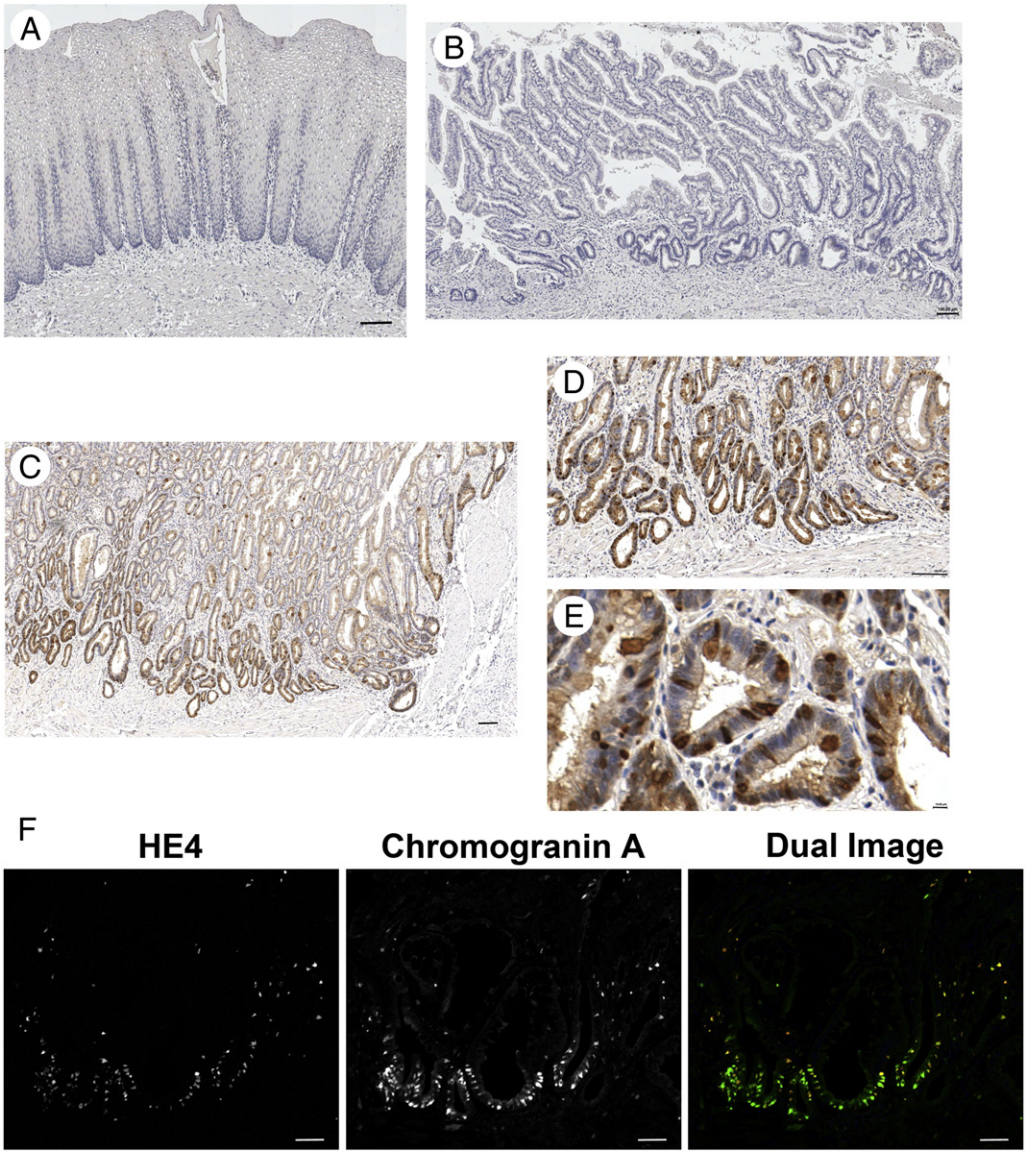
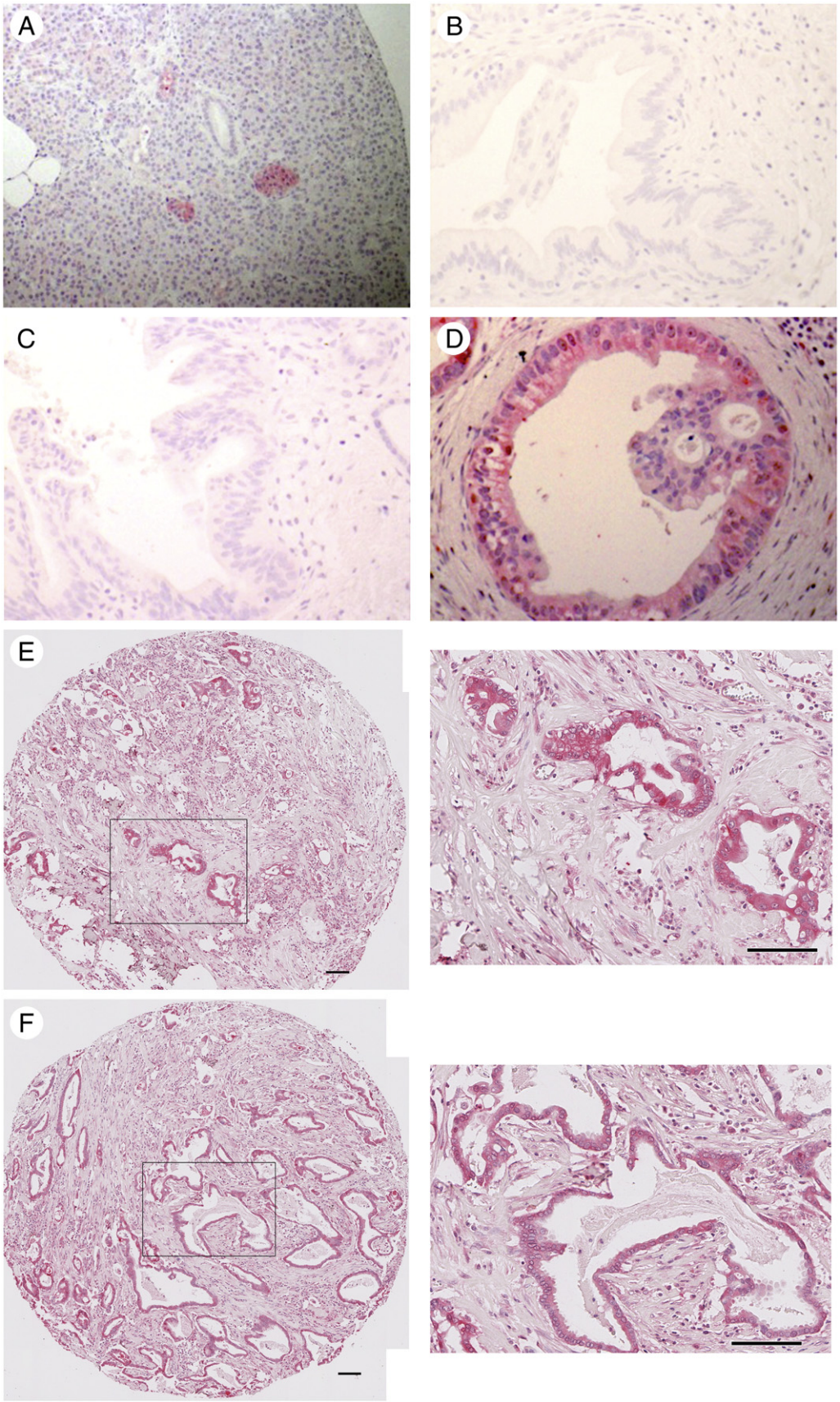


Fig. 2 HE4 in the normal esophageal tissue and Barrett epithelium. HE4 immunostaining was performed in human esophageal tissues. A, Normal esophageal squamous epithelium showed no staining for HE4. B, Barrett epithelium without HE4 staining. C-E, Barrett epithelium with HE4 staining in the bases of some glands. F, Dual-immunofluorescence staining for HE4 and chromogranin A in the Barrett epithelium. HE4 immunoreactive cells in the Barrett epithelium were also immunoreactive for chromogranin A (dual-label–merged image [right]: HE4 pseudocolored red and chromogranin A pseudocolored green with direct overlap seen as yellow), indicating that they are enteroendocrine cells. All scale bars are 100 μm.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staining for intestinal type. The second set of gastric cancer specimens obtained from John Hopkins contained mostly stage 3 or 4 cancers, with multiple cores from 121 patients (Table 2). These cancers were annotated based on Lauren classification as intestinal type or diffuse. Of the 200 cancer patient cores, 34% of the intestinal-type gastric cancers showed high expression for HE4, compared with 40% with low levels of expression and 26% that displayed no detectable expression (Fig. 1D). Among the diffuse-type gastric cancers, 74% showed high expression for HE4, compared with 18% with low levels of expression and 8% that displayed no detectable expression. These patterns were similar to those found in the Korean specimens and were also statistically significantly different ($P < .001$).

3.2. HE4 in the esophagus

No previous investigations have considered HE4 expression in the esophagus. In normal esophagus, no immunohistochemical positivity for protein expression of HE4 in the squamous epithelium of the esophagus was observed (Fig. 2A). We also stained sections from 12 patient cases containing Barrett metaplasia in the esophagus. Of the 12 sections stained, 8 of the sections showed no staining for HE4 in Barrett intestinal metaplasia (Fig. 2B). However, 4 resections showed immunohistochemical staining for HE4 protein expression in small triangular cells usually deep in the glands of the Barrett epithelium (Fig. 2C-E). Dual staining of these cells with chromogranin A antibodies demonstrated that the HE4-expressing cells in the Barrett epithelium were enteroendocrine cells (Fig. 2E and F). It is of note that not all chromogranin A–positive endocrine cells were also stained for HE4.

To assess the possible association of HE4 with esophageal carcinogenesis, we stained TMAs containing 133 samples of esophageal adenocarcinomas. Only 3 cancer cores showed significant staining in cancer cells, although we were able to identify scattered triangular HE4 staining cells in association with cancer, consistent with enteroendocrine cells. All of these results indicate that HE4 expression is not significantly associated with either Barrett intestinal metaplasia in the esophagus or esophageal adenocarcinoma.

3.3. HE4 in pancreas

Pancreatic adenocarcinoma also develops in the setting of noninvasive PanIN lesions. We examined HE4 expression in normal, PanIN, and adenocarcinoma specimens from patients at John Hopkins. No HE4 staining was observed in normal acinar cells or pancreatic ducts. However, prominent HE4 staining was found in pancreatic islets (Fig. 3A). Importantly, we did not observe any staining in either early or late PanIN lesions in the pancreas (Fig. 3B and C). Nevertheless, strong staining was observed in pancreatic ductal adenocarcinoma in patients without staining in adjacent PanIN lesions (Fig. 3D). Strong HE4 staining was observed in pancreatic cancers with varying levels of differentiation (Fig. 3E and F). Among 220 pancreatic cancer samples (representing 172 individual patients) analyzed in this cohort, 103 (46.8%) stained for HE4 in more than 5% of the neoplastic epithelium.

3.4. Survival determination based on HE4 expression

We used patient outcome data for both the pancreatic and the gastric cancer tissue arrays to evaluate whether the expression of HE4 could influence patient survival. Staining patterns were stratified into 3 groups; tumors without any staining and then those with staining were stratified into either high or low staining based on the median level in the samples. The median percent staining for the Johns Hopkins pancreatic cancer cohort was 11%, 7% for the Johns Hopkins gastric cancer cohort, and 4% for the Korean cohort.

In the pancreatic cancer group, there was no impact of staining for HE4 on survival (Fig. 4A). In the gastric cancer cohort from Korea, there was also no effect of survival based on HE4 expression (Fig. 4B). In patients with gastric cancer from Johns Hopkins who predominantly were resected with high stage tumors, the group without HE4 staining showed an early survival advantage, but this difference was not statistically significant overall ($P = .175$; Fig. 4C). Differences in the survival patterns in the 2 gastric cohorts were also reflected in the proportion of the samples with no, low, and high levels of staining, with 24%, 38%, 38%, respectively, for the Korean sample and 19%, 31%, 50%, respectively, for the Johns Hopkins sample. The Johns Hopkins pancreatic cohort had almost equal numbers of subjects within each staining level, 34% with no staining, 35% with low staining, and 29% with high staining. The test statistics for the John Hopkins cohorts (both gastric and pancreatic) were adjusted to account for multiple cores per subject. The degree of the need for this adjustment can be evaluated by examining the ratio in the standard errors for the parameter estimates based on adjusted (robust) versus nonadjusted estimation. As expected (because of the number of cores for each subject), the pancreatic set had a larger degree of difference (highest ratio was between no staining and low staining group at 1.3, meaning the standard error was 30% larger using the robust estimation). The gastric set had ratios very close to 1 (with <1% difference between the 2 estimates).

Fig. 3 Immunohistochemical staining of HE4 in normal pancreas, PanIN, and pancreatic cancer. A, HE4 staining in normal pancreas. Note: strong staining in a pancreatic islet. B, HE4 staining in a low-grade PanIN lesion. C, HE4 staining in a high-grade PanIN lesion. D, HE4 staining in pancreatic adenocarcinoma from the same patient with PanIN lesion in panel C. E and F, HE4 staining in pancreatic adenocarcinomas. Right: higher magnification views. All scale bars are 100 μm.

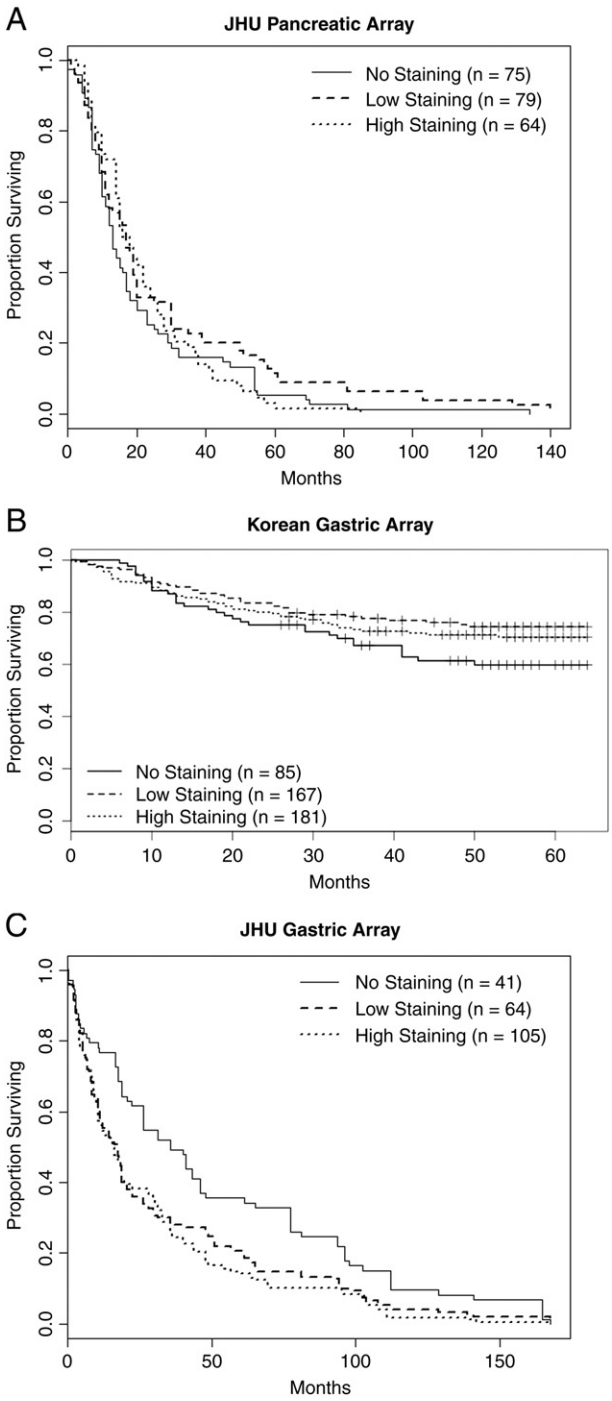

Fig. 4 Outcomes in patients with pancreatic and gastric adenocarcinoma associated with HE4 expression. Samples in all groups were classified based on no detectable HE4 staining or low or high staining based on the median staining observed among positively stained samples. Kaplan-Meier survival statistics were calculated for all groups. A, Survival in patients with pancreatic cancer identified at Johns Hopkins. B, Survival in patients with gastric cancer identified at Seoul National University, Korea. C, Survival in patients with gastric cancer resected at Johns Hopkins.

4. Discussion

Carcinogenesis in the upper gastrointestinal tract is associated with distinct mucinous metaplastic lineages. The presence of these metaplastic lineages suggests that the development of metaplasia is a key event in the evolution of adenocarcinoma in the stomach, esophagus, and pancreas. Despite this general association of adenocarcinoma with preexisting metaplasia, there has been surprisingly little concordance in biomarker expression within these cancers. We have demonstrated here that up-regulation of HE4 is a common feature in at least gastric and pancreatic cancers. However, the patterns for the timing of up-regulation appear to be different. Thus, although HE4 is up-regulated in all metaplastic lineages in the stomach, we did not observe any expression of HE4 in any PanIN lineages in the pancreas. Nevertheless, HE4 expression appeared elevated in most pancreatic adenocarcinomas to a similar extent as in gastric adenocarcinomas. Up-regulation of HE4 did not appear to be a significant part of carcinogenesis in the esophagus. These results indicate that the up-regulation of HE4 is associated with tissue-specific carcinogenesis at variable points in neoplastic progression.

HE4 is a member of a broader family of secreted putative protease inhibitors, all containing 2 to 4 WAP domains [4,5,14]. The exact function of these proteins is poorly understood, but their status as likely extracellular protease inhibitors suggests that they may be involved in the regulation of extracellular matrix, cell migration, and cell invasion. This type of role could also explain the different tissue-specific patterns of expression in metaplasias. The metaplasias in the stomach are organized into distinct glandular structures that require migration of cells bidirectionally toward the lumen or gland bases [15]. In contrast, metaplasias in the pancreas are organized into simpler ductular structures, which are lined by mucinous metaplastic lineages [16]. Thus, in the case of pancreatic cancers, up-regulation of HE4 may be more indicative of changes toward more invasive phenotypes.

The patterns of HE4 up-regulation may also reflect common processes for the derivation of cancerous lineages in the stomach and the pancreas. Thus, increasing evidence suggests that initial preneoplastic lesions in both the stomach and the pancreas are derived from transdifferentiation of zymogenic cells in the stomach or pancreas into mucinous metaplasias [17,18]. It should also be noted that metaplasias of fimbrial secretory cells may also give rise to serous ovarian cancers [19,20]. Thus, the process of up-regulation of HE4 expression may reflect a pattern of cell derivation from acinar cell lineages. The actual lineage of origin for adenocarcinoma through the Barrett epithelium remains obscure, but there is no evidence for transdifferentiation of an acinar-type cell in the genesis of Barrett metaplasia or esophageal adenocarcinoma because, indeed, different patterns for HE4 expression in esophageal and gastric

adenocarcinoma would support different pathways for the evolution of these 2 types of mucosal cancer.

Nevertheless, we have also observed an association of HE4 expression with some endocrine cell populations. HE4 was expressed in islet cells and endocrine cells within the Barrett epithelium. Previous investigations have noted that endocrine cells are often present in the Barrett epithelium [21]. In contrast with the results in esophageal metaplasia and pancreas, we did not observe HE4 expression in enteroendocrine cells in either the human or the mouse gastric mucosa. The function of HE4 in these cells therefore remains unclear.

In summary, we have demonstrated in these studies that up-regulation of the extracellular protease inhibitor HE4 is associated with carcinogenesis in the stomach and pancreas. Although we did not discern a significant impact of HE4 expression on patient outcomes, HE4 may represent an important marker of the neoplastic process in the stomach and pancreas.

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