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Neuroendocrine Cells in Barrett's Mucosa and Adenocarcinomas of the Gastroesophageal Junction

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We estimated the prevalence and prognostic significance of neuroendocrine (NE) cells in a series of 208 resection specimens containing gastroesophageal junction (GEJ) adenocarcinomas, with 56 specimens containing Barrett's mucosa. Immunohistochemically, chromogranin A (CGA) was positive in 49% (102/208) of GEJ adenocarcinomas and in 68% (38/56) of Barrett's mucosas. CGA in GEJ tumors correlated with pTNM stage. CGA in Barrett correlated with pTNM stage and tumor grade of the adjacent carcinoma. Patients with CGA in Barrett had better survival than patients without CGA in Barrett, with 5-year survival percentages of 56% and 9%, respectively. In multivariate analysis, CGA in Barrett was an independent prognostic factor for survival after surgery. Therefore CGA in Barrett adjacent to GEJ adenocarcinoma might be helpful in the assessment of patient outcome. *Int J Surg Pathol 12(2):117–125, 2004*
Key words: Barrett's mucosa, adenocarcinomas of the gastroesophageal junction, neuroendocrine cells, chromogranin A, immunohistochemistry.

Neuroendocrine (NE) cells are defined as argentaffin and argyrophil cells that produce peptides or amines. They belong to the diffuse neuroendocrine system (DNES) and were previously known as amino-precursor-uptake-decarboxylation (APUD) cells. The neuroendocrine system of the normal gastrointestinal tract might regulate proliferation and growth of epithelial and mesenchymal cells and probably function in sensation of hunger during fasting and food-intake [1]. Chromogranin A (CGA), a specific matrix component of endocrine granules, participates in vesicle aggregation, granulogenesis, and hormone secretion and serves as a precursor for bioactive peptides (prohormone function) in endocrine and NE cells [2–4]. CGA is stored in secretory granules of NE cells and is regarded as a general endocrine marker [5–7]. The presence of NE cells in carcinomas of the gastrointestinal tract is well documented but their role remains speculative [8–21]. In a number of immunohistochemical studies a prognostic relevance of NE cells in adenocarcinomas, mostly colorectal adenocarcinomas, has been reported, however, several other studies failed to demonstrate a relation between NE differentiation and biological behavior of colorectal adenocarcinomas [9–11,14–22]. Reports on the prevalence and prognostic significance of NE cells in adenocarcinomas of the esophagus and its precursor lesion, the Barrett's mucosa, have been scarce. Hamilton et al. [23] did not find a significant correlation be-

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tween the presence of chromogranin A (CGA) immunoreactive tumor cells and survival in patients with esophageal adenocarcinoma. We analyzed the presence of NE cells in 208 resection specimens with adenocarcinoma of the gastroesophageal junction (GEJ), i.e., gastric cardia or distal esophagus, and premalignant Barrett's mucosa and correlated immunostaining with tumor characteristics and patient survival in order to evaluate the possible application of NE cells as prognostic marker.

Materials and Methods

Tissues and Patients

Two hundred and eight patients (176 men; 32 women) with GEJ adenocarcinoma who underwent transhiatal resection of the tumor with restoration of continuity of the gastrointestinal tract by a gastric tube with cervical anastomosis were included in this study. Patients were operated on between April 1987 and April 2002 at the Department of Surgery, Erasmus MC, Rotterdam. A standard dissection of the perigastric, left gastric, and celiac nodes was performed. Macroscopic tumor clearance was aimed at in all cases but no extended lymph node dissection was done. Seventeen patients received neoadjuvant chemotherapy and 2 patients received neoadjuvant chemoradiation. Patients' mean age was 63.6 years (range 39–84 years) at the time of diagnosis. All patients were followed up until April 2003 or until death if earlier. All 208 pathology records were reviewed. Barrett's mucosa was diagnosed by the GI-pathologist and was defined as the presence of intestinal-type epithelium with Goblet cells in the tubular esophagus [24].

In 73 patients Barrett's mucosa had been sampled before development of adenocarcinoma. Barrett's mucosa adjacent to tumor could be obtained in 56 of these 73 resection specimens, whereas in 17 resection specimens the Barrett's mucosa could not be detected. Barrett's mucosa showed no signs of dysplasia in 22, low-grade dysplasia in 22, and high-grade dysplasia in 12 resection specimens. A carcinoma was considered to arise from the distal esophagus when premalignant Barrett's mucosa was present and/or the epicenter of the mass was located in the tubular esophagus extending from the tracheal bifurcation to the gastroesophageal junction including the intraabdominal esophagus, according to the TNM classification (International Classification of Diseases for Oncology C15.5). The tumor was considered to be cardiac when the epicenter was immediately below the gastroesophageal junction, extending approximately 2 cm downward. The tumor was classified as a junction carcinoma when the epicenter was just at the GEJ, without predominance for distal esophagus or gastric cardia and no Barrett's mucosa was present. Tumors arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal esophagus were excluded. Of the adenocarcinomas in our patient group, 112 arose from the distal esophagus and 73 arose from the cardia. The exact location of 23 GEJ adenocarcinomas could not be specified as either distal esophagus or gastric cardia and these were classified as junction carcinomas.

Immunohistochemical Analyses

From formalin-fixed, paraffin-embedded tissue blocks of the primary tumor, 4-µm-thick sections were mounted on 3-aminopropyl-triethoxysilane (APES)-coated glass slides. For immunostaining a monoclonal antibody against CGA (Hybritech, San Diego, USA, at a dilution of 1:1,250) was used. Staining was carried out by a standard avidin biotin immunoperoxidase technique, using a commercially available kit (Labvision, Fremont, USA). Deparaffinized sections were treated with methanol containing 3% H_2O_2 for 20 minutes. After washing with phosphate-buffered saline (PBS), blocking serum was applied for 5 minutes. Then, primary CGA antibodies were allowed to react at room temperature for 1 hour. After washing in PBS, biotin-conjugated secondary antibody was applied for 10 minutes followed by peroxidase-marked streptavidin. After rinsing in PBS, peroxidase was visualized by diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.03% H_2O_2 solution for 10 minutes. The slides were counterstained with Mayer's Hematoxylin and dehydrated in alcohol before mounting. Expression of CGA was evaluated by high-power microscopic examination (400×) of the entire tissue section. As negative controls normal mouse immunoglobulins and normal rabbit serum were applied on duplicate sections. Positive controls using normal colonic epithelium were also run with each batch, in addition to using noninvolved normal gastric mucosa in the resection specimens, if present, as an internal positive control. Scoring of cytoplasmic CGA expression in adenocarcinomas was based on the percentage of positive cells: >20% of cells with cytoplasmic staining (2+), 1–20% of cells with cytoplasmic staining (1+), no cells staining (0).

Statistical Analysis

Correlations between CGA immunoreactivity and patient and tumor characteristics were assessed by t test and (a trend version of) χ^2 test. Survival

rates were calculated according to the Kaplan-Meier method and differences in survival were assessed by using the log rank test; p<0.05 was considered statistically significant. The Cox regression model was used to analyze the independent prognostic value of CGA expression after correction for possible confounding factors.

Results

CGA expression was detected in 102/208 (49%) adenocarcinomas of the GEJ (Table 1). The CGA-positive cells mostly presented diffusely scattered throughout the tissue or multifocally located in small nests, with just 8 tumors having >20% (2+) CGA-positive cells (Fig. 1). For statistical comparison we hence combined the groups with 1+ and 2+ staining. Negative controls did not show staining, and positive controls were positive. In 56 of the 208 resection specimens Barrett's mucosa adjacent to adenocarcinoma was detected. Positive staining for CGA was seen in 38/56 cases (68%). CGA immunoreactivity was absent in 18/38 (47%) tumors with CGA-positive Barrett's mucosa (Table 1). There was no correlation between CGA immunoreactivity in Barrett's mucosas and CGA immunoreactivity in adenocarcinomas (p=0.57). In the patients with Barrett's mucosa adjacent to tumor, there was no correlation between CGA immunoreactivity in the Barrett and presence or degree of dysplasia (p=0.97 and p=0.65, respectively).

CGA-positive staining in GEJ tumors correlated with a more favorable pTNM stage (p=0.04, Table 2). CGA-positive staining in Barrett's mucosas correlated with a more favorable pTNM stage and tumor grade (p=0.005 and p=0.024, respectively, Table 2). No difference in survival between patients with CGA-positive and CGA-negative adenocarcinomas was found (p=0.69, Fig. 2). Five-year survival percentages were 30% and 28%, respectively. However, patients with CGA-positive cells in Barrett's mucosa adjacent to the tumor had a better survival than patients without CGA-positive cells in Barrett's mucosa (p=0.0015, Fig. 3). Five-year survival percentages were 56% and 9% for patients with and without CGA expression in Barrett, respectively. Univariate analysis to identify prognostic variables in the total group showed pTNM stage, tumor grade, and radicality of resection to be prognostic factors for survival (p=<0.001, p=0.012, and p=<0.001, respectively). In multivariate Cox regression analysis only pTNM-stage and radicality of resection turned out to be independent prognostic factors for survival in the total patient group (p=0.003 and p=0.006, Table 3A). However, in the group of patients with Barrett's mucosa, univariate analysis showed age, radicality of resection, and CGA immunoreactivity in the Barrett's mucosa to be prognostic for survival (p=0.035, p=0.008, and p=0.003, respectively, Table 3B), which was substantiated by multivariate analysis (p=0.03, p=0.037, and p=0.003, respectively, Table 3B).

Discussion

Our study, which showed that the presence of NE cells in GEJ tumors did not correlate with 5-year survival rate, is in concordance with the results reported by Hamilton et al. [23]. They investigated the expression of CGA in 52 patients with adenocarcinomas of the esophagus and did not find a correlation with survival [23]. We also confirm their findings of CGA-positive Barrett's mucosas with adjacent CGA-negative tumors (namely, in 38% of their tumors and in 47% of our tumors). Obviously neuroendocrine differentiation commonly disappears in invasive adenocarcinomas. We likewise observed NE cells more often in Barrett's mucosa without dysplasia or with low-grade dysplasia than in high-grade dysplastic Barrett's mucosas, although this difference lacked statistical significance. Hamilton et al. found expression of CGA in 62% (21/34)

Table 1. CGA Expression in Adenocarcinomas of the GEJ and Barrett's Mucosa

		Barrett's Mucosa		
	Non-Barrett	CGA Positive	CGA Negative	Total
Adenocarcinomas				
CGA positive	74	20	8	102
CGA negative	78	18	10	106
Total	152	38	18	208

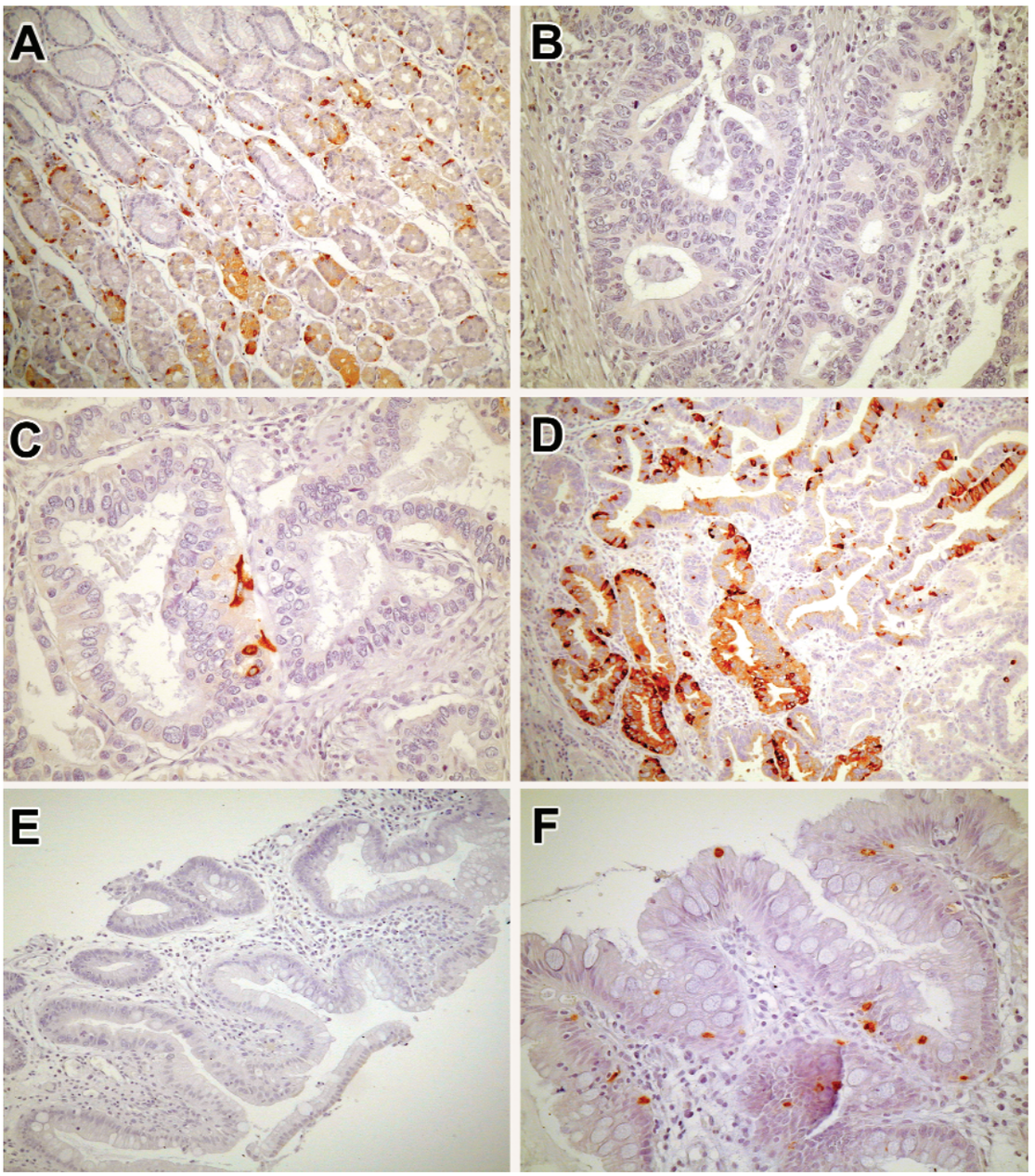

Fig. 1. **A.** CGA immunoreactivity in normal gastric epithelium. **B.** CGA-negative adenocarcinoma. **C.** Scatters of individual tumor cells show CGA staining (1+) within a well-differentiated adenocarcinoma. **D.** Adenocarcinoma shows apparent cytoplasmic CGA staining in >20% (2+) of tumor cells. **E.** CGA-negative Barrett's epithelium adjacent to adenocarcinoma. **F.** CGA-positive cells in Barrett's epithelium most prominently located in the basal layer of the epithelium.

of the Barrett's mucosas, as compared to our finding of 68% (38/56). In our study, patients with CGA-positive Barrett's mucosa had a better survival rate than patients with CGA-negative Barrett's mucosa. Our study differs from the study of Hamilton et al. in several ways. The monoclonal CGA antibody we used differs from the antibody used by Hamilton et al. Our study encompasses 208 patients versus 52 patients in the study of Hamilton et al. Finally, their population contained 37 of 52 patients who underwent preoperative therapy, compared to 19 of our 208 patients, and this might influence CGA stain-

Table 2. Clinicopathological Characteristics of 208 Patients with GEJ Adenocarcinomas and Barrett's Mucosa Adjacent to Tumor (If Present, 56 of 208 Patients)

	Chromogranin A in Tumor				Chromogranin A in Barrett's Mucosa			
Variable	Number of Tumors Positive (%)	Number of Tumors Negative (%)	Total Number of Tumors (%)	p Value*	Number of Barrett's Positive (%)	Number of Barrett's Negative (%)	Total Number of Barrett's (%)	p Value*
Gender								
Male	85 (83)	91 (86)	176 (85)		30 (79)	17 (94)	47 (84)	
Female	17 (17)	15 (14)	32 (15)	0.76	8 (21)	1 (6)	9 (16)	0.25
Mean age, years ± SD	63.6 ± 10.1	63.6 ± 9.2	63.6 ± 9.7	0.94†	64.8 ± 10.0	64.2 ± 10.9	64.6 ± 10.2	0.99†
pTNM-stage								
I	11 (11)	5 (5)	16 (8)		10 (26)	—	10 (18)	
II	34 (33)	33 (31)	67 (32)		16 (42)	4 (22)	20 (36)	
III	48 (47)	50 (47)	98 (47)		7 (18)	11 (61)	18 (32)	
IV	9 (9)	18 (17)	27 (13)	0.04	5 (13)	3 (17)	8 (14)	0.005
Tumor grade								
Well	5 (5)	4 (4)	9 (4)		4 (11)	—	4 (7)	
Moderate	47 (46)	41 (39)	88 (42)		18 (47)	5 (28)	23 (41)	
Poor	50 (49)	61 (58)	111 (53)	0.23	16 (42)	13 (72)	29 (52)	0.024
Radicality								
R0	71 (70)	76 (72)	147 (71)		29 (76)	11 (61)	40 (71)	
R1, R2	31 (30)	30 (28)	61 (29)	0.86	9 (24)	7 (39)	16 (29)	0.39
Tumor location								
Esophagus	58 (57)	54 (51)	112 (54)		38	18	56	
GEJ	8 (8)	15 (14)	23 (11)		—	—	—	
Cardia	36 (35)	37 (35)	73 (35)	0.67	—	—	—	—
Dysplasia								
Absent					15 (40)	7 (39)	22 (39)	
Low grade					16 (42)	6 (33)	22 (39)	
High grade					7 (18)	5 (28)	12 (21)	0.65

*P value by (a trend version of) χ^2 test; †P value by t test.

ing. However, when the 19 patients who received preoperative therapy were left out from the analyses, our results did not change. Moreover, Shia et al. [25] suggested that the increased endocrine differentiation shown in rectal adenocarcinomas treated by chemo(radio)therapy could be related to therapy-induced cytotoxity, which is in contrast to the lower percentage of CGA-positive patients, although there was a higher percentage of pretreated patients in the study of Hamilton et al. as compared to our study. Since adenocarcinomas of the distal esophagus and the gastric cardia are regarded as one clinical entity by some authors [26,27], we investigated the expression of NE markers in adenocarcinomas of the GEJ, whereas in the study of Hamilton et al., only esophageal adenocarcinomas were included. Because the prevalence of NE differentiation in our series was about the same in esophageal and cardia adenocarcinomas, the different results cannot be ascribed to the fact that in our study cardia adenocarcinomas were included.

Several studies concerning colorectal carcinomas showed that the presence of CGA-positive cells does not influence prognosis [14–19], whereas other studies indicate that CGA expression in tumor cells might distinguish a subgroup of colorectal carcinomas with poorer prognosis [10–12,21]. Swatek and Chibowski [13], using immunostaining for CGA, reported that endocrine cells were significantly more frequent in less advanced and better differentiated colorectal carcinomas. Two other studies showed a significantly better survival in patients with NE expression in pancreatic cancer and nonsmall cell lung cancer [28,29].

In the current study we demonstrated that CGA expression in Barrett's mucosa adjacent to the tumor

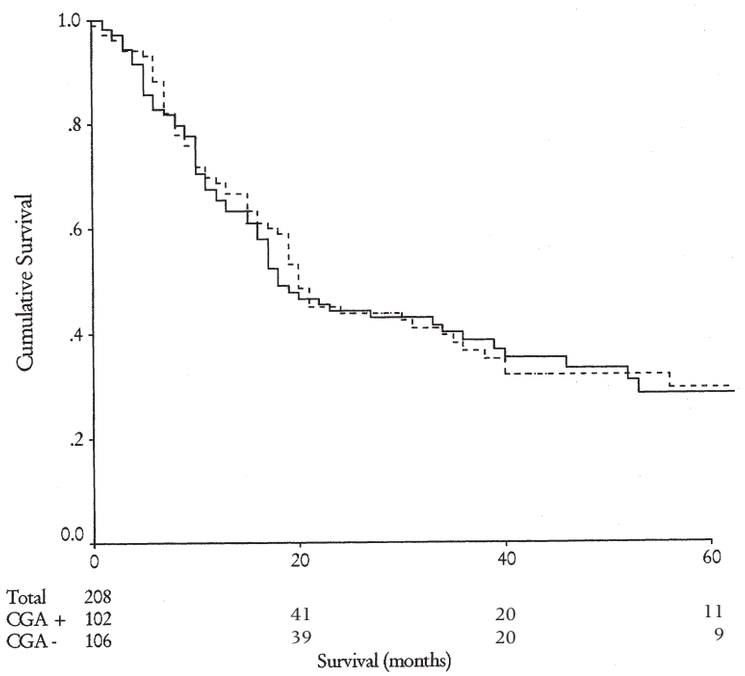

Fig. 2. Cumulative survival of patients with (n=102) and without (n=106) CGA immunoreactivity in adenocarcinomas of the GEJ (p=0.69). Broken line represents CGA-positive tumors, uninterrupted line represents CGA-negative tumors.

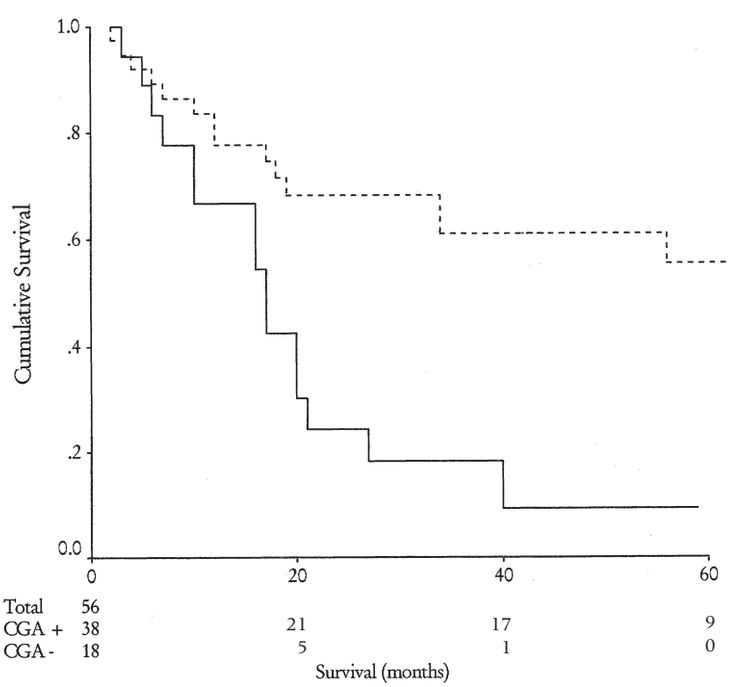

Fig. 3. Cumulative survival of patients with (n=38) and without (n=18) CGA immunoreactivity in Barrett's mucosa adjacent to adenocarcinoma (p=0.0015). Broken line represents CGA-positive Barrett's mucosas, uninterrupted line represents CGA-negative Barrett's mucosas.

Table 3. Results of the Cox Regression Analyses in (A) 208 GEJ Adenocarcinomas and in (B) 56 Barrett's Mucosas (Adjacent to Tumor), as Part of the Series of 208 GEJ Adenocarcinomas

Confounding Variable	Univariate RR* (CI†)	p Value	Multivariate-Adjusted RR‡ (CI)	p Value
A.				
Age	1.01 (1.0-1.03)	0.15	1.02 (1.0-1.04)	0.11
Gender (M, F)	0.94 (0.59-1.52)	0.81	0.75 (0.45-1.25)	0.27
pTNM—stage: I	—	<0.001	—	0.003
II	3.76 (1.16-12.22)	—	2.88 (0.88-9.48)	—
III	8.43 (2.63-27.04)	—	5.35 (1.63-17.56)	—
IV	8.08 (2.38-27.40)	—	5.22 (1.50-18.20)	—
Tumor grade: Well	—	0.012	—	0.118
Moderate	6.84 (0.94-49.51)	—	4.95 (0.68-36.04)	—
Poor	9.98 (1.38-71.98)	—	6.24 (0.85-45.68)	—
Radicality of resection (R0 vs R1, R2)	2.43 (1.69-3.49)	<0.001	1.73 (1.17-2.57)	0.006
CGA tumor (positive vs negative)	1.09 (0.77-1.55)	0.63	1.12 (0.78-1.61)	0.56
B.				
Age	1.04 (1.0-1.08)	0.035	1.04 (1.0-1.10)	0.030
pTNM-stage (I, II vs III, IV)	1.92 (0.91-4.03)	0.086	0.63 (0.22-1.83)	0.395
Tumor grade (well, moderate vs poor)	1.33 (0.65-2.71)	0.435	0.93 (0.35-2.45)	0.883
Radicality of resection (R0 vs R1, R2)	2.63 (1.28-5.40)	0.008	2.91 (1.07-7.95)	0.037
CGA Barrett mucosa (positive vs negative)	3.12 (1.48-6.58)	0.003	4.21 (1.61-11.0)	0.003

*Relative Risk; †95% Confidence Interval. ‡In multivariate analysis, correction was carried out for the confounding variables age, gender, pTNM stage, tumor grade, radicality of resection, and CGA immunoreactivity in the tumors (A), age, pTNM stage, tumor grade, radicality of resection, and CGA immunoreactivity in Barrett's mucosa (B), variables are mentioned in the column "Confounding Variables".

is an independent predictor of improved survival after surgery for GEJ adenocarcinomas. To our knowledge this is the first example that in the concurrent presence of a premalignant lesion and a carcinoma a characteristic of the premalignant lesion has prognostic significance. An explanation for this finding remains obscure.

The presence of NE cells in Barrett's epithelium has been described by several authors [30–34], suggesting that it arises from a multipotent gastrointestinal stem cell probably responsible for the risk of adenocarcinoma [31,34]. Smith and Haggitt [14] have provided 4 explanations for the presence of NE cells in noncarcinoid adenocarcinomas of the gastrointestinal tract: (1) entrapment of normal NE cells within the malignant tumor; (2) benign proliferation of NE cells within a malignant population of intestinal cells; (3) malignant transformation of 2 distinct stem cell lines (1 neuroendocrine and 1 endodermal); and (4) malignant transformation of 1 stem cell line capable of both endodermal and neuroendocrine differentiation. The last hypothesis has found support from several studies [20,35], including that by De Bruïne et al. [36], who demonstrated mucin and CGA expression in the colorectal cell line H716.

Since NE cells comprise an integral part of the intestinal epithelium, the presence of NE cells in Barrett's mucosa can be the mere result of the intestinal-type differentiation. However, additional factors in NE differentiation can be involved. Duodenogastroesophageal reflux is known to be a risk factor for the development of Barrett's mucosa. Reflux disease is often treated by acid-suppressive therapy. Sanduleanu et al. [37] found that serum CGA increases during profound gastric acid inhibition. Fur-

thermore, *Helicobacter pylori* infection was associated with higher serum CGA levels [37]. It should therefore be addressed that both long-term acid-suppressive therapy and *Helicobacter pylori* status might play a role in CGA expression in Barrett's mucosa and adenocarcinomas of the GEJ. Unfortunately, we were not able to obtain reliable information on previous acid-suppressive therapy or *Helicobacter pylori* status in our patient population.

Colombo et al. [38] investigated the effect of CGA on neoplastic growth and morphogenesis by use of mouse models. They found slower progression of mouse mammary adenocarcinoma after transfection of CGA cDNA and suggested that CGA may contribute to regulate tumor growth in a negative manner. Given the fact that distal esophageal adenocarcinomas often develop from a precursor lesion, i.e., Barrett's mucosa, being present for years already before tumor formation, we were able to investigate the significance of CGA on tumor growth in 56 patients with Barrett available adjacent to tumor [39]. CGA expression in these Barrett's mucosas indeed correlated with less advanced pTNM stage and tumor grade of adjacent tumors as compared to Barrett-related carcinomas without CGA in the precursor lesion. Furthermore, CGA expression in the Barrett's mucosa came forward as an independent predictor of survival in Cox regression analysis. Future experiments focusing on transfection of CGA cDNA in Barrett's metaplasia cell lines could possibly gain more insight in the role of CGA in GEJ adenocarcinoma development.

In summary, the current study demonstrates NE differentiation in Barrett's epithelium to be correlated with survival in patients with Barrett's-associated adenocarcinomas of the GEJ. CGA immunoreactivity in Barrett's mucosa adjacent to tumor is an independent prognostic factor for better survival after surgery. It appears from these data, obtained in a large patient group, that CGA expression in Barrett's mucosa might be helpful in the prognostic assessment of patient outcome.

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