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ARTICLE

Relation of Neuroendocrine Cells to Transforming Growth Factor-Alpha and Epidermal Growth Factor Receptor Expression in Gastric Adenocarcinomas: Prognostic Implications

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The presence of neuroendocrine (NE) cells in gastric adenocarcinoma (GCa) is well documented, however, their significance is controversial. There is no evidence in the literature concerning the possible effect of these cells on the expression of TGF-α and EGFR, which are believed to confer growth advantage to tumor cells. 101 partial or total gastrectomy specimens from patients operated for conventional gastric adenocarcinoma were included in the study. In each case immunohistochemistry was performed on sequential tissue sections for chromogranin A (ChrA), TGF-α and EGFR. Samples were graded based on the number of ChrA-positive cells (0-3). TGF-α and EGFR expressions were evaluated according to both the intensity (0-2) and quantification of the positively stained areas (0-3). Follow-up data was available in 54 patients. Twenty-seven patients died of disease, while 27 patients were alive with a follow-up of at least 15 months. ChrA expression was detected in 54.4% of the tumor specimens. TGF-α was stained positively in 42.6% and EGFR in 49.5% of the cases. NE cells in GCa was related to TGF-α ($p<0.0001$) and EGFR expression ($p<0.05$), and TGF-α/EGFR coexpression ($p<0.001$). Among histopathologic variables, the presence of NE cells was significantly related to grade, stage and lymph node status. Although the presence of NE cells had no effect on survival, the expression of EGFR ($p<0.0001$) and TGF-α ($p=0.002$) were related to survival. The results of our study suggest that the presence of NE cells may have an effect on the expression of TGF-α and EGFR in GCa, and the autocrine mechanism between TGF-α and EGFR plays an important role in the prognosis of gastric carcinoma. (Pathology Oncology Research Vol 13, No 3, 215–226)

Key words: neuroendocrine cells, TGF-α, EGFR, gastric carcinoma

Introduction

The presence of neuroendocrine (NE) cells in gastric adenocarcinoma (GCa) is well documented with an estimated frequency ranging from 3% to 50%, depending on the methods used and the numbers of specimens examined.[3,22,27,32,39,42] Neuroendocrine cells have also been found in adenocarcinomas from several organs including the colon, prostate, breast and pancreas.[8,12,30,42] Although the prevailing evidence in some of these tumors implicate an aggressive behavior,[8,12,42] the significance of these cells in gastric adenocarcinoma is not yet settled.[25,27,30,42]

Transforming growth factor-alpha (TGF-α), a 50-amino-acid peptide, is structurally homologous to epidermal growth factor (EGF).[7] As EGF, TGF-α interacts with epidermal growth factor receptor (EGFR) on the target cells.[7] EGFR is a 170-kD protein with an internal domain having tyrosine kinase activity, an external ligand binding domain, and a transmembrane domain.[4] The binding of EGF and TGF-α to EGFR activates a signal transduction pathway, resulting in increased DNA replication stimulating cell proliferation.[5]

Coexpression of TGF-α and EGFR in carcinomas is believed to confer growth advantage to tumor cells by

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autocrine and paracrine mechanisms.[15,36,43,44] With regard to tumors of the gastrointestinal tract, overexpression of TGF-α and/or EGFR was found to correlate with the extent of invasion, staging, and prognosis.[10,11,29,43] In neuroendocrine tumors of the gastrointestinal tract, there is some controversy in the literature about the expression of TGF-α and EGFR.[2,21,28,34]

There is no evidence in the literature concerning the possible effect of the presence of NE cells in conventional adenocarcinomas including GCa on the expression of TGF-α and EGFR.

The aim of this study was to elucidate whether there is an immunohistochemical evidence of an autocrine mechanism involving the TGF-α/EGFR system in the growth of GCa and if their expression is affected by the presence of NE cells. Furthermore, in order to determine the prognostic role of NE cells in gastric carcinomas, we analyzed their correlation with the widely accepted prognostic factors and survival.

Materials and Methods

All partial/total gastrectomy specimens from patients operated for conventional gastric adenocarcinoma in our hospital from April 1989 to April 2004 were retrieved from the archives of the pathology department. Tissues were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin. One hundred-five cases with sufficient clinical (age, sex, type of operation), gross (localization, size, macroscopic type, number of lymph nodes dissected) and microscopic (depth of invasion, number of metastatic lymph nodes, surgical margin) data and evaluable paraffin blocks were selected for further analysis. Hematoxylin-eosin-stained sections of 105 cases were reexamined. In each case all paraffin blocks containing adenocarcinoma foci were selected for chromogranin A (ChrA) immunohistochemistry. From these series of carcinomas, ChrA has previously been studied in 42 cases.[9] To eliminate normal mucosal cells entrapped by cancer, ChrA-immunoreactive cells in the neoplasm infiltrating beyond the muscularis mucosa were accepted as an integral tumor component. Thus, two cases of mucosal cancer and two cases with NE cells comprising more than 30% of the cancer cells were excluded from the study. In 101 cases, further IHC reactions for TGF-α and EGFR were performed in sequential sections of a representative block selected based on the presence of NE cells.

Patient characteristics

Among 101 patients, 63 (62.4%) were male and 38 (37.6%) were female. The mean age was 57.9 years with a range of 34-84 years. Fifty-two patients were older then 60 years of age. None of the patients have received chemotherapy or radiotherapy. Follow-up continued until death or for more than a year for surviving patients. The cases with positive surgical margins were excluded from the analysis of survival data. When death from recurrent gastric carcinoma was specified, the case was included as a tumor-related death. Patients who died of complications within 30 days after surgery were excluded from cases of tumor-related death. Follow-up data was available in the case of 54 patients. Twenty-seven patients were alive, while 27 died of disease with a follow-up of 15-101 (mean=41.4) and 1-131 (mean=17.4) months, respectively.

Immunohistochemistry

IHC was performed on sequential tissue sections, 4 mm in thickness, prepared from formalin-fixed, paraffin-embedded tissue blocks. All immunohistochemical stainings were performed by the streptavidin-biotin peroxidase technique using diaminobenzidine (DAB) as the chromogen, and run in parallel with known positive and negative controls. Briefly, the sections were dewaxed with xylene, rinsed in a graded series of ethanol, and rehydrated in water before blocking endogenous peroxidase activity with 3% H_2O_2 for 10 minutes. Antigen retrieval was achieved by heating the sections in 0.01 M citrate buffer, pH 6.0, at 750 W for about 3 min and then for 15 min at 160 W using a commercial microwave oven. The sections were incubated with normal serum for 30 min at room temperature. Then, the primary antibodies, monoclonal anti-human chromogranin A (AM126-5M, Biogenex), monoclonal antibody to TGF-α (GF10, Oncogene), and polyclonal antibody to intracellular domain of EGFR (AR335-5R, Biogenex) were applied according to manufacturers' instructions. Thereafter, biotinylated secondary antibodies, streptavidin and peroxidase (AP500-5M, Biogenex) were applied for 30 min each at room temperature. Positive controls included breast carcinoma for TGF-α and EGFR, and known carcinoid tumor for ChrA. Negative controls were performed by substituting normal serum for the primary antibody.

In addition, peritumoral gastric mucosa of 68 cases was examined for endocrine cell hyperplasia using ChrA IHC. In the antral mucosa of 47 cases, immunohistochemistry for gastrin (Ab-1, RB-1459-AO, Neomarkers) was performed according to the manufacturer's instructions.

Evaluation of immunohistochemistry

Based on the number of ChrA-positive cells, all samples were graded as follows: negative – no NE cells, (1+) – NE cells in <1%, (2+) – NE cells in 1-10%, and (3+) – NE cells in >10% of the tumor cells. Evaluation of the IHC staining for TGF-α and EGFR was done according to both

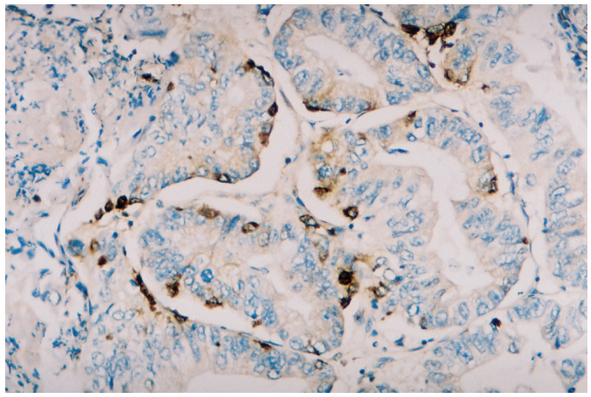

Figure 1. *Neuroendocrine cells intersperse among glandular cells resting on the basement membrane in intestinal type gastric adenocarcinoma (ChrA IHC, x200)*

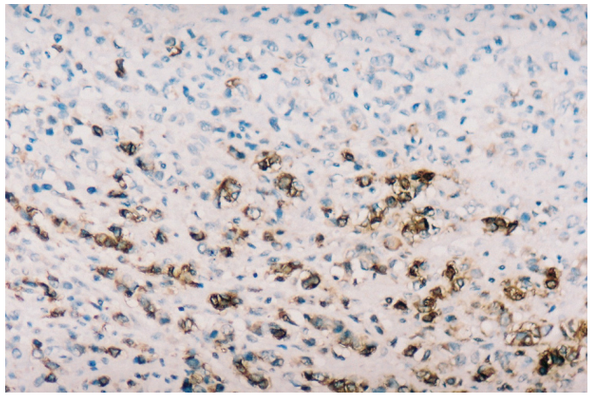

Figure 2. *Neoplastic cells expressing 3 (+) ChrA positivity in diffuse type gastric adenocarcinoma (ChrA IHC, x200)*

the intensity (0-2) and quantification of the positively stained areas (0-3). When a total score (sum of the intensity and quantification measurements) was 4 or greater, the expression was considered as high for either TGF-α or EGFR.[13] According to the results of TGF-α and EGFR staining, the carcinomas were scored as 0 if neither TGF-α and EGFR, as 1 if either TGF-α or EGFR, and as 2 if both TGF-α and EGFR were scored 4 or greater.[19]

In the antral mucosa of 47 cases, G cell hyperplasia was evaluated based on the criteria of Lechano et al.[23] and the cells with gastrin expression were counted under the microscope in 0.5 mm^2 area with the use of an oculometer.

Statistical analysis

Chi-square test was used in order to disclose the relation of ChrA staining with TGF-α and EGFR expression and TGF-α/EGFR coexpression. Differences in positivity according to clinicopathologic characteristics and immunoexpressions of each marker were also compared with chi-square test. The differences in gastrin expression between groups were compared using Student's t test. The cumulative survival rates were calculated by the Kaplan-Meier method and the differences among them were analyzed by the long-rank test.

Results

Histopathologic characteristics

Sixty-five subtotal and 36 total gastrectomy specimens comprised the study. Location of the tumors was as follows: 13 in cardia, 35 in corpus, and 53 (52.5%) in antrum. The main bulk of the tumor was in the lesser curvature in 66 (65.3%) cases. According to Borrmann's classification, the tumors were polypoid in 8, fungating in 28, ulcerated in 57, and infiltrative in 8 cases. The size of the tumor was smaller than 5 cm in 44 cases.

Histologic evaluation using Lauren's classification revealed 61(60.4%) intestinal and 40 (39.6%) diffuse type adenocarcinomas. When a two-tier system based on WHO classification[1] was used, 47 were low-grade (papillary, tubular, well differentiated medullary) and 54 were high-grade (scirrhous, signet ring cell, poorly differentiated medullary) tumors. There was lymphatic, vascular and perineural invasion in 72 (71.3%), 53 (52.5%), and 49 (48.5%), respectively. According to TNM classification,[35] 6 (5.9%) T1, 41 (40.6%) T2, 49 (48.5%) T3, and 5 (5%) T4 tumors were included in the study. Regional lymph node involvement was present in 65.3% of the cases (N0=35, N1=35, N2=25, N3=6). Stage grouping was as follows: stage I=24, stage II=26, stage III=27, stage IV=24. In 14 (13.9) of cases there was tumor at the surgical margins.

Immunoexpression of ChrA, TGF-α and EGFR in gastric carcinomas

ChrA expression was found in 55 (54.4%) of the tumor specimens, with a positivity of 1 in 22, 2 in 19 and 3 in 14 cases (*Figs. 1 and 2*). TGF-α and EGFR showed high expression in 43 (42.6%) and 50 (49.5%) of the tumor specimens, respectively. Synchronous expression of TGF-α and EGFR (score 2) was observed in 30 (29.7%) of the tumors, while 38 (37.6%) belonged to score 0, and 33 (32.7%) to score 1.

Table 1 shows the relationship between the immunoexpression of ChrA with the TGF-α and EGFR status. For statistical analysis, tumors that scored 2 and 3 positive NE cells were grouped together. The presence of NE cells in GCa was related to TGF-α ($p<0.0001$) and EGFR expression ($p=0.012$), and with TGF-α/EGFR coexpression ($p<0.0001$) (*Figs. 3 and 4*). When the cases were grouped according to Lauren's classification, the presence of NE

Table 1. **Relationships between the immunoexpression of ChrA with that of TGF-α and EGFR**

ChrA expression		*TGF-α expression*		*EGFR expression*		*TGF-α/EGFR coexpression score*		
ChrA expression		*low*	*high*	*low*	*high*	*0*	*1*	*2*
0	(n = 46)	38	8	30	16	28	11	7
1+	(n = 22)	13	9	11	11	8	9	5
2+/3+	(n = 33)	7	26	10	23	2	13	18
total	*(n = 101)*	*58*	*43*	*51*	*50*	*38*	*33*	*30*
p value		<0.0001		0.012		<0.0001		

cells correlated with TGF-α expression and TGF-α/EGFR coexpression in both intestinal (p<0.001 for both TGF-α and TGF-α/EGFR) and diffuse (p=0.006 for TGF-α and p=0.004 for TGF-α/EGFR) type GCa. Correlation of NE cells with EGFR reached statistical significance only in intestinal (p=0.006) but not in diffuse (p=0.224) type GCa.

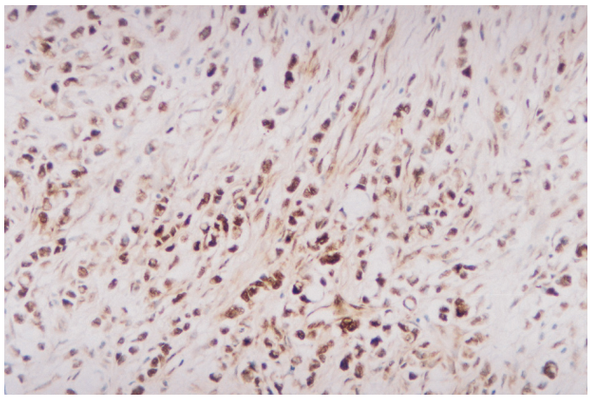

Figure 3. *TGF-α immunoexpression in NE cell 3 (+) diffuse type gastric adenocarcinoma (x200)*

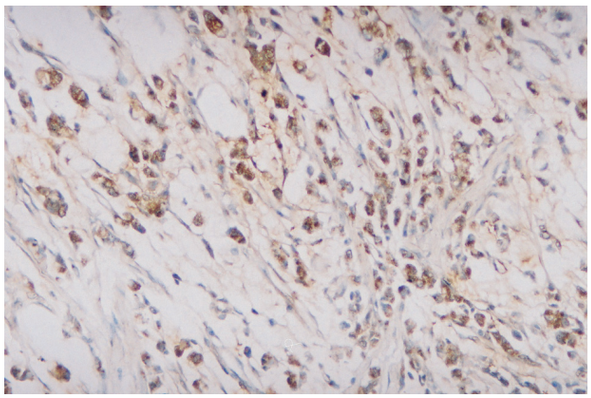

Figure 4. *EGFR immunoexpression in NE cell 3 (+) diffuse type gastric adenocarcinoma (x200)*

Relationship between the patient and tumor characteristics with the immunoexpression of ChrA, TGF-α and EGFR

When the cases were categorized as ChrA (0), (1+), (2+) and (3+), ChrA expression was significantly related only to lymph node status (*Table 2*). However, when the statistical analysis was performed after grouping GCa cases as NE (-) and NE (+), there was significant difference between groups in relation to stage (p=0.022), grade (p=0.015), and lymph node status (p=0.022) (*Table 2*). The percentage of cases expressing ChrA was higher in diffuse type GCa (67.5% vs. 45.9%, p=0.054). Although tumors with antral location seemed more likely to be NE (+) GCa than those of other locations, no statistical significance could be discerned.

TGF-α expression was significantly related both to the lymph node status and perineural invasion (*Table 3*). EGFR expression was more prominent in tumors with lymph node positivity and those with perineural invasion, but there were no statistically significant difference. TGF-α/EGFR coexpression was related to the stage and lymph node status of GCa. We found an association of borderline significance between TGF-α/EGFR coexpression and perineural invasion (p=0.053). Although the expression of both TGF-α and EGFR was more common in stage III and IV cases than in stage I and II ones, no significance could be achieved. EGFR correlated only with antral location (p=0.015) (*Table 3*).

Presence of endocrine cell hyperplasia and gastrin expression in peritumoral mucosa - relationship with the immunoexpression of ChrA, TGF-α and EGFR of the tumor

Endocrine cell hyperplasia in the peritumoral mucosa could be evaluated in 68 cases. Diffuse and linear hyperplasias were detected in 25 and 17 cases, respectively. In 13 cases there was micronodular hyperplasia. No relation could be determined between either the presence or the

Table 2. **Relationship of ChrA positivity with patient and tumor characteristics**

Patient and tumor characteristics	*All GCa (n=101) no. (%)*	*ChrA immunoexpression*				*p value for NE (-) vs. (+)*
		NE (-)	*NE (+)*			
		0 no. (%)	*1+ no. (%)*	*2+ no. (%)*	*3+ no. (%)*	
Age						
<60 years	49 (48.5)	20 (43.5)	11 (50)	10 (52.6)	8 (57.1)	NS
≥60 years	52 (51.5)	26 (56.5)	11 (50)	9 (47.4)	6 (42.9)	
Sex						
male	63 (62.4)	29 (63.0)	12 (54.5)	13 (68.4)	9 (64.3)	NS
female	38 (37.6)	17 (37.0)	10 (45.5)	6 (31.6)	5 (35.7)	
Location						
cardia	13 (12.9)	9 (19.6)	3 (13.6)	1 (5.3)	0 (0)	NS
corpus	35 (34.7)	17 (37.0)	7 (31.8)	5 (26.3)	6 (42.9)	
antrum	53 (52.5)	20 (43.5)	12 (54.5)	13 (68.4)	8 (57.1)	
Gross appearance						
polypoid	8 (7.9)	5 (10.9)	2 (9.1)	1 (5.3)	0 (0)	NS
fungating	28 (27.7)	14 (30.4)	5 (22.7)	5 (26.3)	4 (28.6)	
ulceroinfiltrative	65 (64.4)	27 (58.7)	15 (68.2)	13 (68.4)	10 (71.4)	
Size						
<5 cm	44 (43.6)	19 (41.3)	10 (45.5)	10 (52.6)	5 (35.7)	NS
≥5 cm	57 (56.4)	27 (58.7)	12 (54.5)	9 (47.4)	9 (64.3)	
Grade						
low-grade	47 (46.5)	28 (60.9)	7 (31.8)	8 (42.1)	4 (28.6)	0.015
high-grade	54 (53.5)	18 (39.1)	15 (68.2)	11 (57.9)	10 (71.4)	
Lauren's classification						
intestinal type	61 (60.4)	33 (71.7)	11 (50)	10 (52.6)	7 (50.0)	0.054
diffuse type	40 (39.6)	13 (28.3)	11 (50)	9 (47.4)	7 (50.0)	
Stage						
I+II	50 (45.5)	29 (63.0)	9 (40.9)	6 (31.6)	6 (42.9)	0.022
III+IV	51 (50.5)	17 (37.0)	13 (59.1)	13 (68.4)	8 (57.1)	
Depth of invasion						
T1 + T2	47 (46.5)	24 (52.2)	10 (45.5)	6 (31.6)	7 (50.0)	NS
T3 + T4	54 (53.5)	22 (47.8)	12 (54.5)	13 (68.4)	7 (50.0)	
Lymph node status[¶]						
N0	35 (34.7)	23 (50.0)	6 (27.3)	5 (26.3)	1 (7.1)	0.008
N1	35 (34.7)	14 (30.4)	8 (36.4)	7 (36.8)	6 (42.9)	
N2+N3	31 (30.6)	9 (19.5)	8 (36.4)	7 (36.8)	7 (50.0)	
Lymphatic invasion						
negative	29 (28.7)	17 (37.0)	6 (27.3)	5 (26.3)	1 (7.1)	NS
positive	72 (71.3	29 (63.0)	16 (72.7)	14 (73.7)	13 (92.9)	
Vascular invasion						
negative	48 (47.5)	22 (47.8)	11 (50)	8 (42.1)	7 (50.0)	NS
positive	53 (52.5)	24 (52.2)	11 (50)	11 (57.9)	7 (50.0)	
Perineural invasion						
negative	52 (51.5)	27 (58.7)	12 (54.5)	9 (47.4)	4 (28.6)	NS
positive	49 (48.5)	19 (41.3)	10 (45.5)	10 (52.6)	10 (71.4)	

[¶] ChrA expression was significantly related ($p<0.05$) to lymph node status when the cases were categorized as ChrA (0), (1+), (2+) and (3+). NS=not significant

Table 3. **Relationship of TGF-α and EGFR expression with patient and tumor characteristics**

Patient and tumor characteristics	*TGF-α expression*		*EGFR expression*		*TGF-α/EGFR coexpression*	
	low	*high*	*low*	*high*	*negative*	*positive*
Age						
<60	25 (43.1)	24 (55.8)	25 (49.0)	24 (48.0)	32 (45.0)	17 (56.7)
≥60	33 (56.9)	19 (44.2)	26 (51.0)	26 (52.0)	39 (55.0)	13 (43.3)
Sex						
male	35 (60.3)	28 (65.1)	31 (60.8)	32 (64.0)	44 (62.0)	19 (63.3)
female	23 (39.7)	15 (34.9)	20 (39.2)	18 (36.0)	27 (38.0)	11 (36.7)
Location ¶						
cardia	7 (12.1)	6 (14.0)	10 (19.6)	3 (6.0)	10 (14.1)	3 (10.0)
corpus	21 (36.2)	14 (32.5)	21 (41.2)	14 (28.0)	27 (38.0)	8 (26.7)
antrum	30 (57.7)	23 (53.5)	20 (39.2)	33 (66.0)	34 (47.9)	19 (63.3)
Gross appearance						
polypoid	7 (12.1)	1 (2.3)	5 (9.8)	3 (6.0)	8 (11.1)	0 (0)
fungating	16 (27.6)	12 (27.9)	13 (25.5)	15 (30.0)	19 (26.9)	9 (30.0)
ulceroinfiltrative	35 (60.3)	30 (69.8)	33 (64.7)	32 (64.0)	44 (62.0)	21 (70.0)
Size						
<5 cm	27 (46.6)	17 (39.5)	23 (45.1)	21 (42.0)	33 (46.5)	11 (36.7)
≥5 cm	31 (53.4)	26 (60.5)	28 (54.9)	29 (58.0)	38 (53.5)	19 (63.3)
Grade						
low-grade	31 (53.4)	16 (37.2)	25 (49.0)	22 (44.0)	35 (49.3)	12 (40.0)
high-grade	27 (46.6)	27 (62.8)	26 (51.0)	28 (56.0)	36 (50.7)	18 (60.0)
Lauren's classification						
intestinal type	37 (63.8)	24 (55.8)	31 (60.8)	30 (60.0)	43 (60.6)	18 (60.0)
diffuse type	21 (36.2)	19 (44.2)	20 (39.2)	20 (40.0)	28 (39.4)	12 (40.0)
Stage ‡		I+II	32 (55.2)	18 (41.9)	30 (59.8)	20 (40.0)
40 (56.4)	10 (33.3)					
III+IV	26 (44.8)	25 (58.1)	21 (41.2)	30 (60.0)	31 (43.6)	20 (66.7)
Depth of invasion						
T1+T2	27 (46.6)	20 (46.5)	27 (53.0)	20 (40.0)	34 (47.9)	13 (43.3)
T3+T4	31 (53.4)	23 (53.5)	24 (47.0)	30 (60.0)	37 (52.1)	17 (56.7)
Lymph node status†, ‡						
N0	27 (46.6)	8 (18.6)	22 (43.1)	13 (26.0)	31 (43.6)	4 (13.3)
N1	17 (29.3)	18 (41.9)	16 (31.4)	19 (38.0)	23 (32.5)	12 (40.0)
N2+N3	14 (24.1)	17 (39.5)	13 (25.5)	18 (36.0)	17 (23.9)	14 (46.7)
Lymphatic invasion						
negative	21 (36.2)	8 (18.6)	17 (33.3)	12 (24.0)	23 (32.4)	6 (20.0)
positive	37 (63.8)	35 (81.4)	34 (66.7)	38 (76.0)	48 (67.6)	24 (80.0)
Vascular invasion						
negative	30 (51.7)	18 (41.9)	27 (52.9)	21 (42.0)	36 (50.7)	12 (40.0)
positive	28 (48.3)	25 (58.1)	24 (47.1)	29 (58.0)	35 (49.3)	18 (60.0)
Perineural invasion †						
negative	36 (62.1)	16 (37.2)	30 (58.8)	22 (44.0)	41 (57.8)	11 (36.7)
positive	22 (37.9)	27 (62.8)	21 (41.2)	28 (56.0)	30 (42.2)	19 (63.3)

†TGF-α expression was related to lymph node involvement (p= 0.014) and perineural invasion (p= 0.016); ¶EGFR expression was related to location of the tumor (0.015);

‡TGF-α/EGFR coexpression was related to lymph node involvement (0.015) and stage (p= 0.049).

Table 4. **Presence of endocrine cell hyperplasia and gastrin expression in peritumoral mucosa - relationship with the immunoexpression of ChrA, TGF-α and EGFR of the tumor**

	ChrA		*TGF-α expression*		*EGFR expression*	
	negative	*positive*	*low*	*high*	*low*	*high*
Endocrine cell hyperplasia						
absent	6 (18.2)	7 (20.0)	7 (17.9)	6 (20.7)	6 (20.0)	7 (18.4)
diffuse	12 (36.4)	13 (37.1)	13 (33.3)	12 (41.4)	12 (40.0)	13 (34.2)
linear	12 (36.4)	5 (14.3)	12 (30.8)	5 (17.2)	5 (16.7)	12 (31.6)
micronodular	3 (9.1)	10 (28.6)	7 (17.9)	6 (20.7)	7 (23.3)	6 (15.8)
p value	NS	NS	NS			
G cell hyperplasia						
absent	10 (45.5)	11 (44.0)	13 (48.1)	8 (40.0)	7 (35.0)	14 (51.9)
present	12 (54.5)	14 (56.0)	14 (51.9)	12 (60.0)	13 (65.0)	13 (48.1)
p value	NS	NS	NS			
Mean G cell value	216.04	190.56	190.93	218.15	218.75	190.48
p value	NS	NS	NS			

NS=not significant

type of endocrine cell hyperplasia and the expressions of ChrA, TGF-α and EGFR (*Table 4*).

Gastrin IHC was performed in 47 cases. G cell hyperplasia was detected in 26 (46.7%) of the cases based on the criteria of Lechano et al.[23] (*Fig. 5*). No statistically significant difference could be found either in the presence or the mean number of G cells between NE (-) and NE (+) cases (*Table 4*).

Survival of the patients and the immunoexpression of ChrA, TGF-α and EGFR

Survival data was available for 54 patients who could be followed for at least 15 months. During the follow-up period death occurred in 27 patients. The mean and median survival times for the whole group were 68.8 and 33 years, respectively. While the presence of NE cells had no effect on survival, the expressions of EGFR ($p<0.0001$) and TGF-α ($p=0.002$) were related to survival (*Fig. 6*). The mean survival times for cases with low and high TGF-α expressions were 96.05 and 33.96 months, respectively. Survival rates were 70.4% and 29.6% in cases with low and high TGF-α expression.

The mean survival times for low and high EGFR cases were 78.93 and 33.08 months, respectively. Survival rates of cases with low and high EGFR expression were 76% and 16.7%. Survival rates of NE (-) and (+) cases were 65.0% and 41.2%. Within the group of NE (-) GCa, all patients with high TGF-α (n=3) and EGFR (n=5) expression died of the disease (*Table 5*). Although high expression of both TGF-α ($p=0.009$) and EGFR ($p<0.0001$) had an effect on survival time in NE (-) GCa, in NE (+) GCa we found no relationship between TGF-α ($p=0.087$) and survival time, and an association of borderline significance in the case of EGFR ($p=0.053$) (*Fig. 7*).

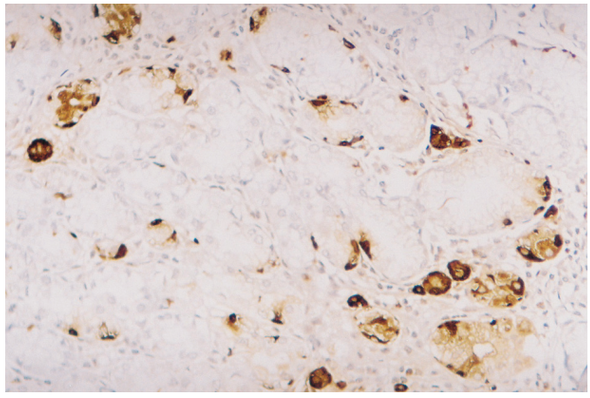

Figure 5. *G cell hyperplasia in peritumoral antral mucosa (gastrin IHC, x200)*

Discussion

Neuroendocrine cells are found in most epithelial surfaces of the body including gastric mucosa, and are known to have important regulatory functions.[24] Like many other cells, NE cells may undergo neoplastic transformation and are the origin of neuroendocrine tumors.[23] However, cells that show immunoreactivity for neuroendocrine markers have also been found in adenocarcinomas of various organs.[8,12,39,42]

There have been several comprehensive studies concerning NE cells in otherwise typical gastric adenocarci-

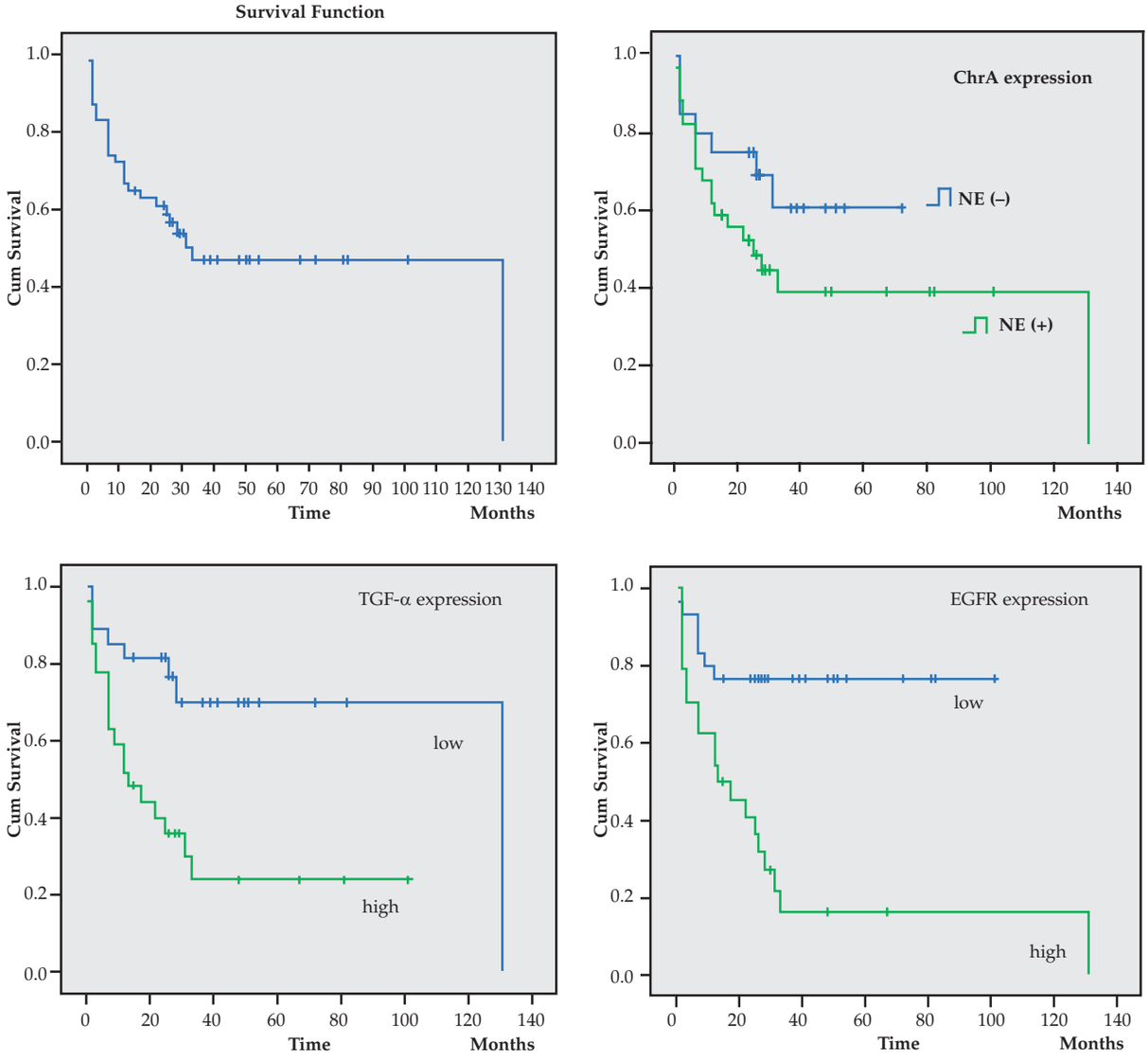

Figure 6. *Kaplan-Meier survival analysis. Correlation of cumulative survival rate and TGF-α, EGFR and ChrA expression. There were significant differences in survival between patients with TGF-α-positive and -negative tumors (p= 0.002) and between EGFR-positive and -negative tumors (p<0.0001). No significant difference could be found in survival between patients with ChrA-negative and -positive tumors.*

noma.[27,32,42] The fact that they form an integral part of the tumor and its metastases has long been established.[3,27] In agreement with the literature, ChrA expression was demonstrated in 55 (54.4%) of our primary tumors, and approximately in one third of their lymph node metastases. In our study, we preferred ChrA as a marker for NE cells because it is accepted as a specific matrix component of endocrine granules.[41]

The role of NE cells in conventional adenocarcinomas of the stomach is not yet settled. In the series of Yao et al., the presence of NE cells was significantly related to poor differentiation of GCa.[42] In the series of Ooi et al., NE cells were more frequent in stage III and IV tumors.[27] Our results were similar to these studies, concerning the grade of differentiation and the stage of the tumor, respectively. The presence of NE cells in our series was also related to lymph node involvement. In their genetic analysis of 8 mixed glandular-neuroendocrine gastric carcinomas, Kim et al. found that most mixed GCa were likely to evolve from a glandular precursor to a genetically heterogeneous adenocarcinoma and then to neuroendocrine differentiation.[20] Presumably, noticeable frequency of NE cells in poorly differentiated and high-stage tumors reflects the morphologic expression of the multi-step progression of

Table 5. **Relationship of TGF-α and EGFR expression with death in NE (-) and NE (+) GCa**

		TGF-α expression		*EGFR expression*	
		low	*high*	*low*	*high*
NE (-) GCa (n=20)	n (%)				
death (-)	13 (65.0)	13 (76.5)	0 (0)	13 (86.7)	0 (0)
death (+)	7 (35.0)	4 (23.5)	3 (100)	2 (13.3)	5 (100)
p value	NS	0.031	0.001		
NE (+) GCa (n=34)					
death (-)	14 (41.2)	6 (60.0)	8 (33.3)	10 (66.7)	4 (21.1)
death (+)	20 (58.8)	4 (40.0)	16 (66.7)	5 (33.3)	15 (78.9)
p value	NS	NS	0.013		

NS=not significant

GCa. Some authors have reported NE cells more commonly in diffuse type carcinomas (according to the classification of Lauren)[3,32,39] and suggested that most of the diffuse type carcinomas must be reclassified as neuroendocrine carcinomas.[32,39] However, as in our series, others have found no definite correlation between histologic type and the prevalence of endocrine cells.[25,30]

Gastrin is the main regulator of enterochromaffin-like-cell (ECL) function and growth,[40] inducing ECL cell proliferation in physiological concentrations. However, prolonged hypergastrinemia, leading to marked ECL cell hyperplasia, results in a normalization or even a reduction of ECL cells, indicating that these cells produce a factor reducing their own proliferation.[38] In our study, neither the peritumoral G cells nor endocrine hyperplasia in the peritumoral mucosa showed any effect on the presence of NE cells in GCa. In the study of Bonar et al., endocrine cell hyperplasia in adjacent gastric mucosa was detected in 9 of 26 NE (+) cases.[3] We found endocrine cell hyperplasia in the surrounding mucosa in 80.0% of NE (+)and 81.8% of NE (-) cases. The difference in the rates of endocrine cell hyperplasia between these two studies can be explained by the different markers used for the detection of endocrine cells. Although Qvingstad et al.[33] suggested that hypergastrinemia induces the presence of NE cells in GCa, we could not find any relationship between NE cells in the GCa and gastrin expression in the surrounding mucosa. Our findings suggest that NE cells in GCa are not under the control of gastrin. However, our results concerning endocrine cell hyperplasia and gastrin expression in the peritumoral mucosa clearly show that they can be involved in gastric carcinogenesis, as implicated by others.[40]

Endocrine cells in normal mucosa are considered to have a local or paracrine activity rather than exerting more general effects.[6] Recently, paracrine or autocrine modes of action of biologically active peptides are becoming a matter of interest in the field of growth factors released by tumor cells.[17] Besides basic fibroblast growth factor and glycoprotein hormones, TGF-α have been reported to be stored in ECL cells.[37] The regulation of the release of these substances has not been clarified.[40] Furthermore, it has been suggested that enhanced production of TGF-α and EGF receptors by tumor cells promote tumor cell growth by autocrine mechanisms.[31,36,44] In Nilsson et al.'s study, the expressions of TGF-α and EGFR were investigated in human neuroendocrine tumors, including midgut carcinoid tumors, pheochromocytomas and medullary thyroid carcinomas.[26] TGF-α expression was demonstrated in biopsies of all tumors examined (n=30), and EGFR in a majority of tumors by Northern analysis and/or immunocytochemistry. They concluded that several human neuroendocrine tumors express both TGF-α and EGFR in vivo and in vitro, suggesting that TGF-α may be the main regulator of tumor cell growth by autocrine mechanisms.[26] A strong expression of TGF-α at the protein level has also be shown for neuroendocrine tumors of the hindgut.[2] Contrary to these findings, in Krishnamurthy et al.'s study, comprised of 25 gastrointestinal carcinoids including 9 foregut tumors, TGF-α was found in 72% of the cases but none of them expressed the intracellular domain of EGFR. They concluded that TGF-α has no role in the growth and progression of GI carcinoids.[21] In neuroendocrine tumors including 39 foregut tumors, it has been shown that the activation of downstream signaling molecules such as Akt and ERK1/2 correlated with the histological score for activated EGFR.[34] Using immunohistochemistry, Western blotting and RT-PCR in 58 gastrointestinal carcinoid tumors and 48 pancreatic endocrine tumors (PET), Papouchado et al. found that EGFR and activated EGFR (P-EGFR) were expressed by both gastrointestinal carcinoids and PET in primary and metastatic tumors.[28]

Compared with neuroendocrine tumors, no investigation has been carried out on the relationship of neuroendocrine cells and their effect on growth factor/growth fac-

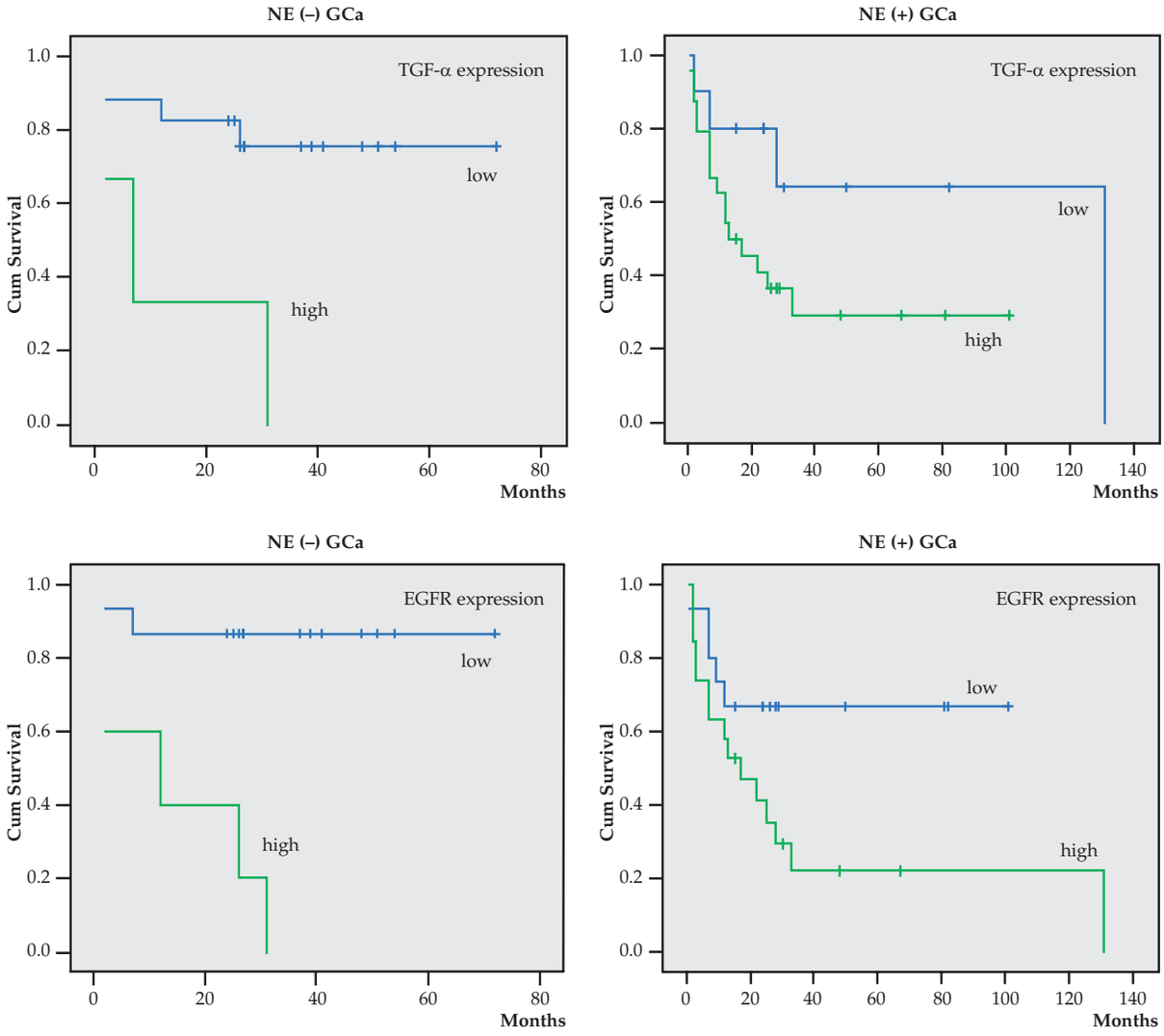

Figure 7. *Relationship of TGF-**a** / EGFR with survival in NE (-) and NE (+) GCa. High expression of both TGF-a (p=0.009) and EGFR (p<0.0001) was related to survival time in NE(-) GCa. In NE (+) GCa, survival time was not related to TGF-a (p=0.087) and there was borderline significance of EGFR (p=0.053).*

tor receptor status in conventional adenocarcinomas with NE cells. In our study we determined a strong correlation between ChrA expression within the tumor especially with that of TGF-α expression and also with EGFR status. Within NE (-) GCa, TGF-α and EGFR expression was associated with the prognosis, but within NE (+) GCa both TGF-α and EGFR had limited role. Furthermore, endocrine cell hyperplasia and gastrin expression in the peritumoral mucosa showed no relationship with the TGF-α and EGFR status of the tumor. These findings suggest that NE cells could have an effect in the regulation of TGF-α and EGFR autocrine mechanisms in GCa. As observed in our cases and others',[3] in intestinal type GCa NE cells intersperse among glandular cells resting on the basement membrane, similarly to that seen in normal gastric mucosa. This can be accepted as the morphological reflection of this regulatory function.

The survival data from this series indicates no significant difference between NE (-) and NE (+) GCa. Survival rate for NE (-) and NE (+) GCa was 65.0% and 41.2%, respectively. Bonar et al. also could not find any significant difference between their NE (+) and NE (-) cases.[3] In the study of Jiang et al,[18] survival data have been analyzed in 42 large cell neuroendocrine carcinomas (LCNEC) (NE phenotype in >50%) and 44 adenocarcinomas with neuroendocrine differentiation (ACNED) (NE phenotype in 1-50%). The prognosis of ACNEDs has been found significantly worse than that of conventional adenocarcinomas (p<0.0001). Furthermore, according to their survival analysis they suggested that some ACNEDs having >20%

positivity of NE markers might actually have been LCNECs.[18] In our study we excluded the cases having more than 30% ChrA positivity. This may explain the difference between these two studies. Besides, we may have missed some NE (+) cases in our study. As is shown by many studies, conventional immunohistochemical techniques could fail to detect specific antigens that are normally present in the tissue sections. When tyramide signal amplification technique was applied there was a considerable increase in the number of GCa containing ChrA immunoreactive cells.[32]

Epidermal growth factor receptor is expressed in many cancers and is associated with poor prognosis.[10,15,16,19,29,36] In concordance with the literature, our data revealed a prominent effect of EGFR on survival. HER-2/neu protein, a homologous protein related to the epidermal growth factor receptor was studied by semiquantitative standardized immunohistochemical staining, chromogenic in situ hybridization, and fluorescence in situ hybridization in 182 gastric cancer patients who underwent curative surgery.[29] Tumors with HER-2/neu amplification were associated with poor mean survival rates and it constituted an independent prognostic factor in gastric cancer patients.[29] In human insulinoma cells, pancreatic carcinoid cells and NE tumor cells of the gut, gefitinib induced a time- and dose-dependent growth inhibition by almost 100%.[14] Data of Hopfner et al. demonstrated that the inhibition of EGFR-TK by gefitinib induces growth inhibition, apoptosis and cell-cycle arrest in NE gastrointestinal tumor cells. Thus, EGFR-TK inhibition appears to be a promising novel approach for the treatment of NE tumor disease.[14]

The results of our study suggest that the autocrine mechanism between TGF-α and EGFR plays an important role in the prognosis of GCa, and NE cells might have an influence on the expression of both TGF-α and EGFR. Increased production of TGF-α and EGFR may serve both as markers for tumor progression and as targets for chemotherapy. These data provide a rationale for reconsidering the treatment options of gastric carcinoma especially with NE cells.

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