Overexpression of Epidermal Growth Factor and Hepatocyte Growth Factor Receptors in a Proportion of Gastrinomas Correlates with Aggressive Growth and Lower Curability

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ABSTRACT

**Purpose:** Growth factor receptor expression and activation, particularly for epidermal growth factor (EGF) and hepatocyte growth factor (HGF), in many endocrine and nonendocrine tumors is important in determining tumor recurrence, growth, and aggressiveness. Whether this is true of neuroendocrine tumors such as gastrinomas is unclear.

**Experimental Design:** To address this question, we analyzed the extent of EGFR and HGFR expression in gastrinomas from 38 patients with Zollinger-Ellison syndrome and correlated it with clinical and tumor characteristics. EGFR (n = 38) and HGFR (n = 22) mRNA levels were determined by competitive PCR, and immunohistochemistry was performed on a subset.

**Results:** In each of the gastrinomas studied, detectable levels of EGFR and HGFR mRNA were present. Low levels of EGFR protein expression were detected in 40% of gastrinomas and HGFR protein expression in 90%.

**Conclusions:** These above results indicate that EGFR and HGFR mRNA are universally expressed in gastrinomas. Furthermore, each is overexpressed in a minority (15–20%) of the gastrinomas, and the overexpression correlates with aggressive growth and lower curability.

INTRODUCTION

NETs (PETs and carcinoids) are generally slow-growing neoplasms; however, a subset, the proportion of which varies with different NET tumors, demonstrates an aggressive growth pattern with a markedly decreased survival (1–4). The most common malignant, symptomatic PET (2, 5) is gastrinoma, and in 25% of patients, the gastrinoma has an aggressive growth pattern with the development of liver metastases and a 10-year survival of 30% (6). In contrast, the 10-year survival rate is >95% in the 75% of patients in which the gastrinomas show indolent growth (6). Furthermore, NETs in different patients vary considerably in their surgical curability (1, 2, 4). For example, only 30–40% of patients with gastrinomas after gastrinoma resection remain disease free for the long term (7). The factors determining this variable growth pattern, curvature, and tumor aggressiveness with gastrinomas as well as other PETs are largely unknown (8). This has occurred because the molecular pathogenesis of NETs is largely unknown and appears to differ significantly from other nonendocrine malignant tumors in which the molecular pathogenesis has been well studied (i.e., colon cancer, breast cancer, and pancreatic cancer; Refs. 8–11).

Alterations of common oncogenes (ras, myc, and others) or alterations in common tumor suppressor genes (p53 and retinoblastoma gene), which are frequent in many common nonendocrine malignancies, are uncommon in typical PETs and carcinoid tumors (8–11). Alterations in the MEN1 tumor suppressor gene on chromosome 11q13 occur in 16–42% of sporadic gastrointestinal NETs (8, 12, 13), and inactivation of the p16INK4a/CDKN2A tumor suppressor gene on chromosome 9p21 has been reported in 17–92% of various PETs (14–16). However, the presence of p16INK4a/CDKN2A or MEN1 gene mutations in the subset of sporadic tumors has not been shown to correlate with the aggressiveness of the NET (12, 15).

Many studies on different nonendocrine and endocrine tumors show that the expression of various growth factor receptors including those for vascular endothelial growth factor, platelet-derived growth factor, EGF, insulin-like growth factor,
and HGF likely play an important role in the molecular pathogenesis of the tumor and the invasiveness and aggressiveness of the tumors (17, 18). The EGFR and HGF families of receptors and their ligands are likely particularly important in a number of neoplasms causing autocrine growth associated with increased invasiveness, and overexpression by the tumor is associated with decreased survival/poor prognosis (17–22). Little is known about the expression of EGFR or HGFR in NETs and whether the extent of expression correlates with aggressive behavior or advanced disease. Amplification of the HER-2/neu proto-oncogene gene, a member of the EGFR family of receptors, was reported in a small number of cases (23) of NETs (9); however, it was unclear whether this was associated with increased expression or correlated with tumor aggressiveness.

The purpose of the present study, therefore, was to determine whether EGFR or HGFR overexpression occurred in a well-studied group of patients with gastrinomas and to correlate the extent of expression with clinical and tumor characteristics, including primary gastrinoma size, location, tumor extent, and curability.

**MATERIALS AND METHODS**

**Patients and Tumors.** Thirty-eight patients who underwent exploratory laparotomy for Zollinger-Ellison syndrome at the NIH between 1990 and 1999 were included in this study. The study protocol was approved by the Clinical Research Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, and all patients gave informed consent. Preoperative measurement of BAO and MAO were performed as described previously (24). Preoperative serum gastrin levels were analyzed by RIA by Bioscience Laboratories (New York, NY) or Mayo Clinic Laboratories (Rochester, MN). The diagnosis of Zollinger-Ellison syndrome was established as reported previously (24). The duration of disease was defined by the clinical history from the time of diagnosis or from the time of disease onset (6). Detailed conventional imaging studies (computed tomography with oral and i.v. contrast, magnetic resonance imaging, ultrasound, and bone scan), selective abdominal angiography with secretin stimulation and hepatic vein gastrin sampling, and somatostatin receptor scintigraphy were performed as described previously (7, 25) to locate the primary tumor and evaluate the extent of disease. All patients underwent an exploratory laparotomy with an extensive intraoperative evaluation for attempted curative resection as described previously (7). The patients were then reassessed within 2 weeks of surgery and 3–6 months postoperatively to determine disease-free status and annually to monitor for progression of disease as described previously (7). Disease-free status was defined by normal fasting gastrin levels (<200 pg/ml), negative results on gastrin provocative testing with secretin (<200 pg/ml increase), and no evidence of tumor on any imaging study (7, 24). Patients were classified as to whether they were disease free immediately postoperatively as well as whether they were disease free at the last follow-up. In all patients, annual detailed imaging studies (computed tomography, magnetic resonance imaging, ultrasound, and somatostatin receptor scintigraphy) were performed, and if the results were unclear, selective angiography was performed. Functional studies (fasting gastrin and secretin test) and imaging studies provided the basis for assessment of tumor growth, recurrence, or progression (7). Consistent absence of imaged lesions or lack of increase in size or number of lesions over the follow-up period was defined as a tumor demonstrating no growth. An increase in size or number of lesions was defined as evidence of tumor growth as described previously (26). The development of liver metastases identified by imaging studies during follow-up served as the definition for the liver metastases group (26, 27). Liver metastases were confirmed by percutaneous liver biopsy. No patient received antitumor treatment (chemotherapy, α-IFN, or somatostatin analogues) before the surgical resection or biopsy; however, after biopsy, 7 patients received antitumor treatment for progressive metastatic disease (3, chemotherapy; 5, α-IFN; 7, octreotide).

Tumor cell lines were obtained from the American Type Tissue Collection (Manassas, VA). Two breast cancer cell lines which are reported to overexpress EGFR [MDA-MB-468 (28, 29), BT-20 (28, 29)], the epidermoid cancer cell line A-431 (30), and the pancreatic cell line PANC1 (31) were used as positive controls in the EGFR quantitative PCR assay. The pancreatic cancer cell lines, CAPAN-2 and BxPc-3, which are reported to overexpress the HGFR (32), were used as positive controls for the HGFR quantitative PCR assay. All cancer cells were grown in DMEM with 10% calf serum, except BT-20 breast cancer cells were grown in Eagle’s minimum essential medium, CAPAN-2 in McCoy’s 5A medium, and BxPc-3 in RPMI 1640, all with 10% FBS. Total RNA or mRNA from three normal pancreases were obtained from commercial sources [Clontech (Palo Alto, CA) and Invitrogen (Carlsbad, CA)].

**Competitive PCR.** Tumor samples were immediately snap frozen in liquid nitrogen either during surgery or after harvesting and stored at −70°C. The histological diagnosis of gastrinoma was made by using standard histological and immunohistological stains (chromogranin A and gastrin). Tumor mRNA was extracted from 8-μm cryosections of the gastrinomas after analyzing an adjacent slide with H&E staining to determine that 80% or more of the section contained tumor tissue. Cancer cell line mRNA was extracted from cell pellets after centrifugation (9000 × g for 10 min). Total RNA was extracted using a commercial kit (RNeasy Mini kit; Qiagen, Inc., Santa Clarita, CA).

Random hexamer-primed, first-strand cDNA was prepared with reverse transcriptase (RNA PCR kit; Perkin-Elmer Corp., Branchburg, NJ). The integrity of the cDNA was assessed by detecting a diffuse smear from 0.6 to 3 kb. After reverse transcription, PCR was carried out for amplification of a 351-bp fragment of the human EGFR or a 242-bp fragment of the HGFR. PCR was performed in a total volume of 15 μl containing 5–10 ng of cDNA, 0.5 μl DNA polymerase (Amplitaq Gold; Perkin-Elmer Corp., Foster City, CA), 10× PCR buffer, deoxynucleotides (Perkin-Elmer Corp.), and gene-specific primers in a DNA thermal cycler (Gene Amp PCR System 9700; PE Applied Biosystems, Norwalk, CT). The conditions for the PCR reactions were the same for all products: initial denaturation at 95°C for 5 min, annealing at 56°C for 35 s, extension at 72°C for 35 s, denaturation at 95°C for 25 s, followed by a final extension at 72°C for 5 min. All reactions underwent 40 amplification cycles. The amplified products were visualized on an agarose gel.
Primers, derived from sequence deposited in GenBank (EGFR, accession NM_005228 and HGFR, accession NM_000245) were designed to avoid pseudogene or genomic DNA amplification. For each gene, the set of primers used and length of the product were as follows: EGFR, sense (EGFR-s) 5N-ATGTCCGGGAACACAAAGAC-3N (EGFR nucleotides 2588–2607) and antisense (EGFR-as) 5N-TTCCGTCDATG-GCTTGGA-3N (EGFR nucleotides 2919–2838), which span a large intron in the cytoplasmic receptor domain; HGFR, (HGFR-s) 5N-GGTCAATTCAGGAAGTCT-3N (HGFR nucleotides 1482–1501) and antisense (HGFR-as) 5N-TTCCGT-GATCTTATCTCAGTTAG-3N (HGFR nucleotides 1701–1721), which also span a large intron in the β chain.

To carry out quantitative PCR, both an EGFR mimic (PCR product-288 bp) and a HGFR mimic (PCR product-324 bp) were made. Different fragments from the HPRT mRNA (GenBank accession no. M31642) were amplified using the conditions described above. For the preparation of the EGFR mimic DNA, a fragment of 248 bp was amplified using a sense primer (HPRT-s) 5N-ACCCACAGTGGTTGGATA-3N (HPRT nucleotides 586–606) and an antisense primer (HPRT-as) of 5N-AAGCAGATGGCCACAGA-ACT-3N (HPRT nucleotides 815–835). For the preparation of the HGFR mimic DNA, a fragment of 284 bp was amplified using a sense primer (HPRT-s) 5N AAAGATGGTCAAG-CTGCAGAA-3N (HPRT nucleotides 551–570) and an antisense primer (HPRT-as) of 5N-AACGAGATGCCCACAGAACT-3N (HPRT nucleotides 815–835).

The product was then amplified using gene-specific primers EGFR mimic-sense 5N-ATGTCCGGGAACACAAAGACAC-CGCCACAGTGGTTGGATA-3N and antisense 5N-ATGTCCGGGAACACAAAGACAC-CGCCACAGTGGTTGGATA-3N and an antisense primer 5N-TTCCGTCDATG-GCTTGGA-3N (EGFR nucleotides 2919–2838) and for the HGFR mimic-sense 5N-ATGTCCGGGAACACAAAGACAC-CGCCACAGTGGTTGGATA-3N and antisense primer 5N-GGTCAATTCAGGAAGTCT-3N. The sequence of all mimics and PCR products was made.

Slides were then immersed in a 10× citrate buffer solution (Biogenex, San Ramon, CA) diluted 1:10 in Tris-buffered saline (TBS) and placed in a pressure cooker containing water. Slides were microwaved for 20 min at high power, allowed to cool in a TBS solution (0.05 M Tris, 0.15 M NaCl, pH 7.6), EGFR immunohistochemistry was performed using a rabbit polyclonal anti-EGFR antibody that was raised against a peptide corresponding to the COOH terminus of the EGFR of human origin (Santa Cruz Biotechnology, Santa Cruz, CA) and an avidin/biotin/immunoperoxidase method. After antigen retrieval, endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide in methanol for 10 min. Endogenous biotin was blocked by using the Avidin/Biotin Blocking kit before incubation in Powerblock (Biogenex) diluted 1:10 in TBS containing 1% BSA (Sigma Chemical Co., St. Louis, MO) and 0.05% Tween 20 (Sigma; TBX) for 15 min. Sections were then incubated for 1 h with the anti-EGFR antibody diluted 1:100 in TBX, followed by washing in TBS (3 × 5 min) and then incubated with biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) diluted 1:200 in TBX for 30 min. After washing with TBS (3 × 5 min), the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for detection with diaminobenzidine tetrahydrochloride (Biogenex) as the substrate. For a negative control, sections of each sample were incubated with nonimmune rabbit IgG (Zymed) in place of the anti-EGFR antibody. The specificity of the anti-EGFR antibody was also tested by absorption with a peptide corresponding to the COOH terminus of human EGFR and by absorption with 1% agarose gel. The results of the competitive PCR were expressed as the ratio of the number of molecules of the EGFR mRNA or HGHR mRNA to β-actin mRNA present.

**Immunohistochemistry.** Immunohistochemical EGFR staining was performed in 15 gastrinomas from patients who had both fresh tissue and paraffin-fixed tissue available. It was performed on an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) according to the company’s protocols, with slight modifications. Briefly, formalin-fixed, paraffin-embedded tissues were cut at 5 μm thickness and mounted on polylysine-charged glass slides. The sections were deparaffinized in Hemo-D (Fisher Scientific, Houston, TX) and rehydrated in a series of graded alcohol/water solutions.

Slides were then immersed in a 10× citrate buffer solution (Biogenex, San Ramon, CA) diluted 1:10 in Tris-buffered saline (TBS) and placed in a pressure cooker containing water. Slides were microwaved for 20 min at high power, allowed to cool in a TBS solution (0.05 M Tris, 0.15 M NaCl, pH 7.6), EGFR immunohistochemistry was performed using a rabbit polyclonal anti-EGFR antibody that was raised against a peptide corresponding to the COOH terminus of the EGFR of human origin (Santa Cruz Biotechnology, Santa Cruz, CA) and an avidin/biotin/immunoperoxidase method. After antigen retrieval, endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide in methanol for 10 min. Endogenous biotin was blocked by using the Avidin/Biotin Blocking kit before incubation in Powerblock (Biogenex) diluted 1:10 in TBS containing 1% BSA (Sigma Chemical Co., St. Louis, MO) and 0.05% Tween 20 (Sigma; TBX) for 15 min. Sections were then incubated for 1 h with the anti-EGFR antibody diluted 1:100 in TBX, followed by washing in TBS (3 × 5 min) and then incubated with biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) diluted 1:200 in TBX for 30 min. After washing with TBS (3 × 5 min), the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for detection with diaminobenzidine tetrahydrochloride (Biogenex) as the substrate. For a negative control, sections of each sample were incubated with nonimmune rabbit IgG (Zymed) in place of the anti-EGFR antibody. The specificity of the anti-EGFR antibody was also tested by absorption with a peptide corresponding to the COOH terminus of human EGFR and by absorption with 1% agarose gel to exclude nonspecific binding of the polyclonal antibody to mucin. Slides were counterstained with hematoxylin, dehydrated, coverslipped, and viewed under a light microscope.

Tissues were graded positive for EGFR if definite brown staining was present in >10% of the cells. Positivity was assigned 1+ if 10–49% of cells stained positive and 2+ if >50% were positive.

Staining for HGFR was performed on 10 gastrinomas using a 1:500 dilution for the HGFR antibody (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA). The rest of the
EGFR and HGFR in Gastrinomas

***Table 1*** Characteristic values, laboratory values, tumor location, and tumor extent in the patients studied

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>38</td>
</tr>
<tr>
<td>Male</td>
<td>21 (55%)</td>
</tr>
<tr>
<td>Age at surgery (yr)</td>
<td>47.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>[16–62]</td>
</tr>
<tr>
<td>Duration of disease (yr)*</td>
<td>8.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>[0.2–24]</td>
</tr>
<tr>
<td>Fasting serum gastrin (pg/ml)*</td>
<td>6264 ± 2934</td>
</tr>
<tr>
<td></td>
<td>[87–110,000]</td>
</tr>
<tr>
<td>BAO (mEq/h)*</td>
<td>47 ± 4</td>
</tr>
<tr>
<td></td>
<td>[10–94]</td>
</tr>
<tr>
<td>MAO (mEq/h)*</td>
<td>67 ± 6</td>
</tr>
<tr>
<td></td>
<td>[24–135]</td>
</tr>
<tr>
<td>Preoperative antisecretory drug</td>
<td></td>
</tr>
<tr>
<td>H + -K + ATPase inhibitor</td>
<td>35 (92%)</td>
</tr>
<tr>
<td>H 2-receptor antagonist</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>MEN1 present</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Primary tumor location*</td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>18 (47%)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>10 (26%)</td>
</tr>
<tr>
<td>Lymph node†</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Others†</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Unknown†</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Tumor extent at surgery†</td>
<td></td>
</tr>
<tr>
<td>Primary only‡</td>
<td>13 (34%)</td>
</tr>
<tr>
<td>Primary + lymph node‡</td>
<td>17 (45%)</td>
</tr>
<tr>
<td>Metastatic lymph node only‡</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>Liver metastases‡</td>
<td>5 (13%)</td>
</tr>
</tbody>
</table>

* Duration of disease was defined as the time from onset of continuous symptoms attributable to Zollinger-Ellison syndrome until surgery as described previously (6).

† Fasting serum gastrin, BAO and MAO were determined preoperatively. Shown are the BAO and MAO from patients without previous gastric acid-reducing surgery (n = 31).

‡ Two patients had more than one primary tumor location.

§ A lymph node primary was defined as previously (7) with only a gastrinoma in a lymph node found at surgery, and the patient was cured.

¶ Other primary tumors include liver (n = 3) and heart (n = 1). A lymph node primary tumor was identified as described previously (7). A lymph node primary tumor was identified as described previously (7). A lymph node primary tumor was identified as described previously (7). A lymph node primary tumor was identified as described previously (7).

‡‡ Each patient is in only one of the four categories.

Primary only refers to patients in whom gastrinomas were only resected from duodenum (n = 5), pancreas (n = 3), lymph node (n = 7), liver (n = 2), omentum (n = 1), or heart (n = 1). A lymph node primary tumor was identified as described previously (7). A lymph node primary tumor was identified as described previously (7). A lymph node primary tumor was identified as described previously (7). A lymph node primary tumor was identified as described previously (7).

∥ Metastatic lymph node only was defined as finding gastrinoma in lymph node(s) without a primary tumor, and the patient was not cured.

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procedure for HGFR (secondary antibody, avidin-biotin complex color development, and counterstain) was similar to that for EGFR above.

**Statistics.** Discontinuous variables were compared using the Fisher Exact test. Continuous variables were compared using the Mann-Whitney U test (two variables), and for more than two variables, an ANOVA with the Bonferroni Dunn test as a post hoc test was used. Values with <0.05 were considered significant.

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**RESULTS**

Gastrinomas from 38 patients were studied (Table 1). This cohort is similar to other large series of patients with Zollinger-Ellison syndrome (34, 35) having a slight male predominance (55%), mean age in the fifth decade (48 years), a long disease duration (8 years), a markedly elevated fasting gastrin level and preoperative BAOs and MAOs (Table 1). Similar to other series (5, 34), all patients required continuous treatment with gastric antisecretory drugs, with the majority (92%) taking H + -K + ATPase inhibitors (omeprazole or lansoprazole). Only 10% of the patients had MEN1, which is less than the 20–25% seen in most series (5, 34) because in our center these patients do not routinely undergo exploratory laparotomy unless a tumor at least 3 cm in diameter is imaged (7, 36). Similar to most recent series (4, 7), duodenal primaries were more frequent than pancreatic primaries, and primary tumors in lymph nodes as well as other sites were also found, including one extra-abdominal gastrinoma in the heart (7, 35). The tumor extent found during surgery was comparable with other recent large surgical series (4, 7, 35) in that approximately one-third were confined to the primary site and approximately one-half were associated with lymph node metastases. In contrast, 13% of patients had liver metastases at surgery, and in 8% only gastrinoma metastatic to lymph nodes was found (Table 1).

In each of the 38 gastrinomas from 38 patients, the 351-bp EGFR mRNA fragment was detected by PCR. The results on the first 11 patients are shown in Fig. 1. That the PCR product was EGFR was confirmed by sequencing. Furthermore, control studies demonstrated that the PCR product was from tumor mRNA because the EGFR primers spanned a long intron and gave no amplification with genomic DNA, and no product was seen if reverse transcription was not performed. Similar to the EGFR, in each of the 22 gastrinomas examined, the 242-bp HGFR product was detected by PCR. It was verified by sequencing and shown not to be present if reverse transcription was not performed and not to be attributable to the presence of genomic DNA because primers spanning a large intron were used and gave no product with genomic DNA.

To quantitate the amount of EGFR and HGFR present in each gastrinoma, a double quantitative PCR was used as described previously (33). The amount of EGFR or HGFR was measured using a mimic as shown for four patients in Fig. 2. To correct for possible variable input, β-actin was also measured by quantitative PCR in each sample, and the final result for both was expressed as molecules of EGFR or HGFR per β-actin molecule (Fig. 3) for each gastrinoma. For both EGFR and HGFR, the levels in different gastrinomas varied widely (Figs. 2 and 3). The EGFR mRNA levels varied 1050-fold, with the lowest level detected being 0.0040 EGFR/β-actin molecule, the highest level 0.42 EGFR/β-actin molecule, and a mean level of 351 bp.
0.054 ± 0.013 EGFR/β-actin molecule (Fig. 3, left panel). The HGFR mRNA levels in different gastrinomas varied 375-fold, with the lowest level being 0.00016 HGFR/β-actin molecule, the highest being 0.060 HGFR/β-actin molecules, with a mean level of 0.0123 ± 0.003 (Fig. 3, right panel).

In other tumors including nonendocrine [breast, pancreatic, urinary, bladder, lung; Refs. 18, 37–40] and some endocrine tissues [thyroid (18, 40–42)], the level of EGFR or HGFR correlates with tumor growth behavior, high recurrence rate, poor survival, or advanced disease. To determine whether the level of expression of EGFR or HGFR correlated with features characteristic of expression of the gastrina, gastrinoma growth, other clinical and laboratory features with prognostic value or associated with gastrinoma biological activity, the levels of EGFR and HGFR mRNA in the gastrinomas were compared in patients with or without these features (Figs. 4 and 5).

Increased age (either at surgery or at disease onset; Ref. 3), presence of MEN1 (5, 6), short disease duration (from either onset or diagnosis to surgery; Refs. 6, 27), or high levels of ectopic hormone release or its effects (i.e., in gastrinomas, fasting serum gastrin, δ secretin, BAO, and MAO; Refs. 2–4, 6) are clinical and laboratory parameters associated with a poor prognosis in various neuroendocrine tumors (carcinoids and pancreatic endocrine tumors) including gastrinomas. The mag-
titude or presence or absence of any of these prognostic variables was not associated with a significantly higher EGFR or HGFR level in the tumor (Fig. 4).

A number of neuroendocrine tumor characteristics, including those in gastrinomas, have been shown to be associated with increased tumor growth, decreased curability, and/or poor survival. These tumor characteristics include an aggressive growth pattern (26, 43), the presence of liver metastases (3–6, 27, 43, 44), the primary tumor location (1, 3, 4, 6), the primary tumor size (3, 4, 6, 43, 45), the tumor extent (localized, lymph node metastases present; Refs. 4, 27), and the presence or absence of factors predictive of long-term disease-free survival (postoperative cure, immediate, long-term cure, disease relapse; Ref. 3). The magnitude, presence, or absence of any of these variables was not associated with a significantly higher EGFR or HGFR mRNA levels in the tumor (Fig. 5). The failure of long-term cure after resection (mRNA levels in the tumor (Fig. 5). The failure of long-term cure after resection was not associated with a significantly higher EGFR or HGFR level also. Each of the positive control cells reported to have increased EGFR levels exceeded this level (Fig. 3), with the PANC1 cell line reported to have >10-fold increase in EGFR receptors (31), having a PCR-determined elevated EGFR/β-actin level of 10. Similarly, each of the two pancreatic cancer cell lines (BxPC-3 and CAPAN-2) reported to have marked overexpression of HGFR mRNA and HGFR (32) had levels more than five times the mean control value and >2-fold above the 95% confidence interval for normal persons.

To attempt to determine whether increased EGFR levels identify a subset of patients with more aggressive tumors, the presence or absence of a number of various factors associated with neuroendocrine tumor and/or gastrinoma growth, prognosis, or biological behavior were analyzed (Table 2). Age, disease duration, magnitude of BAO, fasting serum gastrin level, percentage male gender, or primary tumor size did not differ significantly between patients with or without elevated EGFR levels (Table 2). However, liver metastases (P = 0.034) and percentage of patients with decreased long-term curability (P = 0.027) were significantly more frequent in patients with elevated EGFR gastrinoma levels (Table 2). Furthermore, there was a higher proportion of gastrinomas showing an aggressive postoperative growth of the gastrinoma (57% versus 29%; P = 0.16) and a lower proportion showing decreased curability immediately after resection (29% versus 61%, P = 0.12) with overexpression of the EGFR; however, these differences were not significant (P > 0.05). The proportion of gastrinomas that were duodenal in location, a primary tumor size of ≤1 cm in diameter, or a primary tumor only present did not differ significantly between gastrinomas that did or did not overexpress the EGFR (Table 2). One of the three patients overexpressing HGFR had aggressive disease, whereas the other two were cured 6 and 7 years after resection.
To determine whether EGFR or HGFR overexpression in gastrinomas could be detected by immunohistochemistry, EGFR and HGFR immunohistochemistry studies were performed on gastrinomas from patients in whom both formalin-fixed, paraffin-embedded tissue and frozen unfixed tissue were available. In 6 of 15 gastrinomas, immunohistochemistry detected EGFR. In one gastrinoma, 2/11001 staining was seen, with 50% of the cells showing cytoplasmic and membrane staining (Fig. 6), whereas the remaining 5 positive gastrinomas showed 1/11001 staining with 10–49% of cells staining. In normal pancreas, pancreatic ducts showed strong membranous EGFR staining (Fig. 6), similar to the positive control (i.e., breast cancer; Fig. 6), whereas normal pancreatic acini and islets were negative (Fig. 6). The gastrinoma with the highest EGFR:β-actin mRNA ratio showed the greatest EGFR staining. Nine of the 10 gastrinomas showed detectable HGFR cytoplasmic and membranous staining (data not shown). In all but one patient, the HGFR staining intensity was relatively low (1+) compared with that seen with the positive control (breast cancer).

**DISCUSSION**

PETs, similar to other gastrointestinal NETs such as carcinoids, are generally slow-growing tumors. However, recent studies demonstrate that a significant subset can demonstrate aggressive growth (1, 4, 6, 26, 27, 43, 44). At present, little is known about the molecular basis for the difference in growth behavior of PETs in different patients.

Abnormalities of various growth factors are involved also in the growth, progression, and development of metastases in a number of tumors (17–22). In particular, alterations in genes for either a member of the EGF family (transforming growth factor-α, EGF, cripto, and others) or the HGF family or their receptors are reported to play an important role in the growth of numerous tumors (17–19, 46, 47). Activation of the EGFR is reported to not only increase tumor growth but also to have potent angiogenic effects, promote tumor invasion, adhesion, and motility (17). Similarly, activation of the HGFR can cause a mitogenesis as well as cause increased motility and invasiveness (46).

Studies demonstrate both nonendocrine tumors (18, 37, 40, 48) and endocrine tumors (thyroid, parathyroid, pituitary, adrenocortical cancer, and small cell lung cancer; Refs. 18, 42, 49–55) frequently overexpress the EGFR. Similarly, the HGFR is frequently overexpressed in both nonendocrine (19, 32, 56–61) and in a few endocrine tumors (thyroid and small cell lung cancer; Refs. 62–65). Overexpression of both EGFR and HGFR in various tumors is associated with increased tumor size (19, 42, 61, 66), tumor stage (19, 18, 40, 42, 57, 67, 68), lymph node metastases (18, 19, 40, 42, 59, 67), and a poor prognosis/survival (18, 19, 40, 64, 69, 70).

In general, the expression of EGFR and/or HGFR or the results of their activation in neuroendocrine tumors (PETs or carcinoids) on tumor growth or their possible development have been only minimally studied. Only one study (71) has investigated HGFR expression in NETs demonstrating HGFR mRNA can be detected in only 14% of PETs and 0% of carcinoid tumors. Similarly, only three studies (71–73) have investigated the expression of EGFR in PETs. In two studies (72, 73) using immunohistochemistry, weak staining for EGFR was seen in some tumors, whereas in the third (71) using RT-PCR, 40% of the PETs (n = 26) were found to contain EGFR mRNA. No quantitative comparisons were performed to correlate with prognosis in either study. In contrast, at least nine studies (71–79) have examined various carcinoid tumors for...
EGFR, and in all of the studies, a proportion of the tumors possessed EGFR; however, in no studies was quantitation performed or sufficient numbers included to correlate with clinical or tumor variables.

Because in one study transforming growth factor-\(\alpha\) stimulated carcinoid tumor growth (76), which led to the suggestion that EGFR could be an autocrine growth factor in NETs; because of its established role in other malignancies, the current study was performed. In both endocrine and nonendocrine tissues, the expression of EGFR and HGFR has been assessed by immunohistochemistry, Western blotting, in situ hybridization, Northern analysis, and quantitative RT-PCR (47, 65, 76, 80–82). These studies demonstrated that for both the EGFR and HGFR, in general, there exists a close correlation between Northern analysis, and quantitative RT-PCR (47, 65, 76, 80–82). These studies demonstrated that for both the EGFR and HGFR, in general, there exists a close correlation between mRNA expression and Western blotting or immunohistochemical results (47, 65, 76, 80–82). Because of the small size of many gastrinomas and limited tissue amount and because precise quantitation of the EGFR and HGFR was needed to carry out the correlations for this study, which could not be obtained from an immunohistochemical analysis, quantitative RT-PCR was used to assess the amount of EGFR or HGFR present. Our results demonstrate that by immunohistochemical methods, low levels of EGFR can be detected in 40% of gastrinomas; however, using RT-PCR, EGFR mRNA is detectable in each of the 38 gastrinomas. The results are similar to a recent study (71) in which 9 of 9 (100%) gastrinomas but only 10% of insulinomas (\(n = 10\)) and 0% of nonfunctional PETs (\(n = 7\)) possessed EGFR by RT-PCR. EGFR expression was detected in one-third of midgut carcinoids (\(n = 7\); RT-PCR; Ref. 71), 58% of lung carcinoids (\(n = 19\); immunohistochemistry; Ref. 74), and 90–100% of various gastrointestinal carcinoids in two studies by immunohistochemistry (50, 75, 76). In other endocrine malignancies, EGFR was detected in 50–96% of thyroid papillary cancers (42, 51, 88), 72% of thyroid follicular cancers (88), 0–95% of parathyroid adenomas (52, 89), 94–98% of adrenocortical cancers (53, 90), and 68–100% of pituitary adenomas (54, 55). Our results demonstrated that each of 22 gastrinomas studied also expressed HGFR mRNA and low levels of HGFR protein by immunohistochemistry. This result differs from that of the only other study on PETs using similar methodology (71) in which only 33% of the gastrinomas (\(n = 9\)) and none of the insulinomas (\(n = 10\)), nonfunctional PETs (\(n = 7\)), or carcinoids (\(n = 9\)) expressed HGFR. Similar to our findings with gastrinomas, a high percentage of some other endocrine tumors express HGFR [small cell lung cancer (88%); Ref. 65] and papillary thyroid cancer (77–96%; Refs. 62, 67), in contrast to other endocrine cancers which show a lower percentage of tumors expressing HGFR [follicular thyroid cancer (33–70%; Refs. 62, 64)]. These results are consistent with the conclusion that almost all gastrinomas express EGFR and HGFR mRNA in contrast to a number of other PETs (71) but similar to that reported with a number of other nonpancreatic endocrine tumors.

Numerous endocrine and nonendocrine tumors are reported to overexpress EGFR and/or HGFR (18, 32, 40, 42, 49, 50, 56–58, 62, 65). No previous studies have quantitated the expression of either HGFR or EGFR in NETs, either PETs or carcinoids. Our results demonstrate that EGFR mRNA is overexpressed in almost one-fifth of the gastrinomas (i.e., 18%). This percentage of overexpression for gastrinoma is much less than that reported with many endocrine tumors [40–96%, papillary thyroid cancer (42, 88, 91); 90%, parathyroid adenomas (52); 80%, adrenocortical cancer (53); and 76% of prolactinomas (92)], and with many nonendocrine tumors [30–50%, pancreatic cancer (40); 40–80%, lung cancer (40); 48%, breast cancer (40, 80); 35–70%, ovarian cancer (40)]. However, it is similar to the results reported with EGFR overexpression in esophageal cancer (8–14%; Ref. 18) and follicular cancer of the thyroid (25%; Ref. 40). Similarly, we found that HGFR mRNA was overexpressed in one-seventh (i.e., 14%) of the gastrinomas. This low percentage is similar to the 25% reported in thyroid adenomas (63), 22% of follicular thyroid cancers (64), 0% reported in medullary thyroid cancers (64), and 22–25% in breast cancers in some studies (57, 93). However, this percentage of HGFR overexpression is much less than 74–75% reported with papillary thyroid cancer (63), 88% of pancreatic cancers (94), and 30% of colon cancers (61). These results demonstrate that the percentage of gastrinomas that overexpress EGFR and HGFR are substantially lower than that seen with most more common endocrine and nonendocrine malignant tumors.

Not only did gastrinomas differ in their frequency of overexpression of EGFR and HGFR compared with most common malignant tumors, they also differed in the magnitude of overexpression of these growth factor receptors. The mean level of HGFR overexpression was \(<1\)-fold increase (i.e., 0.63-fold) with a maximal of 1.2-fold. There are no similar data reported by others for other types of NETs (carcinoids and PETs) to compare this magnitude of change. However, in colorectal adenomas and carcinomas by Northern analysis, HGFR mRNA was 2–50-fold increased (95, 96); pancreatic cancer, 7-fold increased (94); hepatocellular cancer, 2–10-fold increased (97); and by semiquantitative PCR, head and neck squamous cell cancers that metastasized showed a 2–8-fold increase in HGFR mRNA (68). Similarly, the magnitude of the overexpression of

<table>
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<th>Table 2 Comparison of clinical, laboratory, and tumor characteristics in patients overexpressing or not overexpressing EGFR in the gastrinoma</th>
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<tr>
<td>Male gender present</td>
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<tr>
<td>Liver metastases present</td>
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<tr>
<td>Aggressive disease course</td>
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<td>Cured immediately after resection</td>
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<td>Cured last follow-up</td>
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<tr>
<td>Duodenal tumor present</td>
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<td>Primary tumor (\geq 1) cm</td>
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<td>Primary tumor only present</td>
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<td>Age onset (&gt;40) yr</td>
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<td>Duration from onset to surgery (\geq 8) yr</td>
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<td>BAO (\geq 45) mEq/h</td>
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<td>Fasting serum gastrin (\leq 1200)</td>
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\(^a\) Only 33 patients had a primary tumor found (6 overexpressed EGFR, and 27 did not), and 31 patients had a BAO without a previous acid-reducing procedure (6, EGFR overexpressed; 25, not overexpressed).

\(^b,c\) Compared with EGFR overexpressed; \(^b\) \(P = 0.034\); \(^c\) \(P = 0.027\).
EGFR mRNA was relatively low with a mean <1.5-fold and a maximal level of 4.4-fold. In contrast, by in situ PCR, EGFR mRNA was 17-fold increased in pancreatic adenocarcinoma (98), 1.8–8.4-fold increased by Northern analysis in renal cell carcinoma (99), and a mean of 19-fold increased (range, 1.3–71) in head/neck squamous cell cancers (100). These data demonstrate that the level of overexpression of HGFR and EGFR in gastrinomas is less than that reported in more aggressive malignant tumors.

The level of expression of EGFR and HGFR have been shown in numerous endocrine (18, 19, 40, 64, 67) and nonendocrine tumors (17–20, 39, 40, 57, 60, 69) to correlate with factors related to tumor growth and invasiveness. One study (71) with HGFR and three studies with EGFR (18, 40, 67) in NETs (PETs and carcinoids) have investigated correlations with some of these variables. In this study (71) involving 28 PETs and 9 gastrointestinal carcinoid tumors, no relationship was seen between EGFR or HGFR expression and presence or absence of metastases. In the present study, we found that EGFR mRNA overexpression was associated significantly \((P = 0.034)\) with the presence of liver metastases and with decreased long-term curability \((P = 0.027)\). The presence of liver metastases is the most important determinant of long-term survival in patients with gastrinomas as well as other NETs (4, 5, 27, 43). Therefore, it is likely that if these patients are followed a sufficiently long period of time, EGFR expression will also correlate with survival. The presence of EGFR mRNA overexpression did not correlate with other clinical and tumor factors that have been shown to have prognostic significance in gastrinomas and other NETs including primary tumor size (6, 27) or location (5, 6, 27), male gender (6), fasting serum gastrin level (6, 101, 102), or disease duration (6, 27). These results with gastrinomas with an association of EGFR overexpression with the presence of an increased occurrence of metastatic disease are similar to that reported in some studies with a number of malignant endocrine tumors [papillary thyroid cancer (42, 50, 88) and adrenocortical

![Fig. 6](https://cancerres.aacrjournals.org/)

Immunohistochemistry for EGFR. **A**, results with a positive control that show EGFR membranous staining of a breast cancer using a rabbit polyclonal anti-EGFR antibody staining. In **B**, EGFR immunoreactivity (brown chromogen) is present in the cytoplasm and membranes of the gastrinoma cells. **C** is from an area showing normal pancreatic ducts and acini. Staining with the anti-EGFR antibody is negative in the histologically normal-appearing acinar cells (arrowhead) and vessel (arrow) while positive in cells of the ducts. In **D**, EGFR immunoreactivity is absent in the islet cells and in the normal-appearing acinar cells. Bar, 50 μm. All slides were counterstained with hematoxylin. Magnification is ×20, except **A** and the insets in **B** and **C**, which are ×100.
cancer (53, 54) and malignant nonendocrine tumors [gastric (18), urinary bladder cancer (18, 40), renal cancer (70, 103), and breast cancer (80)]. However, they differ from results with other studies in which no associations between EGFR and metastases were reported including with thyroid cancer (50, 104), adrenocortical cancer, gastric cancer (105, 106), and breast cancer (107). The decreased rate of long-term curability in patients with overexpression of EGFR is similar to reports of early recurrences in patients with urinary bladder cancers (18, 37), breast cancer (18, 80, 108), thyroid cancer (50), and pituitary adenomas (54). This finding has potentially important clinical significance for the subset of 20% of patients with gastrinomas overexpressing EGFR. At present, there are no known prognostic factors that can reliably predict which patient will develop a recurrence; therefore, all cured patients are assessed by imaging and functional studies every 1 to 2 years (24). Our results suggest the cohort of patients whose gastrinomas have this finding should be evaluated at least yearly for recurrence, whereas the other patients could be evaluated less frequently, such as every 2 years.

The fact that EGFR and HGFR mRNA are present in each of the gastrinomas studied raises the possibility that these growth factor receptors could be involved in autocrine growth cascades in these tumors, as shown in a number of other tumors [i.e., parathyroid adenomas (52), cancers of the breast (40, 83), kidney (40), lung (40), and esophagus (40, 109)]. Furthermore, their presence possibly may be important to the molecular pathogenesis of these tumors. Experimental studies (110) demonstrate that EGFR-deficient mice have decreased differentiation of islets and decreased islet cell proliferation in normal islets in some studies (111) but not others (112). Furthermore, EGFR has been shown to have a stimulating effect on islet growth (113). HGFR is expressed at low levels in normal islets (94). However, HGFR activation has been shown to stimulate the formation of islet-like clusters in fetal pancreatic tissue and increase growth of this tissue (114, 115). Furthermore, HGFR activation stimulates islet proliferation (116). Whether the presence of these growth factor receptors is important in the molecular pathogenesis of PETs, as they are in other tumors (17, 18), is present only speculation. However, with the increased development of antitumor therapies directed against the EGFR (17, 117–119), this speculation raises the possibility that such approaches could be helpful in some malignant PETs for which present treatment regimens are inadequate (2).

REFERENCES


Overexpression of Epidermal Growth Factor and Hepatocyte Growth Factor Receptors in a Proportion of Gastrinomas Correlates with Aggressive Growth and Lower Curability

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