Increased Expression of Insulin-Like Growth Factor I and/or Its Receptor in Gastrinomas Is Associated with Low Curability, Increased Growth, and Development of Metastases

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Abstract

**Purpose:** Growth factors, particularly insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR) in some nonendocrine and a few endocrine tumors, are thought important in recurrence, growth, and aggressiveness. Whether this is true of neuroendocrine tumors such as gastrinomas is unclear. The aim of this study was to address this question in gastrinomas.

**Experimental Design:** IGF-I and IGF-IR expression in gastrinomas from 54 patients with Zollinger-Ellison syndrome were analyzed and correlated with clinical/tumor characteristics. IGF-I and IGF-IR mRNA levels were determined by competitive reverse transcription-PCR. IGF-IR expression, assessed by immunohistochemistry, was done on a subset.

**Results:** IGF-IR mRNA was found in 31 of 32 tumors (97%) by immunohistochemistry. These results indicate that IGF-I and IGF-IR are expressed in almost all gastrinomas. Furthermore, assessment of IGF-I/IGF-IR expression in gastrinomas may be clinically useful in identifying those patients with more aggressive tumors who might benefit from more aggressive treatment.

**Conclusions:**

Gastrointestinal neuroendocrine tumors, comprising pancreatic endocrine tumors and carcinoids, are generally considered slow-glowing neoplasms; however, in a significant subset, aggressive growth occurs resulting in decreased survival (1–3). Twenty-five percent of gastrinomas, the most common malignant symptomatic pancreatic endocrine tumor (1, 4), show aggressive growth resulting in a decreased survival (5). Even in patients who develop liver metastases, recent studies show there is a markedly variable growth pattern in the metastatic gastrinoma in different patients with 40% demonstrating aggressive growth frequently resulting in tumor-related death, whereas in the other 60% of patients the prognosis is excellent (6). At present, the factors responsible for these variable growth patterns with different pancreatic endocrine tumors as well as with gastrinomas, are largely unknown (7). This situation exists because the molecular pathogenesis of neuroendocrine tumors is largely unknown (7).

In contrast to nonendocrine tumors, neither alterations in common oncogenes (ras, myc, etc.) nor alterations in common tumor suppressor genes (retinoblastoma gene, p53) are found in the typical neuroendocrine tumor (7). Alterations in the p16\(^{INKA}/CDKN2A\) tumor suppressor gene occur in 17% to 92% (7, 8), and alterations in the MEN1 gene on chromosome 11q13 occur in 16% to 42% of sporadic gastrointestinal neuroendocrine tumors (7, 9). Unfortunately, neither the presence of p16\(^{INKA}/CDKN2A\) nor MEN1 gene abnormalities in sporadic neuroendocrine tumors correlates with the aggressiveness of the neuroendocrine tumor (7–9). Gene abnormalities at the DPC\(4\) locus on 18q21 are reported in 18% to 88% of neuroendocrine tumors as well as in the region of the VHL locus on 3p25, which correlate with neuroendocrine tumor growth in some studies, but not others (7–9).

Growth factor receptor overexpression is associated with tumor growth and invasiveness in a number of nonendocrine and some endocrine tumors (7, 10, 11). Recently, the level of epidermal growth factor receptor/hepatocyte growth factor receptor expression in gastrinomas was shown to correlate with the presence of liver metastases and decreased curability (12). The epidermal growth factor receptor, even when overexpressed,
may require a functional IGF-I receptor to exert its mitogenic/transforming potential (13). Similarly, activation of the platelet-derived growth factor receptor can influence IGF-I gene activity (14). These studies suggest that the presence of IGF-I receptors may be particularly important for mediating growth factor effects on tumor growth.

IGF-IR is a member of the tyrosine kinase receptor superfamily with a 70% homology to the insulin receptor (15). IGF-IR activation can induce numerous cellular effects including differentiation, transformation, and prevention of apoptosis. The activation of the IGF-IR increases tumor growth and up-regulates vascular endothelial growth factor expression, promoting tumor invasion (16, 17). A number of studies show overexpression of IGF-I and/or its receptors by different tumors and that this expression is associated with aggressive growth, decreased survival, or poor prognosis (17). IGF-I and/or IGF-IR are reported in a few studies to be present in some neuroendocrine tumors, IGF-I can stimulate tumor growth (19); however, no correlation has been found between IGF-IR expression in neuroendocrine tumors and tumor aggressiveness in a small number of patients examined in two studies (18, 20). Therefore, it remains unclear whether increased IGF-I/IGF-IR expression in neuroendocrine tumors, including gastrinomas, is associated with increased tumor growth or decreased survival.

Materials and Methods

Patients and tumors. Fifty-four patients who underwent exploratory laparotomy for Zollinger-Ellison syndrome at the NIH between February 1988 and October 2003 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. The diagnosis of Zollinger-Ellison syndrome or MEN1 was established as reported previously (21). All patients had acid hypersecretion controlled as reported previously (22, 23). Conventional imaging studies (computed tomography, magnetic resonance imaging, sonography, and bone scan), abdominal angiography, and somatostatin receptor scintigraphy were done as reported previously (5, 23–25) to assess tumor location/extent. All patients underwent an exploratory laparotomy for attempted curative resection (23). Disease-free status was defined by normal fasting gastric levels, negative gastrin provocative testing with secretin, and no evidence of tumor on imaging studies (23). Annual imaging studies were done postoperatively. Functional studies (fasting gastrin and secretin test) and imaging studies provided the basis for assessment of tumor growth, recurrence, or progression (12, 23). Relapse was defined as the recurrence of disease after a patient had been disease-free postresection (12, 23). Aggressive disease was defined as a >25% increase in tumor volume per month or the appearance of a new lesion(s). An increase in size or number of lesions on imaging studies was defined as evidence of tumor growth (6).

Competitive reverse transcription-PCR. Tumor samples were snap-frozen in liquid nitrogen during surgery or after harvesting and stored at −70°C. Tumor mRNA was extracted from 5-μm cryosections of the gastrinomas after analyzing an adjacent slide to determine that ≥80% of the section contained tumor tissue as described previously (12). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Inc., Santa Clarita, CA). Random hexamer-primed first-strand complementary DNA was prepared with RT (RNA PCR Kit; Applied Biosystems, Foster City, CA). After RT, PCR was carried out for amplification of a 301-bp fragment of the human IGF-I or a 208-bp fragment of the IGF-IR. PCR was done in a total volume of 25 μl containing 5 to 10 ng of cDNA, 0.5 micromol DNA-polymerase (Amplitaq Gold, Applied Biosystems), 10× PCR buffer, deoxynucleotides (Applied Biosystems), and gene-specific primers in a DNA thermal cycler (GeneAmp PCR System 9700, PE Applied Biosystems). The conditions for the PCR reactions were the following: initial denaturation at 94°C for 5 minutes was followed by denaturation at 94°C for 40 seconds, annealing at 58°C to 62°C for 40 seconds, depending upon the specific primers (IGF-I: 60°C, IGF-IR: 62°C, and β-actin: 58°C) and extension at 72°C for 40 seconds. The final elongation step was extended to 5 minutes at 72°C. All reactions underwent 40 amplification cycles. The amplified products were visualized on an agarose gel (Relian Gel system, 4% NuSieve 3:1 agarose, CAMBREX, Rockland, ME).

IGF-I and IGF-IR primers were derived from sequence deposited in Genbank (IGF-1 accession no. AY260957 and IGF-IR accession no. AY332722). For each gene, the set of primers used and length of the product were as follows: IGF-I: sense (IGF-I-s) 5′-AAAAATCAG-CAGTCTTCAAC-3′ (IGF-I nucleotides 2,100-2,119) and antisense (IGF-I-as) 5′-AGATCAGAGCTCAGGGAGCA-3′ (IGF-I nucleotides 62,852-62,871) which span a large intron. IGF-IR: (IGF-IR-s) 5′-GGGAAATGGAGTCTGATG-3′ (IGF-IR nucleotides 281,072-281,091) and antisense (IGF-IR-as) 5′-AAATGCGCACTTCGTTCCA-3′ (IGF-IR nucleotides 285,766-285,785) which also span a large intron.

To carry out quantitative PCR, both an IGF-I mimic (PCR product, 188 bp) and an IGF-IR mimic (PCR product, 290 bp) were made. For the IGF-I mimic DNA, a fragment of 188 bp was amplified from MEN1 gene (Genbank accession no. BC002544) using a sense primer (MEN1-s) 5′-GAGATCAGAGCGCATGAGG-3′ (MEN1 nucleotides 1,631-1,650), an antisense primer (MEN1-as) of 5′-CCCGTTGGAAAGACAG-3′ (MEN1 nucleotides 1,759-1,778) and the conditions described above. For the preparation of the IGF-IR mimic DNA, a fragment of 290 bp was amplified using a fragment of the human hypoxanthine phosphoribosyltransferase (HPRT) gene (Genbank accession no. M26434). The sense primer (HPRT-s) was 5′-CATGGATCCTCGTTCTC-3′ and an antisense primer (HPRT-as) of 5′-CTCCATGTGTTCCAGTGT-3′ (HPRT nucleotides 452-471). The product was then amplified using gene specific primers IGF-I mimic-sense 5′-AAAAATCAGAGCTCAGAGGATTGAAAGGAGGATG-3′ and antisense 5′-AGATCAGAGCGTCA-3′ which is an antisense primer (MEN1-s) of 5′-CCCGTTGGAAAGACAG-3′ (MEN1 nucleotides 1,759-1,778) and the conditions described above.

Complementary DNA was gel fractionated and purified using the Wizard PCR Prep DNA Purification System (Promega Co., Madison, WI). The molar concentration of the purified mimic solution was measured by UV absorption at 260 nm. Aliquots were frozen at −20°C.

To allow quantitative correction for any differences in input amounts of total RNA as described previously (26), a quantitative PCR for β-actin was done on all samples. The PCR conditions were as described previously (26) with a sense primer 5′-CCTCAGCCTTCTCGAGA-3′ and an antisense primer 5′-GAAATGGAGTCTGATG-3′. The PCR product was 204 bp and was only seen when complementary DNA was used as the template because the two primers spanned two introns (26). A β-actin mimic (PCR product 289 bp; ref. 26) was synthesized as described above for the other mimics using a sense primer 5′-CCTCGGTTTGGAGATCC-3′ and an antisense primer 5′-GAAATGGAGTCTGATG-3′. The PCR product was 204 bp and was only seen when complementary DNA was used as the template because the two primers spanned two introns (26).

Competitive PCR was done by amplifying the target complementary DNA in the presence of increasing concentrations of the corresponding mimic with the respective primer pairs (IGF-I-s/as, IGF-IR-s/as, or β-actin-s/as). The concentration of target complementary DNA was calculated by comparison to the concentration of the mimic, as determined by equal intensity of ethidium bromide staining in a 4% agarose gel. The results of the competitive PCR were expressed as the ratio of the number of molecules of the IGF-I mRNA or IGF-IR mRNA to β-actin mRNA present.
Differential PCR. DNA was extracted from tumor and blood, using the DNeasy Tissue and QIAamp Blood Kits, respectively (Qiagen), of nine patients with the highest IGF-IR mRNA levels. Differential PCR for IGF-IR gene amplification was done using the method of Frye et al. with comparison with the single copy IFN-γ gene (27). Briefly, a 221-bp fragment of the IGF-IR gene was amplified using the following primers: sense TGCTTTCAGACACGATG and antisense CCTGTCAACAGAAATGGCAG; a 150-bp fragment of the IFN-γ gene was produced using the following primers: sense TCTTCTTCCGCAATGGT and antisense CAGGGATGCTCTTCGACCTC (28). Both fragments were amplified in the same vial using the following conditions: initial denaturation at 94°C for 10 minutes followed by denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 2 minutes. The final elongation step was extended to 7 minutes at 72°C. All reactions underwent 36 amplification cycles, which were on the linear portion of the product accumulation curve. The amplified products were separated on agarose gels (Relian Gel System, 2200 Analysis Systems (Alpha Innotech Co., San Leandro, CA).

Immunohistochemistry. IGF-IR immunohistochemistry was done on formalin-fixed, paraffin-embedded tissues. Immunohistochemistry against IGF-I was not done because adequate antibodies for paraffin-embedded material are not available. The breast cancer cell line, MCF-7 (American Type Culture Collection, Rockville, MD), which over-expresses IGF-IR (29), was used as a positive control. IGF immunohistochemistry was done on tissue samples from 32 patients who had mRNA levels analyzed by competitive reverse transcription-PCR, in which formalin-fixed gastrinoma tissue was available. Serial sections (5 μm) of gastrinomas were cut, deparaffinized in xylene and rehydrated in a series of graded alcohol. Sections were then incubated for 30 minutes in 3% hydrogen peroxide diluted with methanol (Fisher Chemicals, Fair Lawn, NJ). Antigen retrieval was done by immersion in slides in 10 mmol/L sodium citrate buffer (pH 6.0), 900 mL of H2O, and 100 mL of 10× Antigen Retrieval Citra (Biogenex, San Ramon, CA) and placing in a pressure cooker containing water. Slides were microwaved for 8 minutes at maximum power (i.e., 1,300 W) and after making sure to boil it, continued for 15 minutes at 260 W. Sections were blocked in PBS containing 3% normal goat serum (Vector Laboratories, Burlingame, CA) for 20 minutes, and by using the Streptavidin/Biotin Blocking Kit (Vector Laboratories) before incubation with the primary antibody. A rabbit polyclonal anti-IGF-IRβ (C-20) antibody was used that was raised against a peptide corresponding to the last 20 amino acid of the NH2-terminus of human IGF-IRβ (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were incubated overnight at 4°C with the anti-IGF-IRβ antibody diluted 1:750 or 1:1,500 in PBS followed by washing in PBS (3 × 5 minutes) and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 in PBS for 1 hour. The Vectastain Avidin-Biotin Complex kit (Vector Laboratories) was used for detection with diamobenzidine tetrahydrochloride (Zymed, San Francisco, CA) as the substrate. Antibody specificity was assessed by preincubating the IGF-IR β antibody with a 5-fold by weight excess of the peptide to which the antibody has been raised. Slides were counterstained with hematoxylin, dehydrated, a coverslip applied and viewed under a light microscope. Distribution of staining was recorded as focal or homogenous, and staining intensity was graded on an arbitrary four-point scale: 0, no staining; 1+, faint or weak staining; 2+, moderate staining; and 3+, strong staining. For quality control purposes, each staining run included a section of normal pancreas containing islets that was scored as 3+.

Statistical analysis. All values were expressed as mean ± SE. Discontinuous variables were compared using the Fisher exact test or the χ2 test, the Mann-Whitney U test (two variables) and for more than two variables, the Kruskal-Wallis test or an ANOVA with the Bonferroni Dunn test as a post hoc test. In tables and figures with multiple tests, Ps < 0.005 were considered significant even after conservative correction.

Results

The clinical/laboratory characteristics of the 54 patients with gastrinomas studied are similar to other large series of patients with gastrinomas (21) in having almost equal frequency in both sexes, a mean age in the fifth decade (49 years), a long disease duration (8 years), a markedly elevated fasting serum gastrin level, and preoperative basal and maximal acid outputs (Table 1). Similar to other series (4, 23, 30), all patients required continuous treatment with gastric antisecretory drugs with the majority (91%) taking H2- and/or ATPase inhibitors. Similar to most recent series (23), duodenal primaries were more frequent than pancreatic primaries, and primary tumors in lymph nodes as well as other sites were found (23). In approximately one third of patients, the tumor was confined to the primary site and approximately one half were associated with lymph node metastases. In contrast, 17% of patients had liver metastases at surgery and in 6% only gastrinoma metastatic to lymph nodes was found (Table 1).

Insulin-like growth factor 1 receptor mRNA expression. In gastrinomas from 48 of the 54 patients, the 301-bp IGF-I fragment was detected by PCR. In all gastrinomas, the 208-bp IGF-IR product was detected by PCR. The results on the first 11 patients are shown in Fig. 1 (top). In 10 of the first 11 patients, IGF-I was detected and IGF-IR was found in each of the patients (Fig. 1). Control studies showed that the PCR products were correct by sequencing and that they resulted from tumor mRNA because both the IGF-I primers and the IGF-IR primers spanned a long intron and gave no amplification with genomic DNA, as well as no product in the absence of reverse transcriptase (data not shown).

The amount of IGF-I or IGF-IR 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 competitive PCR in each sample and the final result for both was displayed by the ratio of molecules of IGF-I or IGF-IR per β-actin molecule (Figs. 2-4) for each gastrinoma. The IGF-I mRNA levels varied >254-fold, from 0.0013 IGF-I/β-actin molecule to 0.333 IGF-I/β-actin molecule (Fig. 3, top). The mean ratio of IGF-I mRNA was 0.043 ± 0.009 IGF-I/β-actin molecule (Fig. 3, top). The IGF-IR mRNA levels in different gastrinomas varied 2,670-fold, from 0.00053 IGF-IR/β-actin molecule to 1.33 IGF-IR/β-actin molecule (Fig. 3, top). The mean ratio of IGF-IR mRNA was 0.173 ± 0.035 IGF-IR/β-actin molecule (Fig. 3, top). In six gastrinomas, the IGF-I mRNA was below the minimum detection levels. The IGF-IR mRNA for a given gastrinoma showed a significant (r = 0.66, P < 0.0001) correlation with the IGF-IR level (Fig. 3, bottom).

Relationships of insulin-like growth factor 1 and insulin-like growth factor 1 receptor with the clinical or laboratory variables. Increased age (3), absence of MEN1 (4), short disease duration (5), female gender (3), or high levels of ectopic hormone release or its effects (i.e., in gastrinomas-BAO, MAO; refs. 1, 3) are clinical and laboratory variables associated with a poor prognosis in gastrinomas and/or various neuroendocrine tumors. However, the presence of none of these clinical or laboratory characteristics correlated with the magnitude of the IGF-I expression (data not shown). Similarly, none of these variables showed a significant correlation with the amount of IGF-IR mRNA expression except possibly for the presence of male gender (P = 0.034), which was associated with a lower
Table 1. Clinical characteristics, laboratory values, tumor location, and tumor extent in the patients studied

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
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<tbody>
<tr>
<td>Patients</td>
<td>54 (100)</td>
</tr>
<tr>
<td>Male</td>
<td>27 (50)</td>
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<tr>
<td>Age at surgery (y)</td>
<td>49.6 ± 1.4</td>
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<tr>
<td>[Range]</td>
<td>[15-75]</td>
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<tr>
<td>Duration of disease (y)*</td>
<td>8.7 ± 0.8</td>
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<tr>
<td>[Range]</td>
<td>[0.2-25]</td>
</tr>
<tr>
<td>Duration of postoperative follow-up (y)</td>
<td>71 ± 0.4</td>
</tr>
<tr>
<td>[Range]</td>
<td>[0.4-15.8]</td>
</tr>
<tr>
<td>Fasting serum gastrin (pg/mL)†</td>
<td>5,500 ± 2,240</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>[87-110,000]</td>
</tr>
<tr>
<td>Secretin (pg/mL)‡</td>
<td>11,100 ± 3,950</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>[55-152,000]</td>
</tr>
<tr>
<td>BAO (mEq/h)†</td>
<td>44.0 ± 3.3</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>[10.2-99.0]</td>
</tr>
<tr>
<td>MAO (mEq/h)†</td>
<td>68.9 ± 4.9</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>[22-136]</td>
</tr>
<tr>
<td>Preoperative antisecretory drug</td>
<td></td>
</tr>
<tr>
<td>H+K+ ATPase inhibitor</td>
<td>49 (91)</td>
</tr>
<tr>
<td>H2-receptor antagonist</td>
<td>5 (9)</td>
</tr>
<tr>
<td>MEN1 present</td>
<td>9 (17)</td>
</tr>
<tr>
<td>Primary tumor location§</td>
<td></td>
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<tr>
<td>Duodenum</td>
<td>30 (56)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Lymph node§</td>
<td>9 (17)</td>
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<tr>
<td>Others**</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Tumor extent at surgery§§</td>
<td></td>
</tr>
<tr>
<td>Primary only</td>
<td>18 (33)</td>
</tr>
<tr>
<td>Primary + lymph node§§</td>
<td>24 (44)</td>
</tr>
<tr>
<td>Metastatic lymph node only§§</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Liver metastases§§</td>
<td>9 (17)</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.
†Fasting serum gastrin was determined preoperatively.
‡Secretin was determined preoperatively (n = 48) and is the increase in fasting serum gastrin (pg/mL) with bolus secretin injection (2 clinical units/kg) over the mean of the two preinjection levels.
§BAO and MAO were determined preoperatively. BAO and MAO from patients without previous gastric acid – reducing surgery [BAO (n = 46), MAO (n = 37)].
§§One patient had two primary tumor locations, a duodenal and pancreatic primary.

A lymph node primary was as defined previously with only a gastrinoma in a lymph node found at surgery and the patient was disease free.
¨Primary tumors include liver (n = 1), bile duct (n = 2), non – small cell lung cancer (n = 1), and omentum (n = 1).
§§Each patient is in only one of the four categories.

Primary only refers to patients in whom gastrinomas were only resected from duodenum (n = 3), pancreas (n = 3), lymph node (n = 9), liver (n = 1), omentum (n = 1), or bile duct (n = 1). Primary with lymph node means both a primary tumor and metastasis in a lymph node were found. The primary tumor was located in the duodenum (n = 22) and pancreas (n = 2).

Metastatic lymph node only was defined as finding gastrinoma in lymph node(s) without a primary tumor and the patient was not disease free.
tumor extent \( (P = 0.0044) \) were all associated with a significantly higher expression of the IGF-IR mRNA in the gastrinomas, whereas disease-free status at the time of the study \( (P = 0.020) \) and occurrence of a relapse postresection \( (P = 0.049) \) also tended toward higher IGF-IR expression (Fig. 4, top).

**Prognostic studies: disease-free survival.** The IGF-I level did not correlate with disease-free survival (Fig. 4, bottom). Specifically, the 5-year disease-free survival rate postresection in patients with gastrinomas with IGF-I ratios above or below 0.0160 were not significantly different (70% [95% confidence interval, 46-87] versus 69% [95% confidence interval, 44-86], respectively; \( P = 0.84; \) Fig. 4, bottom). In contrast, survival was significantly better in patients with gastrinomas with lower IGF-IR levels (Fig. 4, bottom). Specifically, the 5-year disease-free survival rate postresection in patients with gastrinomas with IGF-IR levels below or above 0.040 was (92% [95% confidence interval, 65-99] versus 55% [95% confidence interval, 34-75], respectively; \( P = 0.034; \) Fig. 4, bottom).

**Amplification of the IGF-IR gene.** Rarely increased IGF-IR PCR gene copy number has been reported in other tumors (31). To determine whether IGF-IR gene amplification could contribute to the increased mRNA expression in some gastrinomas, we assessed IGF-IR gene copy number in the nine gastrinomas with the highest IGF-IR mRNA levels using differential PCR (Fig. 1, bottom). Differential PCR of IGF-IR compared with the single copy gene, \( \text{IFN-\gamma} \) (expressed as the IGF-IR/IFN-\gamma ratio), was not significantly different in the gastrinomas compared with the patients’ WBC (mean \( \pm 1 \) SD 1.75 \( \pm 0.14 \) versus 1.68 \( \pm 0.24 \) (Fig. 1, bottom)). Furthermore, no gastrinoma showed increased IGF-IR gene amplification over that seen in WBCs (Fig. 1, bottom).

**Immunohistochemistry.** To examine IGF-IR protein’s expression, immunohistochemistry was done using paraffin-embedded tissue from the breast cancer cell line MCF-7, which is known to overexpress IGF-IR (29) as well as normal pancreas, and 32 gastrinomas that had IGF-IR mRNA levels in which formalin-fixed gastrinoma tissue was available. In the MCF-7 cells, strong membranous staining pattern occurred in virtually all of the breast cancer cells (data not shown). In the normal pancreas, some normal islet cells...
stained strongly, normal acinar cells stained faintly whereas the epithelial cells of the normal pancreatic duct did not stain (Fig. 5A). In 31 of 32 gastrinomas (97%), immuno-histochemistry detected IGF-IR. Twenty-seven samples showed a diffuse and homogeneous distribution pattern (Fig. 5E) and four gastrinomas showed a focal staining pattern (Fig. 5B and C). In gastrinomas, the IGF-IR immunostaining was predominantly localized to the cytoplasm, with no staining in the stroma or in the vascular structures (Fig. 5). No significant staining was seen when the IGF-IR antibody was preabsorbed with the IGF-IR peptide to which it had been raised (Fig. 5D and F). In the 27 gastrinomas showing a diffuse and homogeneous distribution pattern, 7 (26%) showed weak staining, 13 (48%) showed moderate staining, and 7 (26%) showed strong staining. In the four gastrinomas showing a focal staining pattern, three (75%) showed strong staining and one (25%) showed moderate staining. Each of the four patients (100%) with focal staining had tumors showing aggressive growth and postoperative tumor growth compared with 10 of 27 patients (37%) who showed diffuse staining ($P = 0.032$). Furthermore, three of the four patients (75%) with gastrinomas showing an IGF-IR focal staining pattern developed liver metastases, whereas only 5 of the 27 patients (18%) with an homogeneous staining pattern had liver metastases ($P = 0.043$).

Fig. 4. Comparison of IGF-I and IGF-IR mRNA levels in gastrinomas with tumor characteristics and effect of IGF-I or IGF-IR level on disease-free survival of patients. $T_{\text{op}}$, number of patients in each category in parentheses. Disease free was defined as normal fasting gastrin levels, negative secretin test and no imageable tumor and was assessed within 2 weeks of resection (i.e., disease-free immediately) and yearly (23). Relapse refers to a patient who was disease-free postresection and had disease recurrence. Aggressive disease refers to patients postresection in whom new lesions developed, liver metastases developed, or the tumor demonstrated growth on imaging as defined in Materials and Methods (8). Bottom, Kaplan-Meier plots of disease-free survival post-surgical resection according to the IGF-I or the IGF-IR level. Eighteen patients had an IGF-I/β-actin tumor level of >0.016 (median value) and 17 patients had ≤0.016. Twenty-one patients had an IGF-IR/β-actin tumor level of >0.04 (median level) and 14 patients had ≤0.04. Abbreviations: Duod, duodenum; Panc, pancreas; Prim, primary; LN, lymph node. Bars, SE. *, $P < 0.05$; **, $P < 0.005$. 

Content: The text describes the staining patterns of IGF-IR in gastrinomas and the correlation with disease-free survival. It reports that 31 of 32 gastrinomas showed IGF-IR staining, with 27 showing a diffuse and homogeneous pattern and 4 showing a focal pattern. The diffuse staining was predominantly cytoplasmic, while the focal staining was localized. The IGF-IR antibody was still detectable after preabsorption with the IGF-IR peptide. The diffuse staining was categorized into weak (7%), moderate (13%), and strong (7%). The focal staining was categorized into strong (75%) and moderate (25%). The diffuse staining showed higher disease recurrence and postoperative tumor growth compared to focal staining ($P = 0.032$). Furthermore, the diffuse staining was associated with liver metastases ($P = 0.043$).
Gastrinomas, like other neuroendocrine tumors (pancreatic endocrine tumors and carcinoids), are generally slow-growing tumors compared with typical adenocarcinomas (2, 3, 5, 6). However, recent studies show a significant subset (20-30%) has aggressive growth (2, 3, 5, 6). Little is known about the molecular pathogenesis or determinants of aggressive growth of neuroendocrine tumors because neither common oncogenes (ras, myc, etc.) nor tumor suppressor genes (p53, retinoblastoma, etc.), which are important in the pathogenesis of common gastrointestinal adenocarcinomas (gastric, colon, etc.), are typically altered in neuroendocrine tumors (7). At present, there are few prognostic factors that are useful in an individual patient that helps in identifying the subset with aggressive disease. If such prognostic factors could be identified, more aggressive antitumor treatment or surgery could be carried out earlier in this subset with poor prognosis and perhaps, increase survival (3, 6).

Recent studies report that various growth factors such as those for vascular endothelial growth factor, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor, and IGF play an important role in growth, progression, and development of metastases by various tumors (7, 10, 11, 15, 32). IGF-I and/or its receptor’s expression in various tumors, particularly breast, prostate, colorectal, and lung cancers may be especially important in tumor growth/progression (15, 33). Increased serum levels of IGF-I are reported to be associated with increased risks of breast, prostate, colorectal, and lung cancers (15), and the IGF-IR is reported to play an important role in cell growth, proliferation, DNA synthesis, antidiifferentiation, and antiapoptosis in various cancers (15, 33).

Numerous nonendocrine tumors (pancreatic adenocarcinoma, breast cancer, colorectal, and prostate; refs. 33–37), and some endocrine tumors (thyroid, adrenocortical, and pheochromocytomas; refs. 38–41) produce IGF-I and possess or overexpress IGF-IR. Furthermore, numerous studies show IGF-I
and/or IGF-IR activation are mitogens for a wide variety of cancer (33, 34). Expression of IGF-I and/or IGF-IR in various tumors has been reported in some studies to be associated with advanced tumor stage (35, 36, 39), increased tumor size (39), proliferative activity (42), recurrence (43) or metastases (35), and a poor prognosis/survival (31, 42, 44). Other studies have reported no association of IGF-I/IGF-IR with tumor stage (36, 37, 41, 43, 44), size (43), or survival (35, 43).

In general, the expression of IGF-I and/or IGF-IR in neuroendocrine tumors, the effects of activating IGF-IR on neuroendocrine tumor growth, or clinically correlating their presence with tumor behavior, have been investigated in only a few studies involving small numbers of cases. IGF-I or IGF-IR mRNA has been reported in different neuroendocrine tumors (7, 18–20, 45, 46), as well as IGF-I synthesis and release by these tumors (19, 45, 47) and the release of the IGF-I has been shown to have an autocrine growth effect on the tumor cells (19, 47). In our study, IGF-IR and IGF-I mRNA were detected in 100% and 89% of gastrinomas, respectively, and IGF-IR protein in 97% using immunohistochemistry. These results have some similarities and differences from other studies on neuroendocrine tumors. We found IGF-I and IGF-IR mRNA in a higher percentage of pancreatic endocrine tumors than the 65% and 71%, respectively, detected by PCR in two studies (18, 20), and the results from four studies of carcinoid tumors (18–20, 46) where IGF-I was present in 84% and IGF-IR in 40%. These differences may be partially explained by differences in different neuroendocrine tumors (20). In one recent study (20), IGF-I mRNA was detected by PCR in 89% of gastrinomas, 67% to 70% of insulinomas/gastrointestinal carcinoids, and only 44% of nonfunctional pancreatic endocrine tumors. Furthermore, IGF-IR was detected in 89% to 90% of gastrinomas/insulinomas, 67% of gastrointestinal carcinoids, and 33% of nonfunctional pancreatic endocrine tumors (20). These results raise the possibility that importance of IGF-I/IGF-IR as an autocrine growth in these different neuroendocrine tumors may differ. In other endocrine malignancies, IGF-I and IGF-IR were similarly detected in a high percentage of tumors and frequently overexpressed. Specifically, IGF-I and IGF-IR were detected in 80% to 100% and 92% of undifferentiated/poorly differentiated thyroid tumors (39), respectively; 67% to 100% and 50% to 100% of follicular thyroid cancers (39, 40, 43); 39% to 100% and 42-93% of papillary thyroid cancers (39, 40, 43); 80-100% and 50% of medullary thyroid cancer (46); 57% and 71% of pheochromocytomas (38); and 90% and 75% to 94% of adenocortical cancers (38, 41). These results are similar to high percentages of some nonendocrine tumors that possess and/or overexpress IGF-IR (breast, lung, bladder, ovarian [88%], prostate [55%], colon [83-99%], pancreatic [57-75%], and gastric [71%]; refs. 34, 35, 37, 42) but differ from the lower percentages found in other nonendocrine tumors (head-neck cancer, salivary gland cancer, renal cell carcinoma [13-14%]; ref. 37).

Our results show that gastrinomas from different patients show a wide variation in the expression with IGF-I and IGF-IR mRNA levels. In follicular and papillary thyroid cancer, IGF-I and IGF-IR levels increases were reported of 2- to 7-fold, in adrenal cortical cancers IGF-IR was increased 3- to 5-fold (38), whereas in pancreatic cancer they were increased by 32- and 4.4-fold, respectively (34). Furthermore, our study showed a high significance ($r = 0.66, P < 0.0001$) between the level of expression of IGF-I and IGF-IR in a given gastrinoma. This result is similar to reports with adenocortical cancers (41), breast cancer (48), and prostate cancer (36) but differs from results with pheochromocytomas and adrenal adenomas (41). This high correlation between IGF-I and IGF-IR levels for a given gastrinoma suggests that with some gastrinomas enhanced IGF-IR activation could be mediated by two different processes that could be complementary: overproduction of IGF-I and increased IGF-IR. The mechanism of IGF-IR mRNA overexpression was not examined in detail in this study, although it is clear that similar to other nonendocrine tumors (31), it is not generally due to IGF-IR gene amplification. The mechanisms by which IGF-IR activation might stimulate growth of gastrinomas has not been studied, but in other cells, IGF-IR activation stimulates receptor tyrosine kinase activity which activates distinct transduction cascades including Ras and mitogen-activated protein kinases and phosphoinositol-3-kinase (15, 33, 49).

IGF-I and/or IGF-IR expression are reported in some studies of nonendocrine and a few endocrine tumors (31, 35, 36, 39, 42, 44) but not in other studies (35, 37, 41, 43, 44), to correlate with tumor growth, stage, invasiveness, decreased survival, or other clinical features of the tumor. In two studies involving different pancreatic endocrine tumors (7, 18, 20) and three studies involving carcinoid tumors (7, 18–20), the presence or absence of IGF-I and/or IGF-IR did not correlate with tumor aggressiveness. However, in these studies (7, 18–20), no quantitative comparisons were done. In the present study, we found that increased IGF-IR mRNA expression in gastrinoma significantly correlated with increased tumor growth, aggressive disease, and increased tumor extent as did IGF-I expression, to a lesser degree. Furthermore, increased IGF-IR levels were associated with the development of liver metastases ($P = 0.003$) and a lower long-term disease-free rate postresection ($P = 0.02$). Numerous studies have shown that the development of liver metastases in patients with malignant neuroendocrine tumors, including gastrinomas, is the most important determinant of long-term survival (2, 3, 5, 50). Therefore, our results suggest that if patients with gastrinomas are followed long enough the level of IGF-IR would likely correlate with disease-related survival. An additional finding in our study supporting this conclusion was that lower tumor IGF-IR mRNA levels were associated with a significantly higher disease-free survival rate ($P = 0.034$).

Neither gastrinoma IGF-IR nor IGF-I mRNA levels correlated with primary tumor location or size (4, 5), fasting serum gastrin levels (51), or disease duration (21), all of which have been shown to have prognostic significance in gastrinomas and/or other neuroendocrine tumors (2–5). The results with gastrinomas with an association of increased IGF-IR/IGF-I expression with the presence of aggressive disease are similar to reports with some malignant endocrine tumors (papillary thyroid cancer; refs. 39, 43) and nonendocrine tumors [colorectal tumors (35), renal cancer, and breast cancer (44)]. However, these results differ from other studies showing no correlation in the IGF-I/IGF-IR expression and tumor aggressiveness in some nonendocrine tumors (prostate cancer; refs. 36, 42). Furthermore, our results (2–4) are consistent with animal studies which show that the presence of IGF-IR on various tumors increases invasiveness, metastatic potential, and development of liver metastases (17).
The finding that both IGF-I and IGF-IR mRNA expression levels are related to gastrinoma aggressiveness and that IGF-I levels are predictive of disease-free survival could have clinical significance. At present, for an individual patient, there are no reliable prognostic factors that predict tumor aggressiveness or recurrence with sufficient certainty to be clinically useful. Therefore, all patients must undergo regular reassessments at frequent intervals including detailed imaging studies to assess possible progression/recurrence. The assessment of IGF-IR mRNA levels in the gastrinoma may allow stratification of patients to different risk levels that could be used to determine risk and allow identification of patients requiring more careful follow-up.

The fact that we find that IGF-I/IGF-IR mRNA expression correlates with aggressive gastrinoma growth and tumor extent and most gastrinomas possess each, raises the possibility that IGF-IR activation could be involved in an autocrine growth function in gastrinomas, similar to that shown in other endocrine (19) and nonendocrine tumors (34). Furthermore, the expression of IGF-I and/or IGF-IR may be important in the development and/or pathogenesis of pancreatic endocrine tumors. IGF-I has been implicated in islet development and differentiation (52). IGF-I increases islet growth (53). Furthermore, elevated levels of IGF-I have been shown to convey invasiveness and metastatic potential in a mouse model of pancreatic islet tumorigenesis (17). Whether at present the presence of either IGF-I and/or IGF-IR is important in the molecular pathogenesis of human pancreatic endocrine tumors, is at present only speculation. However, with the increased development of possible therapeutic strategies directed against the IGF-IR (10) as well as the effects of such drugs as somatostatin analogues at decreasing IGF-I secretion, the possible involvement of IGF-IR in the molecular pathogenesis of these tumors as well as our findings that expression correlates with tumor aggressiveness, raises the possibility an approach directed against IGF-IR could have therapeutic value in the treatment of these tumors.

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References

Increased Expression of Insulin-Like Growth Factor I and/or Its Receptor in Gastrinomas Is Associated with Low Curability, Increased Growth, and Development of Metastases

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