HER-2/neu Expression and Gene Amplification in Gastrinomas: Correlations with Tumor Biology, Growth, and Aggressiveness

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ABSTRACT

A proportion of gastrointestinal neuroendocrine tumors are aggressive; however, little is known of molecular determinants of their growth, and molecular studies have identified no useful prognostic factors. Overexpression of HER-2/neu is common in some nonendocrine tumors, frequently correlates with increased tumor aggressiveness, and can be used as a basis of treatment with trastuzumab. Little is known of its expression in malignant pancreatic endocrine tumors. In the present study HER-2/neu gene amplification and expression was determined in 43 gastrinomas from different patients. Results were correlated with clinical, laboratory, and tumor characteristics including tumor growth. HER-2/neu gene amplification was assessed by differential PCR, mRNA levels assessed by quantitative PCR, and protein by immunohistochemistry. Fourteen percent of patients had HER-2/neu gene amplification in tumors compared with levels in their WBCs. HER-2/neu mRNA varied over a 700-fold range. However, only 3% exceeded levels seen in normal pancreas, and immunohistochemistry did not show protein overexpression in any tumor (n = 10). HER-2/neu mRNA levels were significantly higher (P = 0.032) in tumors associated with liver metastases but not with tumor location or size. These results show that HER-2/neu amplification/overexpression does not seem to play a role in the molecular pathogenesis of most gastrinomas, as suggested in a previous study involving small numbers of cases. However, mild gene amplification occurs in a subset, and overexpression is associated with aggressiveness. Therefore, HER-2/neu levels could have prognostic significance as well as identify a patient subset with gastrinomas who might benefit from trastuzumab treatment.

INTRODUCTION

PETs,¹ including gastrinomas, resemble GI carcinoid tumors in generally demonstrating slow growth (1–3). However, recent studies show a significant subset of these tumors show aggressive growth, leading to a shortened survival (1, 3–8). In one recent study (8) no tumor-related deaths occurred in patients with slow-growing gastrinomas, whereas 62% of those with aggressively growing tumors had a tumor-related death. At present there are no clinical or laboratory features that can predict in an individual patient whether a PET will pursue an aggressive course (1–3). The identification of prognostic factors might be of great benefit in treating these patients because early aggressive antitumor treatment could be instituted, such as more extensive surgical resection. Such a new approach is particularly needed because the current treatment of patients with advanced malignant PETs is generally unsatisfactory (3, 8, 9).

In a number of nonendocrine tumors recent insights into their molecular pathogenesis have identified cellular mechanisms involved in tumor invasiveness and led to identification of prognostic factors, as well as new forms of treatment. In many nonendocrine tumors, activation of oncopgenes or inactivation of tumor suppressor genes is important in their pathogenesis and/or behavior (10, 11). However, in typical PETs and carcinoids alterations of common oncogenes (ras, myc, src, etc.) or inactivation of common tumor suppressor genes (p53 and retinoblastoma) are uncommon (12–16). Mutations in the MEN1 tumor suppressor gene occur in 16–40% of sporadic PETs and 18% of sporadic GI carcinoids (12, 13, 15–18). Recently, inactivation of the tumor suppressor gene p16INK4a/CDKN2A is reported in some GI neuroendocrine tumors (19, 20), with 50–52% of gastrinomas showing methylation of 5’-CpG islands in the p16INK4a/CDKN2A gene and 0–40% having homozygous deletions in this gene (17, 20, 21). Genetic alterations are reported in the tumor suppressor gene DPC4/Smad4 in 55% of nonfunctional PETs but not in functional PETs (22).

These results show that inactivation of the p16INK4a/CDKN2A gene and MEN1 gene mutations are likely important in the molecular pathogenesis of a subset of PETs and carcinoids. However, in a significant proportion of these tumors no molecular alterations in these genes are found, and the molecular pathogenesis is unknown (16). Furthermore, neither mutations in the MEN1 gene nor alterations in the p16INK4a/CDKN2A gene are predictive of aggressive behavior in PETs (17, 19). Therefore, studies of molecular pathogenesis of PETs/carcinoids have not identified any prognostic factor that is generally useful in an individual patient (16).

The HER-2/neu gene is a member of the ErbB-like oncogene family that maps to chromosome 17q21 (23, 24). This gene encodes a M185,000 protein (p185) with tyrosine kinase activity, which structurally resembles other members of the four-member family of epidermal growth factor receptor-related growth factor receptor family [epidermal growth factor receptor, HER-1 (c-erb-B1), Her-3 (c-erb-B3), and HER-4 (c-erb-B4)]. Overexpression of the HER-2/neu protein has been shown in experimental studies to be an important determinant of malignant transformation, development of metastatic disease, and increased cell proliferation (23, 24). Overexpression of the HER-2/neu protein has been identified in a number of nonendocrine (breast, pancreatic gastric, esophageal, prostate, and colon cancers; Refs. 23, 25–27) and a few endocrine tumors (thyroid, pituitary, and pheochromocytomas; Refs. 28–33). Furthermore, overexpression of the HER-2/neu protein correlates inversely with survival, and directly with increased invasiveness and aggressive growth in a number of these nonendocrine tumors (23, 25–27), and in one study in an endocrine cancer (thyroid cancer; Ref. 33). This has led to the development and approval by the Food and Drug Administration of a humanized monoclonal antibody against the HER-2/neu protein [trastuzumab (Herceptin)] that is now used for treatment of breast cancer (23, 24, 34). The demonstration of overexpression of HER-2/neu in other tumors would potentially make them amenable to treatment with trastuzumab (25, 35, 36).

There are only a few studies involving small numbers of cases of HER-2/neu gene amplification/protein overexpression in PETs and/or carcinoids, and they have given contradictory results (16). Some studies report an increase is frequently present in HER-2/neu gene amplification/protein overexpression, whereas others report either no

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The abbreviations used are: PET, pancreatic endocrine tumor; GI, gastrointestinal; MEN1, multiple endocrine neoplasia type 1; FBS, fetal bovine serum; HPRT, hypoxanthine phosphoribosyltransferase; BAO, basal acid output; MAO, maximal stimulated acid output.
change or only that infrequently was an increase seen (20, 29, 37–41). Therefore, the current study was designed to examine whether HER-2/neu gene amplification and/or overexpression occurred in gastrinomas, which are the most common functional malignant PET (9, 42). Sufficient numbers of cases were included, which had been followed long term to allow correlations with tumor growth patterns. Such correlations allowed us to assess whether levels of expression of this proto-oncogene might be useful either prognostically for identifying patients with aggressive disease or possibly for identifying a subset that possibly might benefit from trastuzumab treatment.

MATERIALS AND METHODS

Patients

Forty-three patients who underwent exploratory laparotomy for sporadic Zollinger-Ellison syndrome at the NIH between 1990 and 1998, and who had a gastrinoma resected or biopsied were included in this study. The study protocol was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases, and all of the patients gave informed consent. The diagnosis of Zollinger-Ellison syndrome was established as reported previously (43). Serum gastrin levels were analyzed by RIA by Bioscience Laboratories (New York, NY) or Mayo Clinic Laboratories (Rochester, MN). The duration of disease was defined as the time from onset of continuous symptoms compatible with Zollinger-Ellison syndrome to surgery (44). A negative family history and lack of laboratory evidence of other endocrinopathies on yearly evaluation, as described previously (45), diagnosed the absence of MEN1. Duration of disease was defined by the clinical history from the time of symptom onset as described previously (7). All of the patients underwent an exploratory laparotomy with an extensive intraoperative evaluation for attempted curative resection, and determination of the primary tumor site and size (46). Tumor volume at time of surgery was determined by measuring the individual tumor lesions removed during surgery (8). Postoperatively the patients were reassessed within 2 weeks of surgery and in 3–6 months to determine disease activity, and then annually to monitor for progression of disease (43). Tumor growth postoperatively was defined as an increase in size or number of lesions on annual imaging studies and continued evidence of active disease. As part of previous studies from our institution some of the tumors had been analyzed for mutations in the MEN1 gene (17) and alterations of the p16 gene (19), and these results were correlated with the HER-2/neu expression levels found in this study.

Cell Lines

The breast cancer cell lines (SK-BR-3, BT474, MDA-MB-453, BT483, BT20, and MDA-MB-468) were obtained from American Type Culture Collection (Rockville, MD). MDA-MB-453 and MDA-MB-468 were grown in DMEM and 10% FBS; SK-BR-3 was grown in McCoy’s 5A medium with 10% FBS; BT474 and BT483 were grown in RPMI 1640 with 10% FBS and 10 µg/ml bovine insulin, and BT 20 was grown in Eagle’s minimal essential medium with 10% FBS.

Tumors

Differential PCR. Tumor samples were immediately snap frozen in liquid nitrogen during surgery and stored at −70°C. Tumor DNA was extracted from 8-µm cryosections of the specimens using a commercial kit (QiAmp Blood kit; Qiagen Inc., Santa Clarita, CA) after analyzing an adjacent slide with H&E staining to determine the extent of normal tissue present. Specimens with a tumor content of <80% of the entire tissue were dissected to enrich the tumor fraction. Germ-line DNA was extracted using the same kit from leukocytes of 17 of these patients. DNA from the breast cancer cell line SK-BR-3 was extracted after the third passage of cells using the same kit. Differential PCR to determine the gene copy number of the HER-2/neu oncogene compared with the single copy gene IFN-γ was performed according to the method described by Frye et al. (47). Briefly, a 98-bp fragment of the HER-2/neu oncogene was amplified using the following primers: sense, CCTCTGACGCTCATCCTC; antisense, ATCTTCCTGTCCGTCGCTT, and a 150-bp fragment of the IFN-γ gene was produced using the following primers: sense, TCTTT-TCTTTCGCCATGAGG; antisense, CAGGGGATGCTCTTGACCTTC. Both fragments were amplified in the same vial using the following conditions: 94°C for 10 min and 35 cycles of 94°C, 50°C, and 70°C for 1 min each in a thermocycler (9700; Perkin-Elmer Corp.). Bands were separated on 10% polyacrylamide gels, stained with ethidium bromide, and band intensities were measured with an image analysis program (NIH-IMAGE).

RNA Quantitation. Tumor RNA was extracted from 8-µm cryosections of the specimens using a commercial kit (RNeasy Mini kit; Qiagen Inc.) after analyzing an adjacent slide with H&E staining to determine that the slides contained >80% tumor tissue. Purified pancreas RNA was purchased from Clontech (Clontech, Palo Alto, CA), and RNA was isolated from four breast cancer cell lines SK-BR-3, BT474, MDAMB-453, and BT483 (American Type Culture Collection) using a commercial kit (Trizol; Life Technologies, Inc. Grand Island, NY). Random hexamer-primed first-strand cDNA was prepared with reverse transcription (RNA PCR kit; Perkin-Elmer, Foster City, CA). 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 270-bp fragment of the human HER-2/neu oncogene cDNA with the following primers: sense (S), 5′-GAAATTTAGACGAAGCATACG-3′ corresponding to nucleotides 2470–2491 of the human c-erb-B-2 mRNA (Genbank accession no. 03363), and antisense (AS), 5′-ACTCTTTGACGACGTTTCC-3′ corresponding to nucleotides 2720–2739. PCR was run under the following conditions: 94°C for 10 min, 32 cycles of 94°C, 60°C and 72°C for 50 s each, and final extension at 72°C for 5 min. Using the same PCR conditions except for the addition of 10% DMSO a 204-bp fragment of the human β-actin gene cDNA was amplified with these primers: S, 5′-CCCTGCGTTTCGCGATACCC-3′ and AS, 5′-GGAATTTTCCTTGCACATACG-3′, which corresponds to 25–42 (sense) and 210–229 (antisense) from Hom sapiens actin β (ACTB) mRNA (GenBank accession no. XM037235-1). The mimics for competitive PCR were constructed using intron sequences of the HPRT gene to make different size products. For preparation of the HER-2/neu oncogene mimic (233 bp) a fragment of the HPRT gene of 193 bp was amplified using sense primers: 5′-TCCCTGAGATTTGTAAGG-3′ and antisense primers: 5′-CCCTGCTTGACTGCTATT-3′, which correspond to nucleotides 16615–16634 (sense) and 27892–27911 (antisense) of the HPRT gene (GenBank accession no. M26434). This product was then amplified using gene-specific HER-2/neu primers: sense, 5′-AACCTTGAAGGATCACGTCGATGAGG-3′ and antisense, 5′-ACTCT-TGACGACGCTTTCCTCCCCCGGACCTAGTT-3′, to obtain the 233-bp HER-2/neu mimic. To obtain the 289-bp β-actin mimics, a 249-bp fragment of the HPRT gene was amplified using sense, 5′-CATTTGAGGCCTCTCTGTGTC-3′ and antisense, 5′-CTGCATGGTTTCTGCCAGT-3′ corresponding to nucleotides 16651–16670 (sense) and 34948–34967 (antisense) of the HPRT gene (GenBank accession no. M26434). This product was then amplified using β-actin gene-specific primers: sense, 5′-CCCTGCGTTTCGCGATATTTGCTGTCG-3′ and antisense, 5′-TGCTATCCTCCTGATCCTCCTCCTCCTCTTTGGCAGTTG-3′ to obtain the 289-bp β-actin mimic. Stock solutions of the mimics were prepared by purifying the PCR solutions with a Microcon 30 Filter (Amicon Inc., Beverly, MA). The concentrations of the mimic stock solutions were determined by measuring the absorbance at 260 nm in a spectrophotometer (Beckman Coulter Inc., Columbia, MD) and serial dilutions prepared. Competitive PCR was performed by adding 1 µl of cDNA solution to 1 µl of serial mimic-dilutions with the respective primer pairs (HER-2/neu/S/AS or β-actin-S/AS) under the appropriate PCR conditions. Initially log dilutions of the mimics were used, then serial dilutions of 25%, 50%, and 75% for each log unit to obtain a more precise standard curve to calculate the amount of mRNA present. The amount of measured target mRNA was determined by calculating the concentration of the mimic that resulted in equal intensity of ethidium bromide staining in a 1% agarose gel. Results of the competitive PCR were expressed as the ratio of the number of molecules of the HER-2/neu mRNA to β-actin mRNA present.

Immunohistochemistry. Immunohistochemical staining was performed using the Hercept-kit (Dako Corp.) and an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) according to the company’s protocols.

Briefly, 8-μm thick paraffin sections were mounted on plain glass slides. After deparaffinization and rehydration the slides were placed in a microwave pressure cooker in 0.01 M citrate buffer (pH 6.0; Biogenex, San Ramon, CA.) containing 0.1% Tween 20 and heated in a microwave oven at maximum power (900 W) for 40 min. Sections were immediately cooled in Tris-buffered saline (0.05 M; pH 7.6). Thereafter, all of the sections were washed in Tris-buffered saline (pH 7.6) containing 5% goat serum (Life Technologies, Inc.) for 30 min. Slides were incubated with the primary polyclonal antibody A0485 (Hercept; Dako Corp.) in a dilution of 1:500 for 12 h at room temperature. The rest of the procedure (secondary antibody, avidin-biotin complex, color development, and counterstain) was performed on the Ventana immunostainer.

RESULTS

The clinical and laboratory characteristics of the 43 patients studied are similar to other series of patients with Zollinger-Ellison syndrome (44, 48–51) with respect to a slight male predominance (62%), mean age of patients at surgery in the fifth decade (48 year), and long duration of disease (9 year; Table 1). All of the patients had either an elevated fasting serum gastrin level or an abnormal secretin-stimulated gastrin provocative test, and the majority had a markedly elevated preoperative BAO and MAO (51, 52). The location and extent of the tumor encountered during surgery was comparable with other recent surgical series (46, 53) in that the duodenum was the primary tumor site in almost half the patients (42%), and in the remaining patients the primary tumor site was equally distributed between the pancreas, lymph nodes, and other sites (Table 1). In 2 patients only lymph node metastases were found at surgery without a primary tumor localized. During the postoperative follow-up of 3.1 ± 0.4, one-half the patients remained disease free (Table 1). Of the 22 patients that were not disease-free after the resection almost two-thirds of the tumors did not show any growth during the postoperative follow-up. In 40% of the patients not cured the tumors displayed growth, and the tumor in 3 of these patients demonstrated aggressive growth with the development of liver metastases during the postresection follow-up time (Table 1).

DNA was extracted from all 43 of the tumors (the primary or a regional lymph node metastasis), leukocytes from 17 of these patients, and from the breast cancer cell line SK-BR-3, which is reported to have amplification of the HER-2/neu gene copy number (54–57) for differential PCR for the HER-2/neu and IFN-γ genes. Fig. 1 (left panel) shows an example of the differential PCR result from 4 patients and from the SK-BR-3 breast cancer cells. Differential PCR demonstrated a 3-fold amplification of the HER-2/neu oncogene over the IFN-γ gene in the breast cancer cell line SK-BR-3 (Fig. 1, top left panel). However, in the 4 gastrinomas (Fig. 1, middle left panel) the intensity of the HER-2/neu band was less than that of the IFN-γ band; therefore, the HER-2:IFN-γ ratio was <1, indicating no amplification of the HER-2/neu oncogene relative to the IFN-γ gene in any of the gastrinomas if this criteria of overamplification is used (58–61). In the WBCs from the same 4 patients (Fig. 1, bottom left panel), similarly no amplification of the HER-2/neu oncogene over the IFN-γ gene was seen using a ratio >1. In the first 17 patients both the gastrinoma and WBC HER-2:IFN-γ ratio were determined using differential PCR, and the presence of the two genes was not significantly different in both tissues (tumor versus WBC: 0.60 ± 0.03 versus 0.62 ± 0.02; Fig. 1, right panel). Using the SD of the HER-2:IFN-γ ratio for the leukocytes in these 17 patients a 97.5% confidence interval was calculated (Fig. 1, right panel). Gastrinomas from 6 patients had a significantly higher HER-2:IFN-γ ratio than the mean ± 3 SD of the leukocytes (i.e., >0.86; P < 0.025; Fig. 1, right panel). In none of the tumor specimens was the ratio >1.5-times
the ratio in nontumor cells, >1, and in no patient gastrinoma did the ratio approach the 3.2-fold amplification seen in the SK-BR-3 breast cancer cell line (Fig. 1, right panel).

RNA of sufficient quality and quantity for amplification of the HER-2/neu and ß-actin transcripts was available from gastrinomas from 33 patients. Fig. 2 shows an example of the competitive PCR for the determination of the HER-2/neu transcript amount from 2 patients. Where the intensity of the ethidium bromide staining of the upper band is calculated to equal that of the lower band is the amount of molecules in the unknown sample. A similar competitive PCR was performed to determine the amount of ß-actin in each sample. Using the dilution of the sample added, the number of molecules of HER-2/neu and ß-actin were determined in all of the samples and expressed as a ratio (i.e., neu/ß-actin ratio; Fig. 3). The amount detected in the gastrinomas varied over a 700-fold range from 0.00029 to 0.19 with a mean of 0.0193 ± 0.007 neu molecules per ß-actin molecule (Fig. 3). In tissue from 4 normal pancreas the ratio for HER-2/neu over ß-actin expression varied over a 4.5-fold range from 0.0275 to 0.125 with a mean of 0.075 ± 0.032 HER-2/neu molecule per ß-actin molecule (Fig. 3, middle column). In contrast, in the four breast cancer cell lines known to overexpress HER-2/neu (SK-BR-3, BT474, MDA-MB-453, and BT483;Refs. 54, 56, 62) significantly more HER-2/neu molecules per ß-actin molecule were found compared with two cell lines not overexpressing HER-2/neu (BT20 and MDA-MB-468; Refs. 54, 62; Fig. 3, right column). When a 95% coefficient interval for the relative expression of HER-2/neu compared with ß-actin was calculated from normal pancreas only 1 of 33 (3%) gastrinomas exceeded this range (Fig. 3, left panel).

HER-2/neu overexpression is known to correlate with tumor aggressiveness, prognosis, and invasiveness in some endocrine (thyroid cancer and pheochromocytomas) and nonendocrine (breast, gastric, pancreatic, and esophageal) cancers (23, 25, 26, 30, 31, 33). Therefore, we compared the amount of HER-2/neu expression with the presence or absence of various clinical, laboratory, and tumoral features reported to correlate with malignant behavior of gastrinomas and/or PETs (Refs. 1, 6, 7; Fig. 4). No clinical (gender, curability, and disease duration) or laboratory (fasting gastrin level) parameter showed a significant correlation with the amount of HER-2/neu mRNA expression in the gastrinomas (Fig. 4, upper right panel). Significantly higher expression of the HER-2/neu mRNA was found in gastrinomas (i.e., the primary or from a regional metastatic lymph node) associated with liver metastases ($P = 0.032$) although the numbers were small. Whether the HER-2/neu expression was the same in the primary and its liver metastases was unknown because insufficient tissue was available from liver metastases because their presence was established by cytology with immunocytochemical analysis. The presence of other tumoral characteristics reported to be associated with aggressive behavior (1, 6, 7) such as nonduodenal primary tumor location, increased tumor size, increased number of tumor sites, or tumor volume were not associated with increased HER-2/neu mRNA expression (Fig. 4, left panel). Similarly, the presence or absence of other genetic abnormalities (p16 INK4a gene mutation or p16(INK4a) gene promoter methylation) known to frequently occur in gastrinomas (15, 17, 19, 20) did not correlate with the amount of HER-2/neu mRNA expression in the tumors (Fig. 4, lower right panel).

To examine protein expression, immunohistochemistry with an antibody directed against the membranous portion of the HER-2/neu protein was performed using paraffin-embedded tissue from the breast cancer cell line SK-BR-3, which is known to overexpress HER-2/neu protein (56, 62, 63), normal pancreas, and 10 gastrinomas. Staining showed a strong membranous staining pattern in virtually all of the

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**Fig. 2.** Ethidium bromide staining of the competitive PCR for the HER-2/neu from the tumors of 2 patients in a 1% agarose gel. The arrow indicates where the calculated amount of unknown equals the amount of competitor and represented the amount of HER-2/neu in the unknown sample. In the top panel this was determined to be 60 molecules in the 1 μl volume of tumor sample added, and in the bottom panel it was determined to be 38 molecules/1 μl volume of tumor sample added.

**Fig. 3.** Distribution of the amount of HER-2/neu mRNA present in gastrinomas, normal pancreas, and positive controls. Quantitative PCR results were obtained from gastrinomas from 33 patients ( ), four normal pancreas ( ), and six breast cancer cell lines (BT20, MDA-MB-468, SK-BR-3, BT474, MDA-MB-453, and BT483, ). Four cell lines (SK-BR-3, BT474, MDA-MB-453, and BT483; half-filled squares) are reported to overexpress HER-2/neu and two cell lines ( ) not to overexpress the HER-2/neu gene (54, 56, 62). The horizontal line with vertical bars represents the mean for the neu/ß-actin ratio determined by quantitative PCR in the gastrinomas; bars, ± SE.
breast cancer cells (Fig. 5, top left). In the normal pancreas removed during surgery from one of the patients with a gastrinoma, a few normal islet cells stained with the antibody (Fig. 5, bottom left). This indicated that our tissue samples still contained the HER-2/neu antigen and therefore any lack of immunostaining in gastrinomas was not likely because of damage to the tissue, which can occur during storage (37, 39, 64). In the normal pancreas some of the islet cells and entire exocrine pancreas were devoid of any positive HER-2/neu staining (Fig. 5, bottom left). Similarly, in each of the 10 gastrinomas examined no immunohistochemical staining with the HER-2/neu antibody could be detected in the tumor. Examples of two of the gastrinomas with negative staining are shown in Fig. 5 (right column). Two gastrinomas that had slightly elevated HER-2/neu:IFN-H9253 ratios (Patients 38 and 40) were included in those studied by immunocytochemistry and each was negative for HER-2/neu staining in the tumor.

**DISCUSSION**

The purpose of the present study was to assess the frequency of amplification of HER-2/neu gene and possible overexpression of its products in gastrinomas. The identification of a subset of patients with gastrinomas overexpressing either the HER-2/neu gene and/or its product might be clinically useful for management of these patients. HER-2/neu amplification/overexpression might prove to be an independent predictor of the growth behavior of the gastrinoma as it has for a number of nonendocrine tumors (23, 25, 26). Secondly, HER-2/neu amplification/overexpression could provide the basis to consider treatment of this subset of patients with the HER-2/neu humanized monoclonal antibody, trastuzumab. Trastuzumab, either alone or in combination with other antitumor agents, is currently being used in patients with breast cancer (23, 34) and considered for use in other cancers with HER-2/neu amplification/overexpression (25, 35, 65).

Our results demonstrate that HER-2/neu gene amplification is uncommon in gastrinomas, and when it occurs it is only a minimal increase compared with the magnitude of the overexpression in other tumors. Differential PCR was used, which is one of the established methods to detect amplification of the HER-2/neu oncogene (47, 58, 66). Using this method, none of the 43 gastrinomas in the present study demonstrated amplification of the HER-2/neu oncogene to a level greater than the single copy IFN-H9253 gene in any of the tumors. However, in 14% of the tumors (i.e., 6 of 43) the ratio of HER-2/neu oncogene to IFN-H9253 exceeded by 3 SDs the mean of the ratio for the leukocyte DNA of the patients (P < 0.025), suggesting mild amplification of the HER-2/neu oncogene was present in these gastrinomas. This level of HER-2/neu amplification is much less than the 2 to 12-fold reported in gastric cancer (67), breast cancer (54, 58–60, 68), urinary bladder cancer (57, 61), and ovarian cancer (69). Furthermore, using the criterion of at least a 2-fold amplification, which is used in many other studies for a positive result for HER-2/neu gene amplification (58–61, 69), our findings in gastrinomas would be interpreted as none of the gastrinomas showing significant HER-2/neu gene amplification.

Our results differ from one study (40) that reported all 12 of the gastrinomas studied demonstrated HER-2/neu gene amplification (2–12-fold). However, they are in agreement with three other studies (70–72) involving small numbers of gastrinomas. In each of these latter three studies (70–72) comparative genomic hybridization was used and identified no overexpression on chromosome 17q in gastrinomas, the location of the HER-2/neu gene. Similarly, in a study of 18 midgut carcinoids, which have many similarities to PETs, by com-
However, overexpression of HER-2/neu protein occurs without HER-2/neu gene amplification. These results suggest that different PETs/carcinoids may differ in the frequency with which HER-2/neu gene amplification occurs and its role in their molecular pathogenesis. Our results with gastrinomas also differ from findings in a number of nonendocrine cancers where HER-2/neu gene amplification is not infrequent (breast, 20%; esophageal, 20%; and gastric, 10–33%; 25); esophageal, 33–53%; (25); and colon, 10% (25)). To investigate the possibility that HER-2/neu mRNA and/or protein overexpression could be occurring in gastrinomas, as well as to determine whether the mild amplification of the HER-2/neu gene in a subset of the gastrinomas resulted in increased expression, the HER-2/neu mRNA levels were determined by qualitative PCR in 33 gastrinomas in which frozen tissue existed and HER-2/neu protein expression was examined by immunocytochemistry in a proportion of the tumors. Quantitative PCR was used because studies demonstrate that immunocytochemical methods may not be reliable for quantitative assessment of low levels of increased HER-2/neu protein expression (23, 88). Furthermore, in both endocrine cancers including PETs and carcinoids (29), as well as some nonendocrine cancers (i.e., gastric (89)) there was a close correlation between HER-2/neu mRNA expression and protein expression. To correct for small variations in both sample input and amount detected, in our study, qualitative PCR was used for both determining the amount of HER-2/neu mRNA and β-actin present, and the result normalized to the β-actin amount. The relative amount of HER-2/neu protein on immunocytochemical analysis including 2 patients who had tumors with slightly increased HER-2/neu gene amplification. These results are similar to two other studies involving small numbers of gastrinomas, which reported 1 of 6 tumors (17%; Ref. 37) and 0 of 20 (0%; Ref. 20), demonstrated HER-2/neu protein overexpression. These results
are also similar to some studies involving other PETs and/or carcinoids, which reported 0–9% (20, 38, 39, 72) but not others, which reported 17–100% of the tumors (29, 37, 41, 90), demonstrated HER-2/neu overexpression with increased membrane staining using immunohistochemical methods. The findings in some of these studies on various PETs/carcinoids at first appear to conflict with results from a study involving 36 PETs examined by comparative genomic hybridization (72) in which 41% had amplification of chromosome 17q, the location of the HER-2/neu gene. However, in that study (72) only 9% of the PETs with HER-2/neu gene amplification had overexpression of HER-2/neu protein by immunohistochemical studies. The above results on HER-2/neu mRNA and/or protein expression in gastrinomas are consistent with the conclusion that amplification of HER-2/neu gene or overexpression of its product do not seem to be involved in the molecular pathogenesis of most gastrinomas.

In a number of both endocrine [thyroid cancer (30, 33) and pheochromocytomas (31)] and nonendocrine cancers [breast (23, 36, 56), gastric (25), colon (25), andovarian (80)] amplification of the HER-2/neu gene or overexpression of HER-2/neu mRNA/protein correlate with aggressive growth, invasiveness, and/or decreased survival. There is very limited information in PETs/carcinoids of the possible role of HER-2/neu overexpression as a prognostic factor for tumor growth behavior, and the two available studies have given conflicting results. One study (41) involving 10 carcinoid tumors concluded that HER-2/neu amplification may be an important prognostic factor for tumor growth behavior. However, another study (37) involving small numbers of PETs/carcinoids showed no relationship between overexpression of HER-2/neu and survival, tumor invasiveness, or presence of liver metastases. In our study, the presence of various laboratory or tumor characteristics reported to be associated with aggressive behavior of gastrinomas (1, 6, 7, 48, 91), such as male gender, high fasting gastrin levels, short disease duration, large primary size, nonnodulon primary location, or increased numbers of tumors, was not associated with higher HER-2/neu mRNA levels. However, a significantly higher expression of HER-2/neu mRNA was found in gastrinomas associated with liver metastases (P = 0.032) although the numbers of tumors in the different groups were small. These results suggest that increased HER-2/neu expression is a factor determining the aggressiveness of some gastrinomas. However, whether assessment of HER-2/neu expression will be clinically useful as a prognostic factor in these tumors is unclear, and until this is resolved, routine assessment of its expression cannot be recommended. This uncertainty is attributable partially to the fact that the extent of overexpression is relatively modest compared with nonendocrine tumors (54, 58–60, 67, 69). This modest increase will make immunohistochemical assessment difficult because this method may be unreliable when HER-2/neu overexpression is low (88), and, thus, to obtain meaningful data careful quantitative assessment will be needed, which is time consuming and expensive. Nevertheless, there are very few prognostic factors useful in an individual patient with a potentially malignant PET/carcinoid, and current antitumor treatments once the disease becomes advanced, are generally unsatisfactory (3, 8, 9, 12). However, if an aggressive phenotype could be recognized earlier, more aggressive surgical resection or early antitumor treatment could be considered, which might improve survival. Therefore, additional studies correlating tumor growth with HER-2/neu mRNA levels in GI carcinoids and PETs should be performed including a larger number of patients.

In previous studies mutations in two tumor suppressor genes, the MEN1 gene and the p16INK4α/CDKN2A gene, were reported in 16–90% of PETs and/or carcinoids (17, 19, 20, 20, 21). However, in none of these studies was the presence of abnormalities in either of these genes associated with increased tumor aggressiveness, liver metastasis, or decreased survival. In the present study we found no correlation between the expression level of HER-2/neu in gastrinomas and the occurrence of abnormalities in either of these two tumor suppressor genes, providing no support for a cooperative interaction of these abnormalities in increasing gastrinoma aggressiveness.

In conclusion, our results do not provide evidence for a general role for overexpression of either the HER-2/neu gene or its product in the molecular pathogenesis of gastrinomas, in contrast to the suggestion from a previous study (40) involving a few cases. However, our results do show that minimal HER-2/neu gene amplification occurs in a small subset (14%) of gastrinomas. Furthermore, significantly higher HER-2/neu mRNA levels are found in more aggressive gastrinomas. Because the current treatments of advanced malignant PETs/carcinoids are generally inadequate and because tumor progression is now the main determinant of survival in these patients (1, 6, 7), the finding of higher levels of HER-2/neu mRNA in more aggressive tumors raises the possibility it may be a useful prognostic factor as well as the possibility these patients might benefit by trastuzumab treatment, either alone or in combination with other agents. However, before either of these two possibilities can be established it is important that HER-2/neu expression be assessed in a study containing larger numbers of patients with progressive liver metastases. Because most deaths occur in the subset with progressive metastatic disease (8), the establishment that increased HER-2/neu expression occurs in this subset or is predictive of entering this subset will strongly support both its clinical importance and its use as a prognostic factor or basis for antitumor treatment.

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HER-2/neu Expression and Gene Amplification in Gastrinomas: Correlations with Tumor Biology, Growth, and Aggressiveness

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