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ORIGINAL PAPER

Signal Transduction Proteins in Tumors From Puerto Rican and Caucasian Gastric Adenocarcinoma Patients: Expression Differences With Potential for Specific Targeted Therapies

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Abstract Overexpression of the *HER2/NEU* gene is associated with aggressive behavior and poor prognosis in breast cancer, making the Her2/neu protein a directed-therapy target. Tumors of two Puerto Rican (PR) patients overexpressed Her2/neu and resulting partial clinical responses motivated us to compare Her2/neu expression in PR ($n = 101$) and Caucasian non-Hispanic ($n = 95$) patients. Immunohistochemistry of tumors showed overexpression of p-Stat3, Cyclin D1, and Her2/neu, compared to non-neoplastic mucosa. Her2/neu and EGF-R protein levels were statistically significantly different with higher levels of both proteins in the PR group. Importantly, Her2/neu expression was strong and diffuse in tumors with signet-ring morphology, while other histo-pathological subtypes showed higher intra-tumoral Her2/neu heterogeneity than typically observed in breast cancer. Targeted therapies in gastric cancer directed at EGF-R and Hers-2/neu pathways warrant further investigation. These therapies may be especially effective in PR patients and in patients with signet-ring cell morphologies with a dismal prognosis.

Keywords Stomach cancer · Gastric adenocarcinoma · Her2/neu · Epidermal growth factor receptor · Stat3 · Cyclin D1

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Abbreviations

PR	Puerto Rico
NCCTG	North Central Cancer Treatment Group
5-FU	5-Flurouracil
FAMe	5-Flurouracil, doxorubicin and methyl-lomustine
FAP	5-Flurouracil, doxorubicin and cisplatin
i.v.	Intravenous
EGF-R	Epidermal growth factor receptor
HER-2	Epidermal growth factor receptor 2
c-*erbB*-2	*HER-2/NEU*
Stat3	Signal transduction and transcription 3
p-Stat3	Phospho-stat3
GE	Gastro-esophageal
PET	Positron emission tomographic
TPF	Docetaxel, cisplatin and 5-FU
MCC	H. Lee Moffitt Cancer Center and Research Institute
FISH	Fluorescence in situ hybridization
RECIST	Response Evaluation Criteria in Solid Tumors
HIER	Heat-induced epitope retrieval

Introduction

Adenocarcinoma of the stomach is the second leading cause of cancer death worldwide with 700,000 new cases reported annually [1]. In the United States, approximately 59,000 new cases and 13,500 deaths are reported every year [2]. Advanced adenocarcinoma of the stomach continues to have a poor prognosis, with a median survival of 6 months. The only potentially curative treatment is complete surgical resection. Unfortunately, over 70% of patients experience postsurgical recurrence.

According to the 2000 US Census Bureau report [3], Puerto Ricans account for 9.7% of the Hispanic population in the United States. Migration studies [4, 5] have shown that, from 1958 to 1971, Puerto Ricans living in New York City had lower mortality and higher survival rates from stomach cancer than Puerto Ricans who resided in Puerto Rico (PR); similar results have been reported for age-adjusted mortality rates [6, 7]. At the University Hospital of Puerto Rico, the largest tertiary hospital on the island, clinical parameters, mortality, and survival were studied in 394 patients with gastric adenocarcinoma [8]. The male to female ratio was 2.5:1, the median age at presentation was in the seventh decade, and the most frequent presenting symptoms were weight loss, vomiting, and abdominal pain. At presentation, approximately 50% of patients had localized disease and 33% had distant metastases. The most common surgical procedure performed was a subtotal gastrectomy. Mortality within 1 month after surgery was 20%; overall 5-year survival was 8.4%. These results are similar to those previously reported in other Hispanic populations, and slightly better than those reported in Caucasians, although ethnicity appears not to have a significant survival impact [9, 10] when controlling for differences in disease site, stage, and comorbidities.

Responses to single-agent chemotherapy are usually not durable and the use of combination chemotherapy, slightly more effective than single-agent therapy in achieving objective radiographic responses, has not led to significant survival advances. Thus, the North Central Cancer Treatment Group (NCCTG), in a prospective study comparing treatment with a single-agent (5-fluorouracil) and three multi-agent systemic chemotherapy regimens (5-flurouracil, doxorubicin and methyl-lomustine [FAMe]; 5-flurouracil, doxorubicin and Cisplatin [FAP]; and FAMe alternating with triazinate), reported no survival benefits [11]. Molecular-targeted agents may lead to significant improvement in our ability to control outcomes in the treatment of gastric cancer, as has been the case for other tumors.

Since its identification more than 20 years ago, intracellular signaling mediated by the epidermal growth factor receptor (EGF-R) has proven to play an important role in cancer-cell proliferation, angiogenesis, and metastasis [12], and is responsible in part for adverse prognosis [13]. The *HER2/NEU* oncogene, on chromosome 17q, shares significant homology with the *EGF-R* gene [14]. Her2/neu testing has become the standard-of-care in the pretreatment evaluation of breast cancer. There is also interest in Her2/neu expression as a prognostic factor and potential target of therapy in tumors of the gastrointestinal tract [15]. In gastric adenocarcinoma, high levels of EGF-R and Her2/neu expression are associated with low overall survival; however, the reported range of Her2/neu expression varies widely [16–21]. This may be due to differences in patient selection, since Her2/neu overexpression appears to be more common in advanced tumors rather than in early disease. The consensus in the literature, however, is that Her2/neu status has significant prognostic value in gastric cancer [22–25].

Partial clinical success with Trastuzumab (Herceptin) in two patients whose tumors overexpressed Her2/neu stimulated us to evaluate Her2/neu expression in a large number of Puerto Rican patients to investigate the potential pathogenic role of EGF-R and Her2/neu pathways in gastric adenocarcinoma. We also analyzed the expressions of Cyclin D1, as a proliferative marker, and of phospho-Stat3 (p-Stat3), since expression of p-Stat3 correlated with that of Her2/neu in the context of a breast carcinoma clinical trial performed by our group [26]. In an effort to understand potential ethnic differences at the molecular level, we compared results obtained in Puerto Rican patients, who resided on the island, with those of a similar group of

Caucasian non-Hispanic patients from H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida.

Methods

Case study # 1

A 39-year-old male, diagnosed at age 14 with stage IIA Hodgkin's disease, was treated with mantle radiation and remained asymptomatic for 25 years. He then began to lose weight and experience progressive dysphagia to solids. An abdominal phospho-Stat3 (p-Stat3) scan revealed thickening of the gastro–esophageal (GE) junction, antrum, and posterior fundus compatible with a gastric neoplasm, as well as nodular densities at the gastro–hepatic ligament and peripancreatic region. An upper endoscopy confirmed the presence of a mass at the GE junction and a biopsy revealed a poorly differentiated adenocarcinoma with signet-ring cell features. An FDG positron emission tomographic (PET) scan demonstrated radiotracer avidity in the stomach and multiple regional gastric nodes, but a chest CT scan revealed no thoracic metastases. Classified as stage T3N2M1, a total gastrectomy with Roux-en-Y pouch reconstruction and D2 lymphadenectomy were performed. During surgery, extensive disease was identified at the celiac axis, gastric lesser curvature, and porta hepatitis. Pathologic examination revealed a poorly differentiated invasive adenocarcinoma with signet-ring features, involving the upper third, middle, and lesser curvature of the stomach (cardia, fundus, and corpus). The tumor invaded the esophageal adventitia and showed vascular invasion and involvement of the surgical esophageal margins. There were metastases in 12 of 27 lymph nodes with 1/3 of the retro-pancreatic lymph nodes positive. Immunohistochemistry showed that the tumor cells were positive (2–3+) for Her2/neu and focally positive for EGF-R. He received postoperative radiation to the tumor bed at a dose of 48 Gy, delivered in 27 fractions. One month following completion of radiation therapy, four chemotherapy cycles of Docetaxel, Cisplatin, and 5-Flurouracil (5-FU) were administered every 4 weeks [27]. Also, weekly Trastuzumab (Herceptin) was given for 13 cycles. Given the expression of Her2/neu in tumor cells, Trastuzumab (4 mg/kg i.v. loading and 2 mg/kg i.v. weekly thereafter) was administered for 4 months following the cytotoxic chemotherapy. Three years later he was recurrence-free as evidenced by normal serum carcinoembryonic antigen, negative thoracic and abdomino-pelvic CT scans, and a normal PET scan.

Case study # 2

A 32-year-old male marathon runner, with no history of prior illness, presented with phlebitis and bilateral deep vein thromboses of the lower extremities during training prior to a marathon competition. Abdominal and thoracic CT scans revealed bilobar hepatic lesions and thickening of the gastric fundus at the level of the GE junction. Endoscopy revealed a polypoid mass at the distal esophagus extending into the GE junction and cardia. Biopsy of the mass demonstrated a well-differentiated adenocarcinoma, intestinal type. He was treated with one cycle of 5-FU (425 mg/m^2 i.v.) and leucovorin (20 mg/m^2 i.v.) daily for 5 days. His course was complicated by a pulmonary embolism treated with an inferior vena cava filter. Immunohistochemistry of the tumor showed positivity (2+) for Her2/neu. Chemotherapy was started with Docetaxel, Cisplatin, and 5-FU (TPF) every 4 weeks for six cycles in combination with weekly Trastuzumab for 28 weeks. A follow-up CT scan 2 months later showed interval improvement and partial radiographic remission by Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The patient remained asymptomatic and was able to run a marathon the following year. Eight months later, he presented with vestibular symptoms and ataxia. An MRI revealed brain metastases that were treated with palliative external whole-brain beam radiation with a total dose of 30 Gy in 10 fractions. Four months later, he developed radiographic evidence of progression of his hepatic disease. After recurrence, weekly Paclitaxel and Trastuzumab were given for five cycles, but his condition deteriorated until death due to disease progression.

Selection of cases

One hundred cases of gastric adenocarcinoma were retrospectively selected at the Universidad de Puerto Rico in San Juan (PR) (Hispanic population), along with 95 cases at the H. Lee Moffitt Cancer Center and Research Institute (MCC), Tampa (FL) (Caucasian population). Clinico–pathological information was obtained for all cases after institutional review board approval at both institutions and adherence to HIPAA guidelines. From the available material, paraffin blocks containing representative areas of both tumor and adjacent normal tissue were selected. Confirmation of the diagnosis and quantification of marker signal were performed independently in the two patient groups by two pathologists (BC and CMC) with experience in gastro–intestinal pathology.

Immunohistochemistry (IHC)

Immunohistochemistry conditions for all markers were optimized at the MCC Pathology Core. For Her2/neu, antigen retrieval was performed in a microwave oven with Heat-induced epitope retrieval (HIER)-citrate buffer. The

rabbit polyclonal antibody (DakoCytomation) was used at a concentration of 1:300 for 30 min, and detection was performed using the EnVision + detection kit. For EGF-R, antigen retrieval was performed with proteinase K digestion (25 μg/ml for 17 min). The clone 111.6 mouse monoclonal antibody (Signet Laboratories) was used at a concentration of 1:50 for 30 min, and detection was performed using the DakoCytomation LSAB + Detection Kit with avidin/biotin blocking (Fig. 1). For Cyclin D1, antigen retrieval was performed in a microwave oven using a HIER-EDTA buffer. The clone AM29 mouse monoclonal antibody (Zymed Laboratories) was used at a concentration of 1:100 for 30 min, and detection was performed using the DakoCytomation LSAB2 Detection Kit with avidin/biotin blocking. For p-Stat3, antigen retrieval was performed as previously described [28] in a microwave oven with HIER-citrate buffer and mild trypsinization. The Tyr705 rabbit polyclonal antibody (Cell Signaling, USA) was used at a concentration of 1:400 overnight, and detection was performed using the Vector Laboratories (USA) Vectastain Elite Detection Kit with avidin/biotin blocking. Her2/neu and EGF-R were located on the membrane, and Cyclin D1 and p-Stat3 in the nucleus.

Quantification of signals

A semi-quantitative method was used. For each of the markers, the percentage of positive cells, signal intensity, and tissue distribution of positive cells were scored independently by two investigators using an Olympus BX40 microscope, with Olympus Plan lenses, at final magnifications ranging from 200× to 1000× (see Figs. 1 and 2 for details), with comparable results. For each slide and for each marker, signal intensity was assigned to one of four categories already familiar to the practicing surgical pathologist: negative (0), mild (1), moderate (2), and high (3). The percentage of positive cells in each of the four intensity categories was estimated and this percentage was then multiplied by its corresponding intensity score (0–3). The sum of the four products was reported as the final score and used for statistical analysis. Her2/neu expression was also quantified according to the Dako FDA-approved method. Following the approach recommended for breast cancer, a score of 1+ was considered negative and a score of 3+ positive. Fluorescence in situ hybridization (FISH) to detect amplification of the *HER2/NEU* gene was performed on cases with a score of 2+ using the PathVysion Her2DNA Probe Kit at USLABS (Irvine, CA).

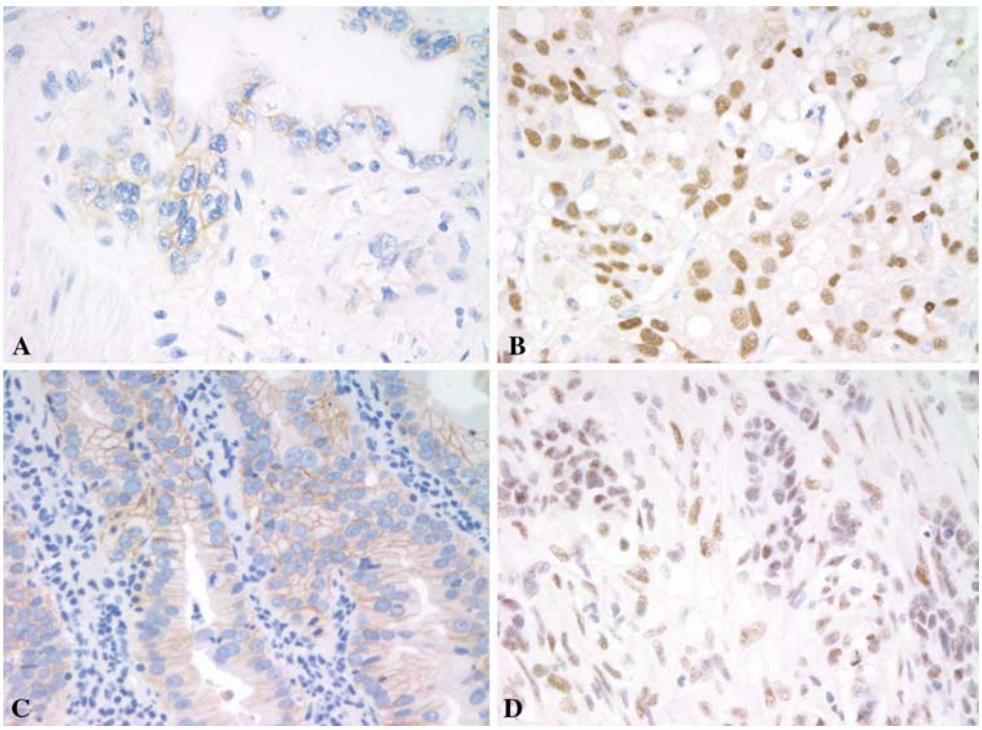

Fig. 1 Immunohistochemical detection of proteins in gastric adenocarcinoma. EGF-R (**a**), Cyclin D1 (**b**), Her2/neu (**c**), and p-Stat3 (**d**) in tumors. EGF-R is detected in a membranous location with variations in intensity. In the figure, panel A shows a low-level of expression while panel B shows a high level of expression. Cyclin D1 was observed in nuclear location. Expression of p-Stat3, the activated form of Stat3, was observed with moderate to strong intensity in the nuclei of the vast majority of tumor cells

Statistical methods

The demographic and clinico–pathological parameters of the two ethnic groups were summarized using descriptive statistics. The differences in these parameters between the two groups were compared using Pearson's χ^2-square test for categorical variables and Wilcoxon Mann–Whitney test for continuous variables. The mean and standard deviation of protein expression in tumor and in adjacent normal mucosa were calculated for each ethnic group. Protein expression was explored with graphical illustrations and was tested for the normality assumption. For each protein expression the difference between tumor and normal tissue was compared using Wilcoxon signed rank test. Then, the difference in each protein expression between the two ethnic groups was tested using the Wilcoxon Mann–Whitney test. For tumor tissue, the associations of each protein expression with tumor location, stage, grade, and tumor size were analyzed using the Kruskal–Wallis Test (for categorical variables) and Spearman correlation coefficient (for continuous variables). A *P*-value of less than 0.05 was considered statistically significant for all the statistical analyses; no multiple comparisons were adjusted for *P*-values. SAS 9.1 software was used for the data analysis.

Table 1 Demographics and tumor characteristics by ethnicity

	Caucasian ($n = 95$)	Puerto Rican ($n = 101$)
Gender, N (%)		
Male	54 (56.8)	101 (100)
Female	41 (43.2)	0
Tumor size, mean (SD), cm	4.0 (3.7)	3.8 (3.2)
Tumor location, N (%)*		
Antrum	14 (14.7)	35 (35.4)
Fundus	10 (10.5)	27 (27.3)
GE junction	30 (31.6)	12 (12.1)
Body	24 (25.3)	19 (19.2)
Other	16 (17.7)	6 (6.0)
Tumor differentiation, N (%)		
Well differentiated*	8 (8.4)	27 (26.7)
Moderately differentiated*	34 (35.8)	15 (14.9)
Poorly differentiated	53 (55.8)	59 (58.4)
Disease stage, N (%)		
I	22 (23.2)	10 (9.9)
II	14 (15.1)	13 (13.1)
III	40 (42.1)	23 (22.8)
IV	17 (18.3)	53 (53.5)

* Statistically significant difference between Caucasians and Puerto Ricans ($P < 0.05$). Data were not available for tumor stage and/or location for 2 patients from each group

Results

At the University of Puerto Rico, the tumors of two Puerto Rican patients were found to overexpress Her-2/neu and these patients were treated with Herceptin. The resulting partial clinical responses motivated us to evaluate Her2/neu expression to investigate the potential pathogenic role of the EGF-R and Her2/neu pathways in gastric adenocarcinoma. Demographics and descriptive statistics of the tumors analyzed are depicted in Table 1. Puerto Rican patients were treated at the Veterans Affairs Hospital and all were males. In the Caucasian group from H. Lee Moffitt Cancer Center and Research Institute, 57% were males and 43% females. The effect of this gender difference was evaluated: no differences in gene expression were found between males and females, suggesting that the high proportion of males in the Puerto Rican group would not affect the overall analysis. No significant difference was found in tumor size between the two patient groups (*P* value = 0.358).

Statistically significant differences were found with regard to location within the stomach, disease stage at presentation, and in degree of differentiation ($P < 0.01$). However, the frequency of poorly differentiated tumors was similar in both groups (56% in Caucasians and 58% in Puerto Ricans). Similarly, no statistically significant difference was found in the number of patients presenting with stage II (13% of Puerto Ricans and 15% of Caucasians).

Figure 1 shows representative tissue expression of p-Stat3, Cyclin D1, and EGF-R. When compared with adjacent nonneoplastic mucosa, p-Stat3, Cyclin D1, and Her2/neu were overexpressed in the tumors of both populations ($P < 0.01$, data not shown). The expression of EGF-R in the tumors was higher and significantly different from that of adjacent nonneoplastic mucosa only in the Puerto Rican patients ($P = 0.014$). In Caucasian patients, expression of EGF-R protein was observed neither in nonneoplastic mucosa nor in tumors. The percentage of tumors positive for p-Stat3, Cyclin D1, EGF-R, and Her2/neu were, respectively, 98%, 48%, 3%, and 8%, in Caucasian patients, and 85%, 45%, 15%, and 13% in Puerto Rican patients (Table 2). These differences were statistically significant only for p-Stat3 ($P = 0.003$) and EGF-R ($P = 0.005$). Semiquantitative analysis of the levels of expression of the proteins in the tumors revealed statistically significant differences between the two groups only for EGF-R ($P = 0.004$) and Her2/neu ($P = 0.003$), with higher levels of expression of both proteins in tumors from Puerto Rican patients (Table 3). Positive Her2/neu expression by immunohistochemistry was subsequently analyzed by Fluorescence in situ hybridization (FISH).

Table 2 Protein expression in tumors

Protein	MCC %	PRCC %
p-Stat3*	98	85
Cyclin D1	48	45
EGF-R*	3	15
Her2/neu	8	13

Protein expression (independent of the intensity) was used to determine percent positivity for p-Stat3, Cyclin D1, and EGR proteins. For Her2/neu cells positivity was determined by a combination of immunohistochemistry and FISH analysis as described in materials and methods

* Statistically significant difference between Caucasians and Puerto Ricans ($P < 0.05$)

Table 3 Levels of protein expression in tumors of both ethnic groups

	Caucasian			Puerto Rican		
	N	Mean (SD)	Min–Max	*N*	Mean (SD)	Min–Max
p-Stat3	93	1.86 (0.99)	0.0–3.00	98	1.71 (1.15)	0.0–3.00
Cyclin D1	94	0.19 (0.48)	0.0–2.97	98	0.29 (0.54)	0.0–2.40
EGF-R*	95	0.01 (0.06)	0.0–0.50	98	0.15 (0.52)	0.0–3.00
Her2/neu*	95	0.16 (0.57)	0.0–2.97	101	0.53 (0.94)	0.0–3.00

A semi-quantitative method was used to examine levels of protein expression. For this, signal intensity was assigned one of four categories: negative (0), mild (1), moderate (2), and high (3). The percentage of positive cells in each of the four intensity categories was estimated and this percentage was then multiplied by its corresponding intensity score (0–3)

* Statistically significant difference between Caucasians and Puerto Ricans ($P < 0.05$). Data for some specimens were excluded due to difficulties with interpretation or staining quality

Figure 2 shows representative Her2/neu expression detected by immunohistochemistry and amplification of the *HER2/NEU* gene detected by FISH. When the results of both immunohistochemistry and FISH were evaluated, Her2/neu was considered positive in 8% of Caucasians and in 13% of Puerto Ricans, a difference that is statistically significant ($P = 0.003$). Table 4 shows the correlation between protein expression and clinico–pathological parameters. In Puerto Ricans, expression of p-Stat3 appears to be higher in larger and poorly differentiated tumors and in patients with a higher disease stage. These correlations were not found in the Caucasian group. No correlations were found for the other three proteins.

Importantly, a significant degree of intra-tumoral heterogeneity was observed in Her2/neu and EGF-R expression (Fig. 2). Some tumors were diffusively positive while others were positive in some regions and entirely negative in others, with no clear relationship between the superficial and invasive components of the tumor. Of interest, however, is that Her2/neu was uniformly overexpressed in tumors with signet-ring cell morphology (data not shown). Not enough lymph node metastases were available for analysis to identify any relationship in expression between the primary tumor and its metastases.

Discussion

In Western countries, the overall incidence of gastric cancer is declining while that of proximal gastric cancer is increasing [28]. Ethnic variations in disease features may be in part responsible for these results. Thus, native white Americans are at low risk, while American Indians, Blacks, and Hispanics, as well as immigrants from Russia, Scandinavia, Japan, and some Latin American countries are at high risk [9, 10, 28, 29]. From 1958 to 1979, the incidence of gastric cancer in Puerto Rican-born residents in New York City was slightly higher than in Puerto Ricans living in Puerto Rico, and comparable to that of other Hispanic groups that migrated to New Mexico and Los Angeles [4–8]. This rising incidence of gastric cancer in Puerto Ricans has been accompanied by a decrease in overall mortality [2, 3, 11, 30]. Although environmental factors [31] and favorable disease biology [32–34] have been implicated to explain this trend, our study shows that the disease at presentation is more advanced in Puerto Ricans than in Caucasians. Further investigation into the epidemiology, pathogenesis, and molecular biology of gastric cancer in Hispanic patients is therefore warranted [33–37].

One factor that may affect the geographical and ethnic variation in gastric cancer epidemiology is infection by *H. pylori*, now categorized by the International Agency for Cancer Research as a group 1 carcinogen, an agent carcinogenic to humans [38]. Several reports from the United States have found the highest frequencies of gastric cancer in geographic areas and populations with the highest rates of acquisition of *H. pylori* infection [39, 40], and a high prevalence of *H. pylori* infection has also been documented in blacks and Hispanics in general [41, 42]. Since a relationship between *H. pylori* infection and Her2/neu expression and other parameters has been previously reported [39], we are currently evaluating our cases to investigate the potential role of *H. pylori* infection in gastric oncogenesis in the two ethnic groups.

Recently, the development of targeted therapies has made the identification of potential molecular targets in tumors a necessity. The *HER2/NEU* gene, located on chromosome 17q21, is related to the *v-erbB* oncogene of the avian erythroblastosis virus. It encodes a trans-membrane glycoprotein receptor with intrinsic tyrosine kinase activity homologous to EGF-R but unable to bind EGF.

Table 4 Mean difference in protein expression in relation to location, grade, and stage by groups

Protein	Characteristic		Caucasians		Puerto Ricans	
			Mean (SD)	*P*-value	Mean (SD)	*P*-value
Her-2/neu	Location	Antrum	0.42 (1.06)	0.842	0.64 (1.02)	0.697
		Body	0.09 (0.27)		0.54 (0.83)	
		Fundus	0.22 (0.66)		0.46 (0.76)	
		GE junction	0.12 (0.54)		0.57 (1.05)	
		Other	0.07 (0.22)		0.18 (0.49)	
	Grade	MD	0.15 (0.55)	0.229	0.47 (0.68)	0.108
		PD	0.16 (0.62)		0.42 (0.87)	
		WD	0.14 (0.29)		0.82 (1.16)	
	Stage	I	0.28 (0.85)	0.807	0.67 (1.11)	0.767
		II	0.06 (0.18)		0.36 (0.54)	
		III	0.15 (0.52)		0.29 (0.59)	
		IV	0.13 (0.51)		0.67 (1.08)	
p-Stat3	Location	Antrum	1.87 (1.04)	0.477	1.71 (1.21)	0.323
		Body	1.94 (1.00)		1.62 (1.14)	
		Fundus	2.18 (1.03)		1.70 (1.17)	
		GE junction	1.60 (0.98)		2.13 (0.93)	
		Other	1.98 (0.93)		1.31 (1.21)	
	Grade	MD	1.68 (0.98)	0.384	1.76 (1.21)	0.027
		PD	1.93 (0.99)		1.97 (1.05)	
		WD	2.15 (1.01)		1.15 (1.16)	
	Stage	I	1.81 (1.03)	0.901	1.43 (1.20)	0.034
		II	2.06 (0.76)		1.08 (1.17)	
		III	1.77 (1.00)		1.51 (1.05)	
		IV	1.83 (1.13)		2.00 (1.13)	
Cyclin D1	Location	Antrum	0.33 (0.73)	0.789	0.18 (0.37)	0.653
		Body	0.13 (0.22)		0.26 (0.52)	
		Fundus	0.21 (0.47)		0.35 (0.64)	
		GE Junction	0.19 (0.59)		0.43 (0.69)	
		Other	0.12 (0.29)		0.43 (0.61)	
	Grade	MD	0.13 (0.29)	0.926	0.21 (0.38)	0.596
		PD	0.25 (0.60)		0.33 (0.57)	
		WD	0.05 (0.07)		0.26 (0.55)	
	Stage	I	0.22 (0.63)	0.654	0.28 (0.60)	0.299
		II	0.12 (0.25)		0.30 (0.42)	
		III	0.18 (0.34)		0.11 (0.31)	
		IV	0.22 (0.72)		0.36 (0.63)	
EGF-R	Location	Antrum	0.01 (0.04)	0.469	0.13 (0.38)	0.062
		Body	0.00 (0.00)		0.34 (0.95)	
		Fundus	0.00 (0.00)		0.05 (0.17)	
		GE Junction	0.02 (0.09)		0.00 (0.00)	
		Other	0.00 (0.00)		0.44 (0.78)	
	Grade	MD	0.02 (0.09)	0.502	0.09 (0.31)	0.123
		PD	0.00 (0.02)		0.21 (0.62)	
		WD	0.00 (0.00)		0.07 (0.35)	
	Stage	I	0.01 (0.03)	0.709	0.00 (0.00)	0.371
		II	0.01 (0.04)		0.23 (0.83)	
		III	0.01 (0.08)		0.30 (0.71)	
		IV	0.00 (0.00)		0.10 (0.35)	

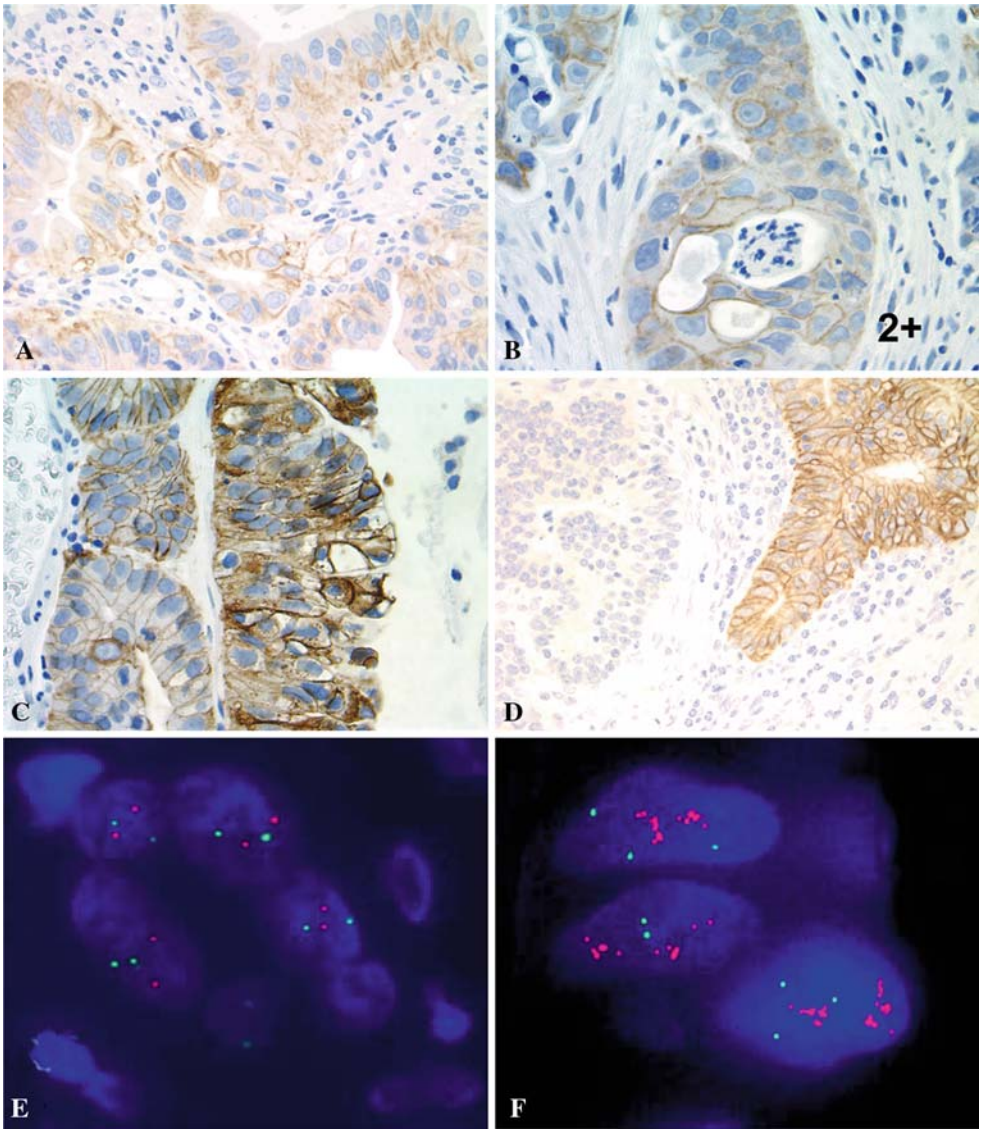

Fig. 2 Analysis of Her2/neu by immunohistochemistry (**a**–**d**) and FISH (**e**, **f**). Positive Her2/neu expression (score 3+) at low power (**a**) and high power (**b**, **c**) in tumors from Puerto Ricans (same patterns were observed in tumors from Caucasian patients). Heterogeneity of expression is evident within a single gland (**b**, **c**). Differences of expression can be observed between adjacent glands of equivalent degree of differentiation. Panel (**d**) shows negative malignant glands (*left*) and positive (3+) glands (*right*). FISH showing no gene amplification (**e**), and multiple copies of the *HER2/NEU* gene (**f**)

Amplification of the *EGF-R* and/or the *HER2/NEU* oncogenes, and overexpression of their proteins, have been reported at various levels in gastric adenocarcinoma [15–25, 43]. In fact, correlations between Her2/neu protein overexpression and *HER2/NEU* gene amplification have been reported [44–47], and overexpression of Her2/neu appears to be an independent prognostic factor in gastric cancer [18, 48–50].

In our study, both Her2/neu and EGF-R were expressed at higher levels in Puerto Ricans than in Caucasians. Studies using cell lines [46, 51] have revealed a potential therapeutic use in targeting the Her2/neu signaling pathway. Specifically, treatment of gastric cancer cell lines with Trastuzumab resulted in decreased proliferation [52, 53], suggesting a growth dependency on this pathway in this tumor type and a potential application of Trastuzumab in this disease known to carry a dismal prognosis. The results obtained with the two Puerto Rican patients described here, treated with Trastuzumab, are the first reported with this type of treatment.

Previous reports of Her2/neu expression in human gastric tumors range from 6 to 23% of the tumors overexpressing Her2/neu [54–56]. The overexpression that we observed for Caucasians (6%) and Puerto Ricans (13%) falls within this range. Some of this variability is probably due to geographic and ethnic factors, with a higher

incidence seen in the Japanese than in other groups [56]. Ex vivo analysis of our tumors reveals a high degree of intra-tumor heterogeneity in Her2/neu expression. With the exception of signet-ring cell carcinomas, where Her2/neu tends to be diffusively and strongly expressed, a very high degree of variation in the expression of Her2/neu is observed in most cases. Thus, within a given tumor, areas of very high Her2/neu expression are often seen adjacent to completely negative areas, without obvious morphological differences between the two regions. This intra-tumor heterogeneity has been previously reported [44], and may be a significant impediment in achieving successful therapeutic response, since Her2/neu-negative tumor cells are expected to be insensitive to the target therapy and with time will repopulate the tumor.

Previous reports of gastric tumors for the expression of the other biomarkers report similar levels of expression as those examined in this study. For example, overexpression of Cyclin D1 was seen in 35% of gastric tumors from Brazilian patients (compared to 48% and 45% reported in this study for Caucasian and Puerto Rican patients, respectively) [57].

The prominent overexpression of p-Stat3 in the vast majority of tumors of both patient populations suggests a pathogenic role of the Stat3 pathway in gastric adenocarcinoma that merits further investigation. Since there were no observable differences between the two patient groups, Stat3 may play a more universal pathogenetic role than Her2/neu. Stat3 inhibitors, however, are not yet available for therapy. It is important to emphasize that evaluation of Her2/neu expression, not only in the primary tumor but also in its metastases, may add very valuable information with regard to prognosis and therapy selection. Furthermore, given the concomitant overexpression of EGF-R identified in our cases and in previous reports [14, 58, 59], improved survival in gastric adenocarcinoma could be achieved with the use of multiple targeted therapies, either simultaneously or sequentially.

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