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HER Family Protein Expression in a Greek Population with Gastric Cancer. A Retrospective Hellenic Cooperative Oncology Group Study

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Abstract. *Background: Gastric cancer is a relatively common malignancy. Recently, the presence of the human epidermal growth factor receptor 2 (HER2) was identified as a molecular target in a proportion of patients who benefited from the addition of appropriate anti-HER2 treatments. Our study explored the clinical and prognostic role of known HER family members, human epidermal growth factor receptor 1 (EGFR or HER1), HER2, HER3 and HER4. Patients and Methods: Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples from 249 gastric cancer patients were studied by immunohistochemistry for protein expression of EGFR, HER2, HER3 and HER4. Results: Of the 249 evaluable patients, 32 did not have complete data of treatment details and/or follow-up and were excluded from the survival analyses. Of the 217 patients with complete treatment and follow-up data, 178 were operated and treated for early disease (group 1), while 39 for advanced disease (group 2). The frequency of positive EGFR, HER2, HER3 and HER4 protein expression in all patients was 17.5%, 11.8%, 14.8% and 32.9%, respectively. There were no differences in protein expression of any of the markers between the two groups. There were, however, statistically significant associations between HER4 and all other HER family members, as well as between HER2 and HER3 expression. Of note, EGFR-positive membranous protein expression was significantly associated with the presence of lymphovascular invasion (p=0.027) and HER3*

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and HER4 negative cytoplasmic protein expression with well/moderately-differentiated tumors (p=0.030 and p=0.014, respectively). None of the HER family members were of prognostic value for OS in univariate analysis. Conclusion: The present study confirmed the known protein expression frequencies of HER family members in gastric cancer in a Greek population. Several associations were observed among the HER family members and between clinicopathological characteristics and HER family members. Further research is needed on their exact prognostic role, as well as their therapeutic targeting.

Gastric cancer is one of the most common malignancies. Almost one million new cases of gastric cancer were estimated to have occurred in 2012 (952,000 cases, 6.8% of the total), making it the fifth most common malignancy in the world after cancers of the lung, breast, colorectum and prostate. Gastric cancer is the cause of 723,000 deaths from cancer (8.8% of the total), making it the third leading cause of cancer-related death worldwide (1).

The epidermal growth factor family of receptor tyrosine kinases (ErbBs) consists of four members: EGFR (ErbB1, HER1), ErbB2 (HER2, *neu* in rodents), ErbB3 (HER3) and ErbB4 (HER4). These receptors play an essential role in regulating cell proliferation, survival, differentiation and migration and are involved in the pathogenesis and progression of several solid tumors that include cancer of the breast, lung, colon, ovary and stomach (2). Several malignancies have been associated with mutations in ErbB receptors or increased expression of members of the ErbB family including lung, breast, stomach, colorectal, head and neck and pancreatic carcinoma (3).

The expression of several members of the ErbB family of receptors in gastric cancer has been examined in several studies, in different patient populations and with different methods. This probably has led to different percentages of expression and therefore the prognostic utility of these markers has not been clearly established. Few studies have examined all four members simultaneously.

The aim of this study was to retrospectively evaluate the expression of all four HER family receptors in gastric cancer tumor tissue samples from Greek patients that have been collected in the tumor tissue repository of the Hellenic Cooperative Oncology Group, correlate their expression with other clinicopathological factors, and evaluate their possible prognostic role.

Patients and Methods

Patients. The present study included 249 patients that underwent surgery for gastric cancer in Greece from 1991 to 2008 and for whom tissue was available at the Hellenic Cooperative Oncology Group tumor tissue repository. This study has been approved by the institutional review board of the Papageorgiou Hospital, Thessaloniki, Greece. TNM staging was according to AJCC 7th edition (4). The REMARK diagram for the study is shown in Figure 1.

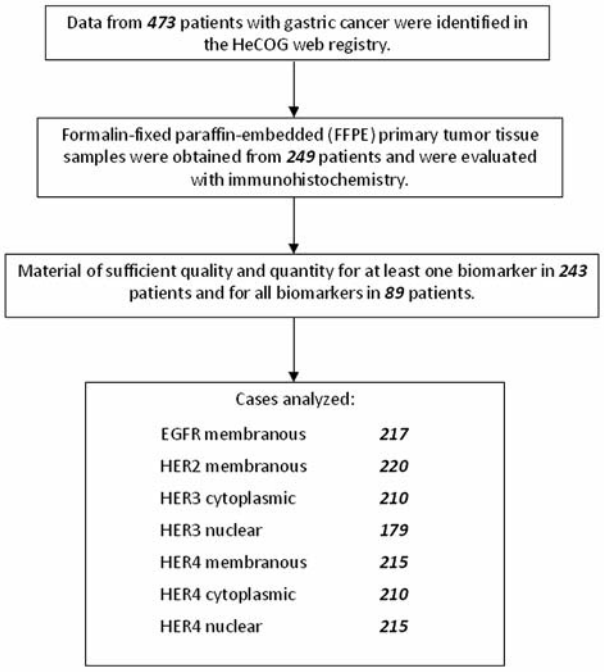

Figure 1. *REMARK diagram for the study.*

Patient characteristics are presented in Table I. Of the 249 evaluable patients, 32 did not have complete data of treatment details and/or follow-up and were excluded from the survival analyses. The 217 patients with complete treatment and follow-up data (eligible cohort) were separated in two groups according to the use of systemic therapy for advanced disease at some point of their disease course. Group 1 included 178 of the 249 patients (71.4%) that had early-onset disease (surgery with or without adjuvant treatment) and group 2 included 39 patients (15.6%), who received systemic treatment for advanced disease. Only one patient did not have surgery and received chemotherapy for advanced disease, and for this analysis he was included in group 2. All patients entered the study before the introduction of trastuzumab treatment, therefore none of them were treated with trastuzumab.

Basic patient and disease characteristics. Basic patient and tumor characteristics in the whole sample, the eligible cohort and the early and advanced disease groups are presented in Table I. No differences were observed in any of these characteristics when the whole sample was compared to the eligible cohort of patients. The median age in the early disease group was 67.1 years whereas in the advanced disease group it was 64.4 years. The male to female percentage did not differ in the two groups (66.3% male *vs*. 33.7% female and 66.7% male *vs*. 33.3% female, respectively). The most common histological type in both groups was the intestinal (51.7 and 48.7%, respectively), while poor differentiation was the commonest histological grade (68.6 and 61.1%, respectively).

Lymphovascular invasion was identified in more patients with advanced disease compared to the early disease group (p=0.036). Other variables, such as age, gender, tumor location, histological grade or histological type did not differ between the two groups.

Table I. *Basic patient and tumor characteristics*.

			Group	
	Whole sample	Eligible cohort	Early disease	Advanced disease
Patients				
N	249	217	178	39
Age				
Median	66.0	66.0	67.1	64.4
Mean (SD)	64.7 (10.9)	64.7 (10.6)	65.7 (10.0)	59.9 (12.2)
Min-Max	21-90	21-90	26-90	21-76
	N (%)	N (%)	N (%)	N (%)
Gender				
Male	162 (65.1)	144 (66.4)	118 (66.3)	26 (66.7)
Female	87 (34.9)	73 (33.6)	60 (33.7)	13 (33.3)
Tumor location (N=243)				
Proximal	51 (21.0)	42 (19.8)	33 (18.8)	9 (25.0)
Andrum	137 (56.4)	118 (55.7)	99 (56.2)	19 (52.8)
Distant	55 (22.6)	52 (24.5)	44 (25.0)	8 (22.2)
Histological type				
Intestinal	128 (51.4)	111 (51.2)	92 (51.7)	19 (48.7)
Diffused	99 (39.8)	87 (40.0)	73 (41.0)	14 (35.9)
Mixed	22 (8.8)	19 (8.8)	13 (7.3)	6 (15.4)
Histological grade (N=242)				
Well-Moderately differentiated	75 (31.0)	69 (32.7)	55 (31.4)	14 (38.9)
Poorly differentiated	167 (69.0)	142 (67.3)	120 (68.6)	22 (61.1)
Lymphovascular invasion (N=239)				
Not present	110 (45.8)	102 (48.8)	91 (52.0)	11 (32.4)
Present	130 (54.2)	107 (51.2)	84 (48.0)	23 (67.6)
Residual tumor (N=239)				
No (R0)	227 (95.0)	196 (94.2)	168 (94.4)	28 (71.8)
Microscopic (R1)	11 (4.6)	11 (5.3)	8 (4.5)	3 (7.7)
Cannot be assessed (Rx)	1 (0.4)	1 (0.5)	2 (1.1)	8 (20.5)
TNM stage (N=239)				
I	22 (9.2)	20 (9.6)	17 (9.8)	3 (8.8)
II	69 (28.9)	63 (30.3)	57 (32.8)	6 (17.6)
III	141 (59.0)	118 (56.7)	98 (56.3)	20 (58.8)
IV	7 (2.9)	7 (3.4)	2 (1.1)	5 (14.7)
TNM stage (grouped) (N=239)				
I-II	91 (38.1)	83 (39.9)	74 (42.5)	9 (26.5)
III-IV	148 (61.9)	125 (60.1)	100 (57.5)	25 (73.5)

TNM, Tumor node metastasis.

Tissue Microarray (TMA). Formalin-fixed paraffin-embedded (FFPE) primary tumor tissue samples were selected from the archival tissue material of the Hellenic Cooperative Oncology Group tumor repository. Tissue specimens were prepared in a TMA format at the TMA lab of Yale University (New Haven, CT, USA) containing one 0.6 mm tumor tissue core for each primary tumor. In total two duplicate TMA blocks were created. In each TMA block, 493 tissue cores were included, of which, 18 cores were from normal gastric specimens and 14 cores from the FFPE cell line pellets MCF7, HT-29, MB-435, H1666-162, 2CHO HER2 B, SW480, A431 and H1666-162 [obtained from the American Type Culture Collection (Manassas, VA, USA) or donated by other labs].

Immunohistochemistry (IHC). IHC staining for HER1 (EGFR), HER2, HER3 and HER4 was performed on serial 3-μm thick sections from both duplicate TMAs at the Laboratory of Molecular Oncology of the Hellenic Foundation for Cancer Research/Aristotle University of Thessaloniki School of Medicine. The EGFR (clone 31G7; Invitrogen, Carlsbad, CA, USA, at dilution 1:50), HER2 (code Nu. A0485, Dako, Glostrup, Denmark, at dilution 1:500), HER3 (clone SGP1; Thermo Fisher Scientific, Fremont, CA, USA, at dilution 1:80) and HER4 (83B10; Cell Signaling Technology, Danvers, MA, USA, at dilution 1:400) antibodies were subjected to IHC labeling using the Bond Max™ (Leica Microsystems, Wetzlar, Germany) and i6000 (Biogenex, San Ramon, CA, USA) autostainers. For identification of tumor cells the sections were also stained with an in-house cytokeratin cocktail. DAB (3,3-diaminobenzidine) was used as a chromogen and hematoxylin as a counterstain.

IHC evaluation. IHC staining was evaluated at the pathology department of the Patras University Hospital by an experienced pathologist (ACT). In each case, the intensity of the staining, the

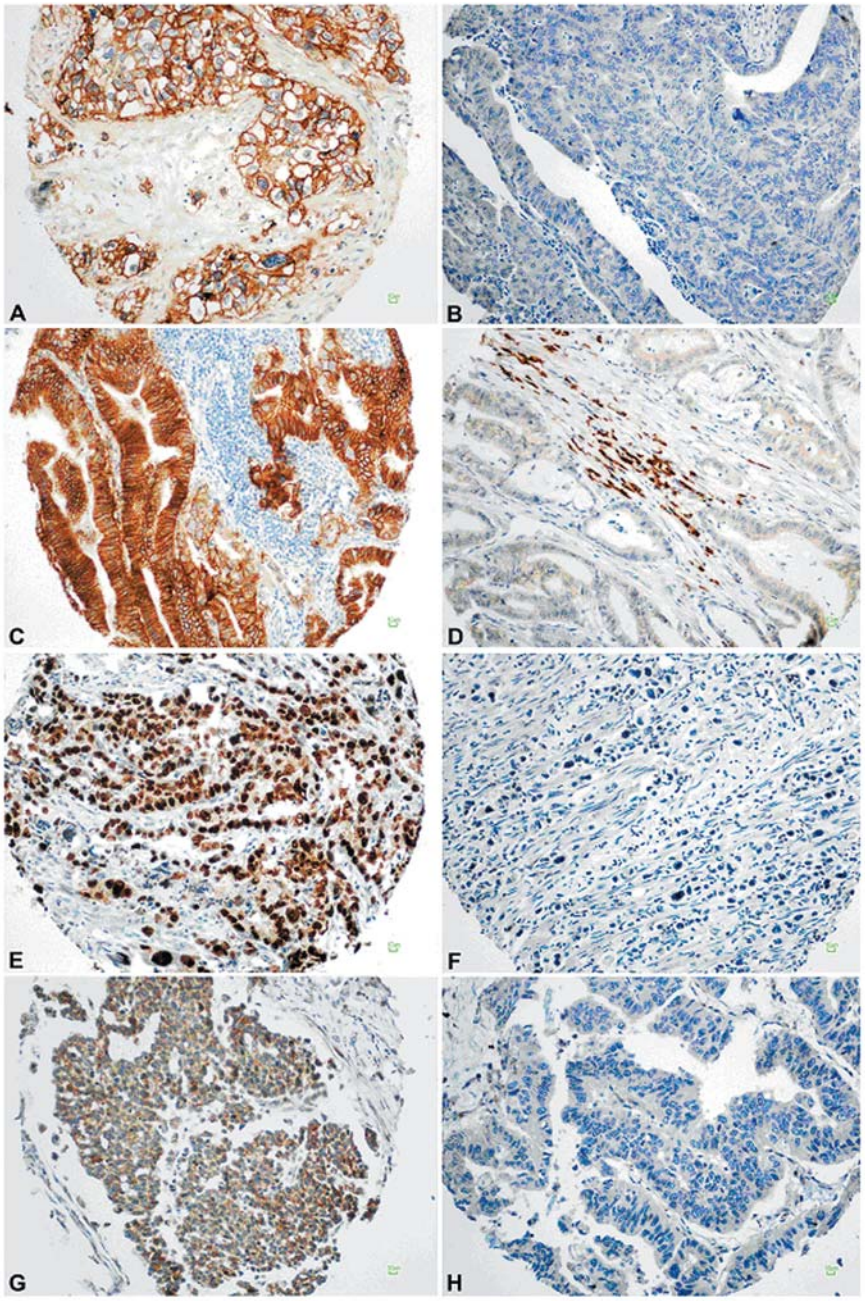

Figure 2. *Human epidermal growth factor receptor (HER) family immunohistochemical images. Immunohistochemistry was performed on tissue microarrays from gastric carcinoma cases for all four HER family members. A. EGFR-positive tumor revealed strong membranous positivity; B. EGFR-negative tumor showed absence or mild cytoplasmic staining; C. HER2 strong circumferential and membranous staining; D. HER2 mild cytoplasmic staining interpreted as negative; E. HER3 strong nuclear and mild cytoplasmic staining of tumor cells; F. HER3 negativity in non-cohesive gastric carcinoma; G. HER4 moderate cytoplasmic staining; H. HER4 negative tumor. Scale bar: 10 μm.*

percentage of positive tumor cells and the localization of the stain were indicated. The intensity was scored as 0=no staining, 1=weakly positive, 2=moderately positive, and 3=strongly positive. The percentage of positive tumor cells was calculated in 5% increments. The EGFR and HER2 proteins were evaluated based on the established scoring criteria (5-7). EGFR intensity of reactivity was also scored using a four-tier system as: 0 (negative), no staining or background staining; 1+, weak discontinuous membranous staining; 2+, moderate complete or incomplete membranous staining; and 3+, strong and complete membranous staining.

Additionally, the scores for EGFR and HER2 were also grouped resulting to negative (0, 1+) and positive (2+, 3+) expression. For HER3, evaluation included neoplastic cells that expressed nuclear or cytoplasmic staining (8). The cut-off percentage was 5%, which means that tumors with <5% of positive tumor cells were considered as negative and tumors with ≥5% of positive tumor cells were considered as positive. Similarly the cytoplasmic and nuclear evaluations of HER4 protein expression were considered positive if expressed in ≥5% of the cells (9). Presence of membranous HER4 protein expression was considered positive. Membranous, cytoplasmic and nuclear protein expressions were evaluated separately. Examples of the different immunohistochemical stainings (positive and negative for each HER family member) are shown in Figure 2.

Statistical analysis. This analysis included patients in early stage of the disease, as well as patients with advanced/metastatic disease. They were categorized in two groups according to treatment: a) undergone surgery followed by adjuvant treatment or observation and b) receiving treatment for metastatic disease irrespectively of having received adjuvant treatment or not. Categorical variables were presented as frequencies and percentages, while continuous data by the use of various measures (mean, standard deviation, medians and corresponding ranges). In order to examine possible associations between clinical characteristics and immunohistochemically evaluated markers, as well as possible associations among markers, the chi-square test was used. Patients' categorization was also examined for potential associations with clinical characteristics and IHC markers.

According to the stage of the disease, overall survival (OS) was measured from the date of the histology report or treatment initiation for the adjuvant patients and from the date of the last chemotherapy initiation for the advanced, until death or last contact. Disease-free survival (DFS) was measured for those patients who had surgery and subsequently were followed or received adjuvant treatment, as the time from the histology report or treatment initiation until documented relapse of the disease, death or last contact, whichever occurred first. Progression-free survival (PFS) was measured for those who received treatment for metastatic disease, as the time from first treatment initiation until documented progression, death or last contact.

Every marker was examined for possible prognostic value in terms of OS, DFS and PFS. Survival status was updated in June 2013. Time-to-event distributions were estimated using Kaplan-Meier curves. The log-rank test was used to examine the prognostic significance of the markers for OS, DFS and PFS. For all univariate tests significance level (α) was set at 0.05. The analysis was fully compliant with the reporting recommendations for tumor marker prognostic studies (10). The SAS software was used for statistical analysis (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA).

Results

As shown in the REMARK diagram (Figure 1), data from 473 patients with gastric cancer were identified in the HeCOG web registry. FFPE primary tumor tissue samples were obtained from 249 patients and were evaluated with IHC for protein expression of EGFR, HER2, HER3 and HER4. Material of sufficient quality and quantity for at least one biomarker was available for 243 patients and for all biomarkers in 89 patients.

Table II. *Marker protein expression frequencies in evaluable cases.*

	Total	
	N	%
EGFR membranous	217	
Negative	179	82.5
Positive	38	17.5
HER2 membranous	220	
Negative	194	88.2
Positive	26	11.8
HER3 cytoplasmic	210	
Negative	179	85.2
Positive	31	14.8
HER3 nuclear	179	
Negative	108	60.3
Positive	71	39.6
HER4 membranous	215	
Negative	209	97.2
Positive	6	2.8
HER4 cytoplasmic	210	
Negative	141	67.1
Positive	69	32.9
HER4 nuclear	215	
Negative	215	100.0
Positive	0	0.0

EGFR results. Results for the HER family marker protein expression frequencies are presented in Table II, while in Table III these results are presented according to the treatment group (early disease or group 1 and advanced disease or group 2).

Of the 249 specimens, 217 (87.1%) were evaluated with IHC for expression of EGFR on the membrane. Thirty-eight of the tumors had positive EGFR expression (score 2+ or 3+), a positivity rate of 17.5% of the evaluable population. The expression rate of EGFR in the two patient groups was 13.5% in group 1 and 21.2% in group 2 (Table III). There were no significant differences among the two patient groups (p=0.26).

The association of EGFR protein expression with clinicopathological characteristics was also evaluated and no association with most of the factors examined (gender, tumor location, grade, histological type, TNM stage, T stage, N status, M status) was found. The only statistically significant association found was between EGFR positivity and presence of lymphovascular invasion (p=0.027).

HER2 results. Of the 249 specimens, 220 (88.3%) could be evaluated with IHC for membranous expression of HER2. Twenty-six specimens were scored as 2+ or 3+ for a positive expression rate of 11.8% of the evaluable cases. There was no statistically significant difference of HER2 protein expression between the 2 patient groups, as shown in Table III (p=0.33). Interestingly, HER2 2+ or 3+ expression was associated with better tumor differentiation (p=0.002) and intestinal histological

Table III. *Marker protein expression in the two groups of patients*

	Group 1 Early disease	Group 2 Advanced disease	Chi-square test *p*-value
EGFR membranous			
Negative	134 (86.5)	26 (78.8)	0.26
Positive	21 (13.5)	7 (21.2)	
HER2 membranous			
Negative	143 (90.5)	28 (84.8)	0.33
Positive	15 (9.5)	5 (15.2)	
HER3 cytoplasmic			
Negative	127 (84.7)	29 (93.5)	0.19
Positive	23 (15.3)	2 (6.5)	
HER3 nuclear			
Negative	78 (61.4)	16 (55.2)	0.54
Positive	49 (38.6)	13 (44.8)	
HER4 membranous			
Negative	151 (96.2)	28 (100.0)	0.29
Positive	6 (3.8)	0 (0.0)	
HER4 cytoplasmic			
Negative	104 (68.4)	19 (67.9)	0.95
Positive	48 (31.6)	9 (32.1)	
HER4 nuclear			
Negative	157 (100.0)	28 (100.0)	-

Table IV. *Significant associations between markers*

	EGFR membranous		
	Negative	Positive	*p*-Value
HER4 cytoplasmic			
Negative	17 (100.0)	0 (0.0)	0.011
Positive	6 (66.7)	3 (33.3)	
In advanced disease only			
	HER2 membranous		
	Negative	Positive	*p*-Value
HER4 cytoplasmic			
Negative	124 (69.7)	11 (45.8)	0.020
Positive	54 (30.3)	13 (54.2)	
In the whole population			
	HER2 membranous		
	Negative	Positive	*p*-Value
HER3 nuclear			
Negative	69 (62.7)	3 (30.0)	0.043
Positive	41 (37.3)	7 (70.0)	
In early disease only			
	HER4 membranous		
	Negative	Positive	*p*-Value
HER3 cytoplasmic			
Negative	113 (84.9)	3 (50.0)	0.024
Positive	20 (6.1)	3 (50.0)	
In early disease only			

type (p=0.003). There was no significant association with age (p=0.39), gender (p=0.86), lymphovascular invasion (p=0.26), lymph node status (p=0.55), T stage (p=0.74), or tumor location (p=0.18).

HER3 results. HER3 expression was examined in the cytoplasm and the nucleus. Of the 249 specimens, nuclear protein expression of HER3 could be evaluated in 179 cases (71.9%), while cytoplasmic expression was evaluated in 210 specimens (84.3%). Nuclear staining was considered positive if 5% or more of the nuclei were positive, which was observed in 71 cases (39.6% of the evaluable cases), while positive cytoplasmic expression was noted in 31 cases (14.8% of the evaluable cases).

Both nuclear and cytoplasmic protein expression of HER3 did not differ significantly between the 2 patient groups (p=0.54 and p=0.19, respectively), as shown in Table III.

Nuclear expression of HER3 was associated with stage III-IV as compared to stage I-II (p=0.009) and also with tumor location in the antrum, compared to the distal or proximal stomach (p=0.048), while it was not associated with age (p=0.62), gender (p=0.95), histological grade (p=0.19), histological type (p=0.11), lymphovascular invasion (p=0.99), or lymph node status (p=0.29). Cytoplasmic expression of HER3 was associated with better-differentiated tumors (p=0.030) and with intestinal histological sub-type (p<0.001). It was not associated with age (p=0.54), gender (p=0.26), lymphovascular invasion (p=0.64), lymph node status (p=0.50), earlier disease stage (I-II) (p=0.079), T stage (T1/T2 *vs*. T3/T4) (p=0.60), or tumor location (p=0.19).

HER4 results. HER4 nuclear protein expression was evaluated in 215 cases but was not found positive in any of the cases, while cytoplasmic staining was evaluated in 210 cases and was positive in 69 (32.9%). Membranous staining was positive in only 6 of the 215 evaluable cases (2.8%). No differences in membranous or cytoplasmic HER4 protein expression was observed between the 2 patient groups (p=0.29 and p=0.95, respectively), as shown in Table III.

Clinicopathological features significantly associated with cytoplasmic expression of HER4 included better-differentiated tumors (p=0.014) and intestinal histology (p=0.03), while it was not associated with characteristics such as age (p=0.34), sex (p=0.08), lymphovascular invasion (p=0.18), lymph node status (p=0.25), T stage (p=0.52), or tumor location (p=0.18).

Associations between HER family member expression. Each HER family member expression was examined for associations with the expression of the other members. EGFR

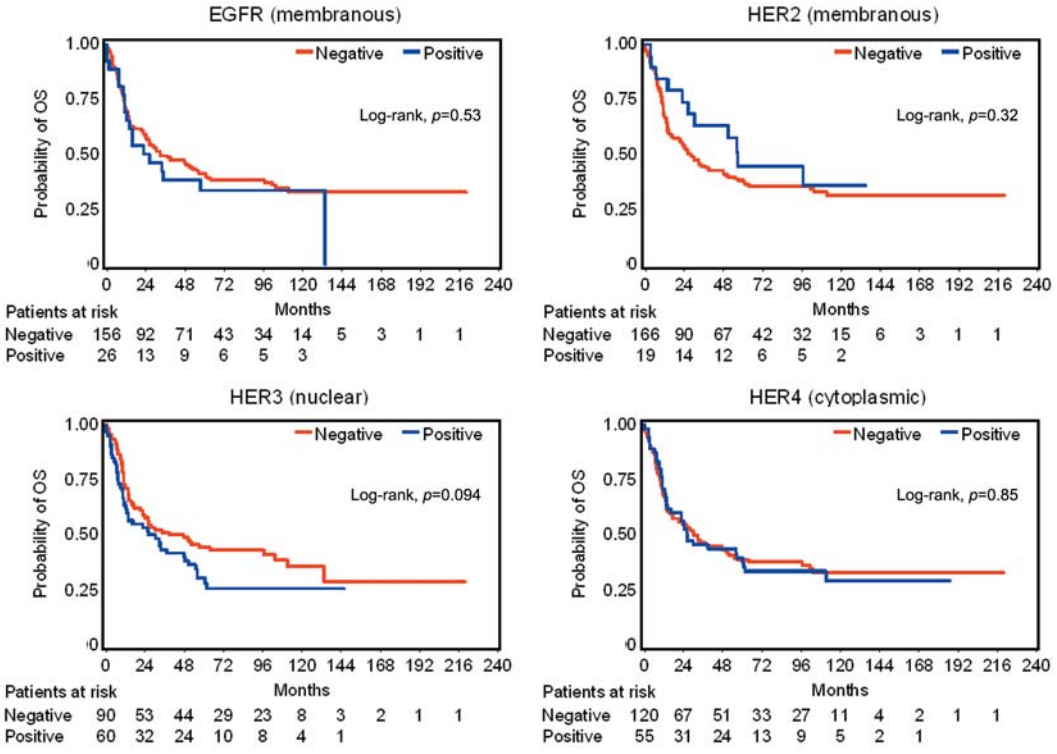

Figure 3. *Overall survival (OS) in early-disease patients according to human epidermal growth factor receptor (HER) family member protein expression.*

expression was associated with the expression of cytoplasmic HER4 (p=0.011) in patients with advanced disease, while membranous HER2 protein expression was associated with cytoplasmic expression of HER4 (p=0.020) in the whole population and with nuclear HER3 expression in early-disease patients (p=0.043). The cytoplasmic expression of HER3 was associated with membranous expression of HER4 in early disease patients (p=0.024). The above statistically significant associations are shown in Table IV.

Prognostic factors for survival. Overall survival data were available for 210 of the 249 patients in this study. The median overall survival was 30.4 months (95% CI 23.1-50.3). In univariate analysis, clinicopathological factors that were associated with longer OS included absence of lymphovascular invasion (log-rank, p=0.007), negative lymph nodes (p<0.001), lower TNM stage (p<0.001), lower T stage (p<0.001) and no metastatic disease (p<0.001), while no associations with survival was observed for age (p=0.36), sex (p=0.19), histological grade (p=0.26), histological type (p=0.14), or tumor location (p=0.51).

None of the HER family members were of prognostic value for OS in the univariate analysis. Representative Kaplan-Meier curves for OS according to protein expression of EGFR, HER2, HER3 and HER4 are shown in Figure 3.

DFS was evaluated in 161 patients, with the median value not being reached yet at the time of follow-up. Clinicopathological factors that were associated with longer DFS were: absence of lymph node involvement (log-rank, p<0.020), negative lymph nodes (p=0.003), lower TNM stage (p<0.001) and early *versus* advanced disease (p=0.001). Age, gender, histological grade, histological type, lymphovascular invasion and tumor location were not associated with DFS.

In 37 patients (of the 39 who received treatment for advanced disease), PFS was evaluated and the only statistically significant predictor for better PFS was lower histological grade (log-rank, p=0.024). No HER family member protein expression had any impact on PFS.

Evaluation of overall survival in the two different groups of patients showed that group 1 had the longest median OS (48.3 months), while in group 2 median OS was shorter (22.9 months) (log-rank, p<0.001).

Discussion

Gastric cancer is an aggressive and heterogeneous disease for which biomarkers are beginning to change our understanding of prognosis and management. Surgery is the main treatment and can lead to cure of patients with early-stage disease. However, survival of advanced resectable gastric cancer

patients remains poor despite the introduction of new treatment strategies, including adjuvant chemoradiation or perioperative chemotherapy. Metastatic or advanced unresectable gastric cancer is an aggressive malignancy with a poor prognosis, despite chemotherapy treatment resulting in a median survival of 7.5 to 12 months that is reduced to a median survival of 3 to 5 months if managed by best supportive care only (11).

The epidermal growth factor family of receptor tyrosine kinases (EGFR, HER2, HER3 and HER4) have similar molecular structures with an extracellular ligand-binding domain, a short transmembrane domain and an intracellular domain with tyrosine kinase activity (excluding HER3) (12). Binding of the ligand induces homodimerization of the receptor, as well as heterodimerization with other types of HER proteins. The dimerization of the HER family receptors activates downstream signal transduction pathways and promotes tumor progression. HER2 does not bind to any known ligand, it is however the preferred heterodimerization partner for the other members of the HER family, while the HER3 receptor is kinase impaired and serves as an allosteric activator or regulator of the other HER family members or their downstream pathway molecules (13). In addition to the well-studied roles of the ErbB receptors functioning from the plasma membrane, there is also evidence that these receptors are translocated to the nucleus, where they participate in cell signaling (14) and could thus affect cell proliferation, DNA replication, DNA damage repair, transcription, development and cancer growth or spread (15).

The important role that the HER family of receptors play in several types of solid tumors has led to development of targeted therapies that have revolutionized the treatment of breast cancer (by targeting HER2), non-small cell lung cancer by targeting HER1 (EGFR) and colorectal cancer with the use of monoclonal antibodies against EGFR. In advanced gastric cancer, the ToGA trial showed that the addition of the anti-HER2 monoclonal antibody trastuzumab to chemotherapy in HER2 overexpressing cases, increases response rates and improves progression-free survival and overall survival (6).

However, there is no clear evidence with regard to the prognostic role of HER2 overexpression in gastric cancer, as well as that of other members of the HER family of receptors. In addition, there could be regional differences in the expression rates of these receptors. We undertook this retrospective study in an attempt to evaluate the rates of expression of the HER family of receptors in Greek patients with gastric cancer and investigate any possible prognostic role, as well their associations with other clinicopathological factors.

In gastric cancer there is a wide variation of EGFR expression ranging from 2 to 44% in several reports (16-23) and has been associated with increased invasion (18), a poorly differentiated histology (22) and shorter survival (17-20, 22).

In our study the expression rate was 17.5%, that is in agreement with other reports (17, 21, 24). There was significant association between EGFR expression and lymphovascular invasion (p=0.027), but we did not find any prognostic value of EGFR expression neither in the two patient groups we examined nor in the whole population of our study. Currently, there is no consensus on the prognostic value of EGFR expression, as there are data suggesting no effect in prognosis (21, 24), while one study found an association of positive EGFR expression with improved prognosis (25). However, in a recent meta-analysis of 7 studies it was found that increased EGFR levels were significantly associated with decreased OS, with a pooled hazard ratio (HR) estimate of 1.66 (95% CI: 1.35-2.02) (26). It is possible that the different techniques and scoring systems used in the various studies contribute to these contradictory results.

Positive HER2 protein expression has been reported to occur in 8-34% of the cases (5, 27, 28). Additionally in a recent systematic review of 49 studies totaling 11,337 patients, with the majority having undergone curative surgery, the median rate of HER2 overexpression was 18% (range, 4-53%) and it appeared to be associated with poor survival and with intestinal-type gastric cancer (29). Higher rates of HER2 expression have also been reported in the gastroesophageal junction, as compared to distal gastric tumors (6, 30). In our study we evaluated HER2 expression according to the established Hofmann criteria and the rate of HER2 expression (2+/3+) was 11.8%, which is within the limits of reported studies. The Chinese results of the HER-EAGLE study, which included tumor samples from 734 gastric cancer (GC) or gastroesophageal junction cancer (GJC) patients, were recently reported. HER2 status was assessed by IHC, followed by dual-color silver-enhanced *in situ* hybridization (DSISH) in IHC 2+ cases. HER2-positive tumors were identified in 12.0% (88/734) of the GC and GJC cases. There were significantly higher rates of HER2 positivity in patients with GJC (GJC: 18.1%, GC: 9.7%, p=0.002), and intestinal-type cancers using the Lauren classification (intestinal: 23.6%, diffused/mixed: 4.3%, p<0.0001). No significant differences in HER2 positivity were identified between resection and biopsy samples, or between early and advanced disease. Additionally the agreement between local laboratories and the central laboratory on HER2 status scoring was good (kappa=0.86) (31).

Our results showed lower frequency of positive HER2 expression (11.8%) than those of the ToGA study (IHC 3+ or IHC 2+/FISH+: 16.0%) and the Spanish results (32) of the HER-EAGLE study (14.1%). This may be due to differences in patient characteristics, in particular the Lauren classification and the location of the tumor. A higher percentage of intestinal-type tumors were analyzed in the ToGA study (51.4%) and the Spanish HER-EAGLE study (58.2%), while this tumor type was detected in 48.7% of our metastatic disease cases (51.4% in the whole population) and there were no patients with GE junction tumors in our study.

We also found an association with intestinal histological type (when +2 and +3 expression was taken into account) and well-differentiated tumors, while we did not identify any associations between the location of the tumor and HER2 expression.

We did not find any associations of HER2 protein expression with survival end-points. The role of HER2 as a prognostic factor in gastric cancer has not been fully elucidated, since some of the initial studies failed to find an association with prognosis, while other authors reported a direct association between HER2 overexpression and poor survival (12). A recent meta-analysis of eight studies evaluating the role of HER2 in gastric cancer showed that high HER2 levels predicted poor overall survival (HR=1.43, 95% CI=1.09-1.88) (26), while in another meta-analysis of fifteen studies with 5,290 patients, HER2 overexpression also had an unfavorable prognostic role for patients with gastric cancer (33).

The role of HER3 protein expression in gastric cancer is not clear as yet. In a study from China, tumor samples were obtained from gastric adenocarcinomas of 134 patients who underwent a gastrectomy from 1999 to 2002. HER3 protein expression, which was noted on the membrane in 13% and in the cytoplasm in 58% of the cases, was significantly associated with several adverse characteristics, including the depth of tumor invasion (T1 *vs*. T2-T4), number of involved lymph nodes, distant metastases, tumor stage and recurrent disease. HER3 overexpression was associated with a significantly worse survival and was an independent prognostic factor in the multivariate analysis (HR=2.38, 95% CI=1.01-5.63, p=0.048) (34).

In the present study we noted positive cytoplasmic HER3 expression in 14.8% of the cases, which is lower than the one reported by Hayashi *et al*. (34) and also lower than other studies that have evaluated HER3 expression: Begnami *et al*. reported HER3 expression in 64% of their cases (24) and Jacome *et al*. found a rate of 62% (21). This could be the result of differences in technique, in the antibody used for IHC or the use of different criteria for interpretation of the stains. For example, we considered HER3 protein expression as positive if 5% or more of the cells showed expression of HER3, while Begnami *et al*. used the Rajkumar score and Hayashi *et al*. and Jacome *et al*. used in their studies a score similar to the one used for EGFR and HER2 evaluation. In contrast to other studies (24, 34) we did not find a prognostic role of HER3 protein overexpression.

The role of HER4 in gastric cancer is unclear, as well. Positive HER4 protein expression by IHC has been reported to be associated with favorable features, such as the intestinal sub-type, well- and moderately differentiated tumors, lower stage and absence of lymph node metastases, as well as absence of vascular, lymphatic and perineural invasion (24). However there was no association with overall survival. In our study, we did find an association with moderately or well-differentiated tumors, but again no association of HER4 expression with prognosis. There is some recent evidence that HER4 and its ligand neuregulin 4 (NRG4) are downregulated in cancerous gastric tissue, a finding that could imply a potential protective or anti-carcinogenic function (35).

In conclusion, this retrospective study in a Greek patient population diagnosed with gastric cancer at various stages of the disease confirms results from other published studies on the frequency and role of EGFR, HER2, HER3 and HER4 protein expression. It is the first study to evaluate protein expression of the HER family members and their possible prognostic value in a Greek population. Some of the differences noted between our results and those of other studies might be justified by the absence of gastroesophageal cancer patients within our cohort, as well as the various stages of disease. It is clear, that prospective data from larger studies are needed to clarify the exact role of each of the HER family members at various stages of this aggressive disease and guide our therapeutic approaches accordingly.

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