

Assessment and prognostic analysis of EGFR, HER2, and HER3 protein expression in surgically resected gastric adenocarcinomas

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Aim: To investigate the significance of epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER)2, and HER3 expression on survival outcomes in Chinese gastric cancer patients.

Materials and methods: Formalin-fixed, paraffin-embedded specimens from 121 patients who underwent gastrectomy at Shanghai Renji Hospital from 2007–2010 were retrospectively examined. Fluorescence in situ hybridization and immunohistochemistry techniques were used to identify gene amplification and protein overexpression. Correlations between the expression or amplification of *HER* family genes and clinicopathological parameters were then determined using statistical analysis.

Results: EGFR protein overexpression, an increase in *HER2* copy number and gene amplification, and HER3 protein overexpression were identified in 33.1%, 17.4%, and 62.0% of samples, respectively. Statistical analysis showed a significant association between EGFR expression and tumor invasion depth or tumor stage. HER2 was also shown to be significantly associated with the tumor grade. In addition, EGFR protein overexpression was found to be significantly associated with worse overall survival ($P=0.03$).

Conclusion: The HER family members showed a high expression in gastric cancer. EGFR protein expression was associated with overall survival.

Keywords: gastric cancer, clinicopathologic significance, immunohistochemistry, fluorescence in situ hybridization

Introduction

Gastric cancer is one of the most frequently diagnosed cancers and is the leading cause of cancer-related death worldwide.¹ To date, the prognosis of patients with advanced gastric cancer is still poor even after surgery or radical resection.² Adjuvant systemic therapies, including some new biological agents, have been implemented to improve outcomes.³

The type I human epidermal growth factor receptor (HER) family consists of four homologous members: ErbB-1 (epidermal growth factor receptor [EGFR]); ErbB-2 (HER2); ErbB-3 (HER3); and ErbB-4 (HER4). All of these are transmembrane receptor tyrosine kinases consisting of three functional domains: an extracellular ligand-binding domain; a lipophilic transmembrane segment; and a cytoplasmic tyrosine kinase domain.⁴ Different from other HER family members, HER3 lacks a functional cytoplasmic tyrosine kinase domain and participates in intracellular signaling through heterodimerization with other HER family members.⁵ Under physiological conditions, while bound to the ligand, those receptors dimerize and activate downstream signaling

pathways, which leads to cell differentiation, migration, proliferation, or survival.⁶

Genetic alterations in the *HER* gene family are shown to be related to tumorigenesis and tumor progression in different types of cancer.⁷ EGFR and HER2 overexpression are considered as prognostic factors in gastric cancer and are currently the targets of several novel biological agents,⁸ while HER3 expression is frequently observed in advanced gastric cancer with poor prognosis.⁹ However, the clinical significance of such overexpression is not fully understood, and previous studies showed conflicting results in the association between overexpression of HER family members and poor prognosis.^{10,11} In the present study, we further explored the protein expression and gene amplification of EGFR, HER2, and HER3 in surgically resected gastric adenocarcinoma from a local Chinese cohort, which could hopefully shed some light on the problem.

Materials and methods

Patient samples

A total of 121 gastric adenocarcinoma tissue samples were randomly collected from patients who underwent total or subtotal gastrectomy at the Shanghai Renji Hospital from 2007–2010. Tissue samples were fixed in 10% neutral formalin and embedded in paraffin before further investigation. Tumor histological subtype was determined according to Lauren's classification¹² after review by two pathologists. Each tumor sample was classified according to the tumor–node–metastasis classification advocated by the International Union against Cancer.¹³ Follow-up data were available from all patients, who were assessed via phone call at 3 months, 6 months, and 12 months after gastrectomy, and then every 6 months thereafter for 5 years or until death. A total of 68 patients received chemotherapy, including FOLFOX (folinic acid, fluorouracil, and oxaliplatin) (37 cases), paclitaxel (two cases), Chinese medicine (one case), and combination therapy (28 cases). Twenty-three patients did not receive chemotherapy, as they were not able to bear the side effects. Chemotherapy information from the rest of the patients was not available. Tumor specimens were collected after obtaining informed consent from the patients, and the protocol of this study was approved by the ethics committee of the Shanghai Renji Hospital.

Immunohistochemistry

All tumor sections (thickness =3–5 μ m) were stained as per the manufacturer's protocol (Dako Denmark A/S, Glostrup, Denmark). The following primary antibodies

were used: EGFR (M7239 mouse monoclonal antibody; Dako pharmDx™ Kit; Dako Denmark A/S), HER2 (K5204 mouse monoclonal antibody; Dako Denmark A/S), and HER3 (M7297 mouse monoclonal antibody; Dako Denmark A/S). Positive controls were selected from breast or lung carcinomas, which were stained positive in previous tests. For negative controls, primary antibodies were simply replaced by phosphate-buffered saline. The immunostaining was then scored by two independent pathologists who were blinded to the clinical information. Tumor membranous staining was scored using a four-grade scale (0, 1+, 2+, or 3+), and the intensity of cytoplasmic staining for HER3 was also carefully determined. The following scoring criteria were used: 0, if no staining was observed; 1+, if more than 10% of the tumor cells had weak staining on the membrane (or cytoplasm for HER3); 2+, if more than 10% of the tumor cells had moderate staining on the membrane (or cytoplasm for HER3); and 3+, if more than 10% of the tumor cells had strong staining on the membrane (or cytoplasm for HER3).

Fluorescence in situ hybridization

HER2 gene amplification was analyzed by fluorescence in situ hybridization using the Vysis Path Vysion *HER2*/DNA probe kit (*HER2* SpectrumOrange/*CEP17* SpectrumGreen, Vysis 30-161060) as per the manufacturer's protocol (Abbott Laboratories, Abbott Park, IL, USA). For each specimen, the numbers of gene signals were counted in 50 nuclei, and the mean *HER2* or *CEP17* copy number was calculated by dividing the total number of gene signals by 50. The *HER2*/*CEP17* ratio was calculated by dividing the total number of *HER2* signals by the total number of *CEP17* signals. Finally, amplification of *HER2* was defined as a mean *HER2* copy number of >10 or as a *HER2*/*CEP17* ratio of ≥ 2.0 .

Criteria for defining biomarker positivity

For EGFR and HER3, scores of 0 or 1+ were considered negative, and a score of 2+ or 3+ was classified as positive or overexpression. For HER2, an immunohistochemistry (IHC) score of 3+ or an IHC score of 2+ with amplification of *HER2* was defined as positive, and the IHC scores of 0 and 1+ or a score of IHC 2+ without an amplification of *HER2* were defined as negative.

Statistical analysis

Survival curves were estimated using the Kaplan–Meier product-limit method, and significant differences between the survival curves were determined using the log-rank test. Survival analyses were conducted using a Cox proportional

hazards model. The χ^2 test was used to assess the possible association between the overexpression of each HER family receptor and the clinicopathologic parameters. Results were considered statistically significant if $P < 0.05$. All statistical analyses were conducted using the SPSS 10.0 statistical software program (IBM Corporation, Armonk, NY, USA).

Results

EGFR protein overexpression

In the 121 samples we examined, EGFR expression results were as follows: grade 0, 62 (51.2%); grade 1+, 19 (15.7%); grade 2+, 19 (15.7%); and grade 3+, 21 (17.3%). Forty cases (33.1%) were classified as positive for EGFR protein expression and 81 (66.9%) cases as negative. Representative images of EGFR immunostaining are shown in Figure 1A–D.

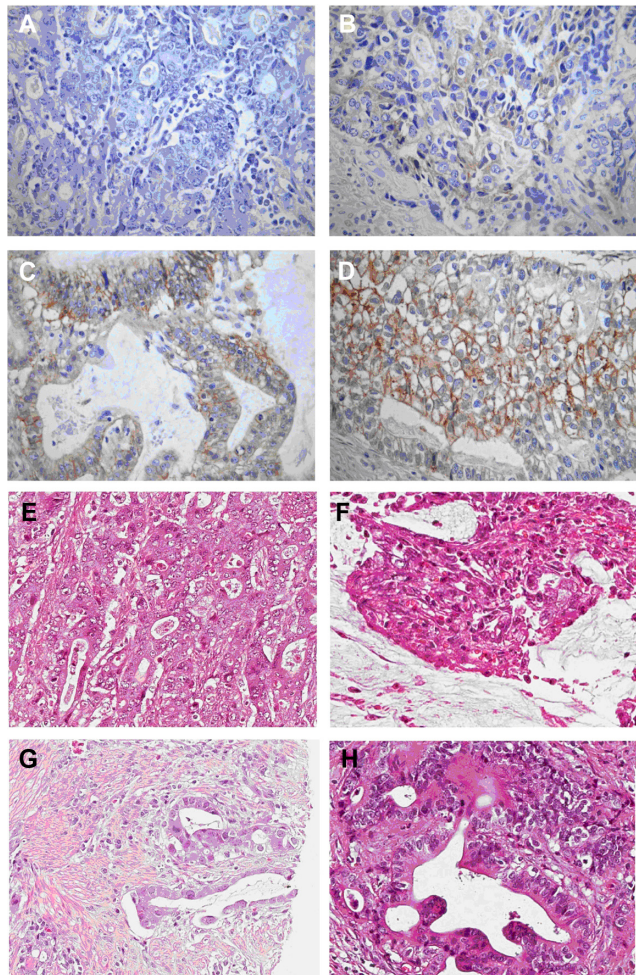


Figure 1 Representative image showing epidermal growth factor receptor protein expression in gastric cancer samples.

Notes: (A) Immunonegativity; (B) 1+ reactivity intensity; (C) 2+ reactivity intensity; (D) 3+ reactivity intensity; (E–H) representative hematoxylin and eosin staining images for samples in (A–D), respectively (original magnification $\times 200$).

Corresponding hematoxylin and eosin (HE) staining images are shown in Figure 1E–H.

HER2 protein overexpression and gene amplification

HER2 protein overexpression was observed in 28.1% of all tumors (16.7% scored 2+ and 11.4% 3+). A total of 48.2% of tumors showed totally negative staining (score 0), and the remaining 23.7% showed weak staining (score 1+). *HER2* gene amplification was observed in 19 (15.7%) of 121 tumors. *HER2* gene amplification was present in six of 19 tumors with a score of 2+ and in ten of 19 tumors with a score of 3+, which was generally consistent with IHC scores. In the 87 samples that exhibited 0 or 1+ HER2 IHC scores, only three showed *HER2* gene amplification. Twenty-one cases (17.4%) were classified as positive for HER2 expression and 100 (82.6%) cases as negative. Representative images of HER2 immunostaining and *HER2* gene amplification are shown in Figure 2A–D and Figure 2E and F, respectively. Corresponding HE staining images for samples in Figure 2A–D are shown in Figure 2G–J.

HER3 protein overexpression

HER3 protein staining was predominantly located in the cytoplasm. In 115 specimens, HER3 immunostaining results were: grade 0, 18 (15.7%); grade 1+, 20 (17.4%); grade 2+, 44 (38.3%); and grade 3+, 32 (27.8%). Seventy-six cases (66.1%) were classified as positive for HER3 protein expression and 38 (33.0%) as negative. Representative images of HER3 immunostaining are shown in Figure 3A–D. Corresponding HE images are shown in Figure 3E–H.

Coexpression of the HER family members

The most commonly coexpressed family members were EGFR and HER3, found in 23.1% of tumors (number [n] = 28). HER2 and HER3 coexpression was shown in 11.6% of tumors (n = 14), in which five (4.1%) also overexpressed EGFR. Only eight tumor samples (6.6%) were positive for both EGFR and HER2 expression (Figure 4).

Clinicopathologic parameters and HER expression

Statistical analysis showed that EGFR overexpression was significantly associated with the depth of tumor invasion and tumor stage (Table 1). HER2 overexpression was also significantly associated with tumor grade, and HER2 positivity

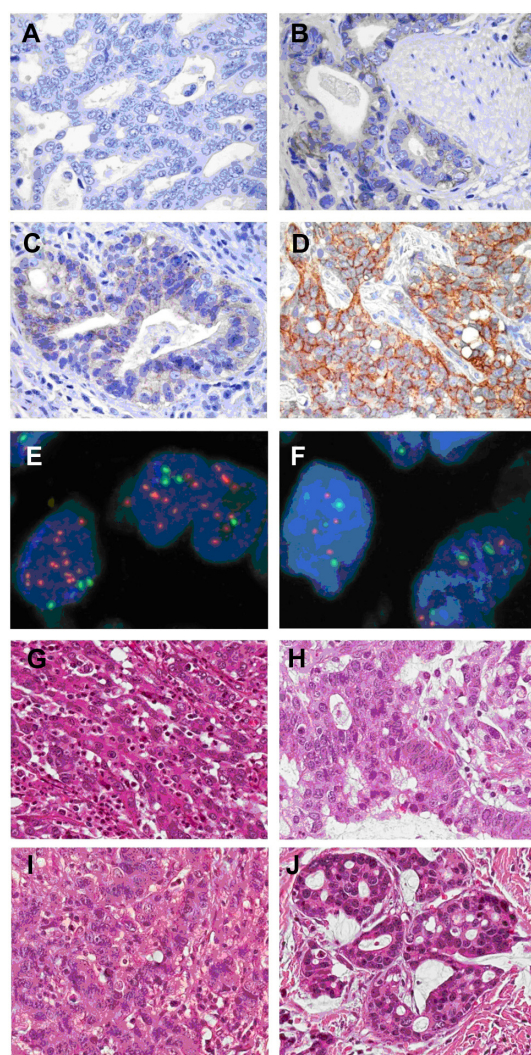


Figure 2 Representative image showing human epidermal growth factor receptor 2 protein expression and gene amplification in gastric cancer samples.

Notes: (A) Immunonegativity; (B) 1+ reactivity intensity; (C) 2+ reactivity intensity; (D) 3+ reactivity intensity; (E) FISH positive; (F) FISH negative; (G–J) representative hematoxylin and eosin staining images for samples in (A–D), respectively (original magnification $\times 200$).

Abbreviation: FISH, fluorescence in situ hybridization.

was more commonly observed among well- and moderately differentiated types than undifferentiated types ($P=0.0001$; χ^2 test). However, no association was shown between HER3 overexpression and sex, age, histological type, or tumor-node-metastasis classification.

After standardizing chemotherapy status, a significant relationship was found between EGFR protein overexpression and the overall survival rate ($P=0.03$; Figure 5A). No significant relationship was shown between the overexpression of other HER family members and overall survival rates (HER2, $P=0.71$; HER3, $P=0.84$; Figure 5B and C). In addition, overall survival rates were found to be shorter in patients with overexpression of both EGFR and HER3, although with a boundary

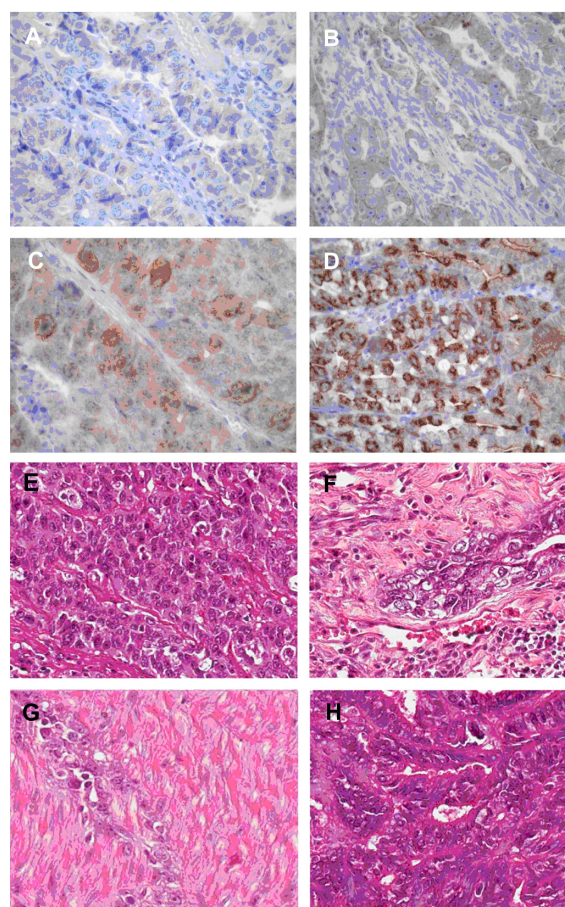


Figure 3 Representative image showing human epidermal growth factor receptor 3 protein expression in gastric cancer samples.

Notes: (A) Immunonegativity; (B) 1+ reactivity intensity; (C) 2+ reactivity intensity; (D) 3+ reactivity intensity; (E–H) representative hematoxylin and eosin staining images for samples in (A–D), respectively (original magnification $\times 200$).

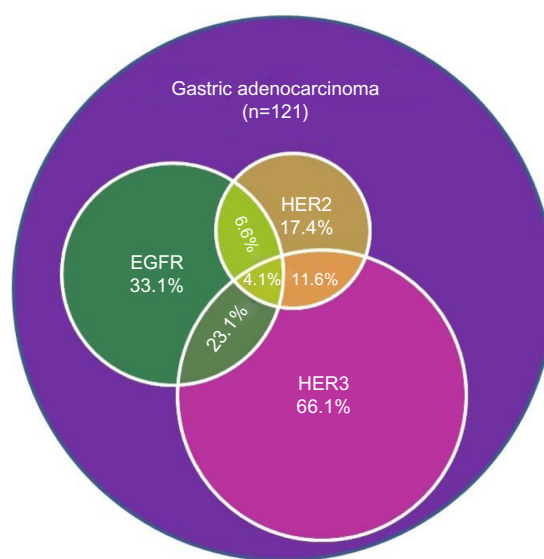


Figure 4 An illustration of the coexpression of HER family members in gastric adenocarcinoma.

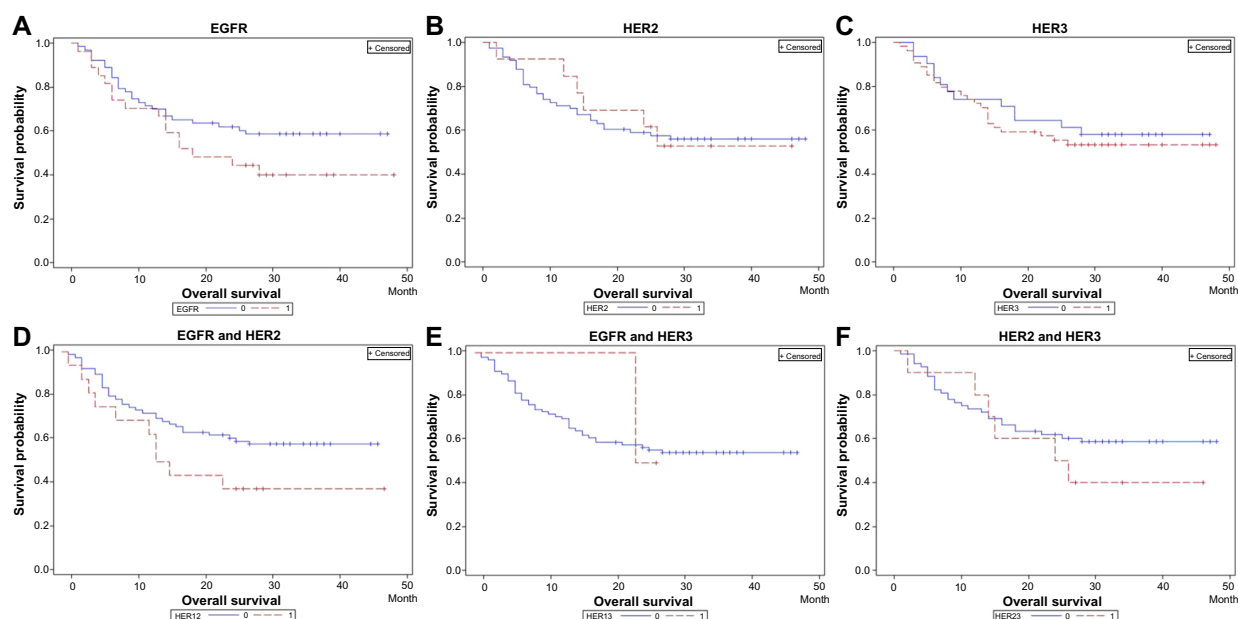
Abbreviations: n, number; HER, human epidermal growth factor receptor; EGFR, epidermal growth factor receptor.

Table 1 Association between clinicopathological parameters and EGFR, HER2, and HER3 expression in 121 cases of gastric cancer

Parameters	n	EGFR-positive (%)	P-value	HER2-positive (%)	P-value	HER3-positive (%)	P-value
N	121	40 (33.1%)		21 (17.4%)		76 (66.1%)	
Sex							
Male	85	28 (32.9%)	0.8062	16 (22.9%)	0.1971	52 (65.8%)	0.6439
Female	36	12 (33.3%)		5 (15.6%)		23 (67.6%)	
Tumor grade							
II	23	6 (26.1%)	0.439	11 (55.0%)	0.0001	18 (78.3%)	0.285
III	87	30 (34.5%)		10 (13.7%)		53 (64.6%)	
IV	11	3 (27.3%)		0 (0%)		5 (62.5%)	
Tumor stage							
I and II	36	6 (16.7%)	0.0146	5 (15.6%)	0.2689	23 (65.7%)	0.9817
III	61	23 (37.7%)		14 (28.6%)		37 (67.3%)	
IV	24	11 (45.8%)		2 (9.5%)		15 (65.2%)	
Depth of invasion							
T ₁ and T ₂	11	0 (0%)	0.0001	4 (40.0%)	0.3389	8 (66.7%)	0.6825
T ₃ and T ₄	110	40 (36.4%)		17 (18.5%)		67 (66.3%)	
Lymph node metastasis							
0	31	6 (19.4%)	0.0783	3 (11.1%)	0.1874	17 (58.6%)	0.3989
1	48	15 (31.3%)		12 (30.0%)		32 (71.1%)	
2	22	10 (45.5%)		4 (23.5%)		12 (60.0%)	
3	20	9 (45.0%)		2 (11.1%)		14 (73.7%)	
Metastasis							
Negative	112	36 (32.1%)	0.8115	21 (22.1%)	0.1049	70 (67.3%)	0.672
Positive	9	4 (44.4%)		0 (0%)		5 (55.6%)	
Subtype							
Intestinal type	24	10 (41.7%)	0.282	9 (37.5%)	0.064	20 (83.3%)	0.0859
Diffused type or mixed type	95	28 (29.5%)		12 (15.4%)		55 (63.2%)	

Note: T₁–T₄ is the tumor stage based on tumor-node-metastasis classification advocated by International Union against Cancer.

Abbreviations: EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; n, number.

**Figure 5** Association between patient overall survival and the overexpression of HER family members.

Notes: (A) EGFR overexpression was significantly associated with worse survival ($P=0.03$; log-rank test). (B) No significant relationship was found between HER2 and overall survival ($P=0.71$). (C) No significant relationship was found between HER2 and overall survival ($P=0.84$). (D) Co-overexpression of EGFR and HER3 was related to worse survival, although with a boundary significance ($P=0.07$). (E) No significant relationship was found between overall survival and co-overexpression of EGFR and HER2 ($P=0.49$). (F) No significant relationship was found between overall survival and co-overexpression of HER2 and HER3 ($P=0.98$). All results were obtained after standardizing chemotherapy status.

Abbreviations: EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor.

significance ($P=0.07$; Figure 5D). No significant association was shown between the expression of other combinations of EGFR, HER2, HER3 and overall survival (EGFR and HER2, $P=0.49$; HER2 and HER3, $P=0.98$; Figure 5E and F).

Discussion

Overexpression of the HER family members can lead to the activation of downstream signaling pathways, resulting in the cellular transformation and proliferation events associated with tumorigenesis.⁶ HER overexpression in gastric cancer has been reported in many previous studies. However, conclusions regarding the associations between HER positivity and patient survival were conflicting.^{10,11} This study analyzed the expression of the HER family members and assessed their potential influence on patient outcomes. Our study has demonstrated an association between HER overexpression and tumor stage, grade, or overall survival in those gastric cancer patients.

EGFR gene amplification and/or protein expression have been observed in a variety of solid tumors, including lung, colorectal, urinary bladder, breast, head and neck, and esophageal carcinomas.^{14–19} The frequency of EGFR protein expression and/or gene amplification in gastric cancer ranges from 2%–35%.²⁰ Possible explanations for this large variation include differences in fixation conditions, antibodies, scoring systems, subjectivity of pathologist interpretation, and intratumoral heterogeneity. In the present study, we detected EGFR positivity in 33.3% of gastric cancer, which is similar to the findings of previous studies,^{20,21} and we also identified a significant association with the depth of tumor invasion, tumor stage, and overall survival. Indeed, a previous study showed that EGFR protein overexpression was significantly associated with response to cetuximab therapy in metastatic colorectal cancers.²² Our study showed more evidence that EGFR positivity could be a predictor of response to EGFR-targeted therapy in gastric cancer.

HER2 expression in gastric cancer was first reported in 1986,²³ which was followed by a large number of related reports.^{24–27} The incidence of HER2 protein expression in gastric cancer varies between studies, ranging from ~9%–38%.²⁸ In our study, the HER2 protein overexpression and gene amplification was identified in 28.1% (34/121) and 15.7% (14/69) of gastric cancers, respectively, leading to a HER2-positive rate of 17.4% (21/121) in patients with gastric cancer. A recent study by Xu et al²⁹ showed a similar positivity rate (11.2%). Furthermore, the HER2 positivity rate appears to be higher in differentiated-type tumors when compared to undifferentiated-type tumors, and similar results have been reported by other

investigators.⁸ The underlying cause for this selectively high expression of HER2 in differentiated-type tumors remains unclear. The role of HER2 as a prognostic factor in gastric cancer also remains unclear. A recent systematic review assessing the impact of HER2 overexpression on survival found that 20 studies (57%) reported no difference in overall survival, while two (6%) studies showed significantly longer overall survival in patients with HER2 overexpression, and 13 (37%) found significantly worse overall survival in patients with HER2 overexpression.³⁰ In addition, a recent study by Xu et al²⁹ found a significant relationship between HER2 positivity and the overall survival rate. Our results, on the other hand, provided further evidence that HER2 status does not influence the outcomes of patients with surgically resected gastric adenocarcinomas, which is in contrast to breast cancer.

Although the tyrosine kinase domain of HER3 is nonfunctional, its dimerization with another HER protein is required for the activation of downstream signaling pathways.⁵ Several studies have shown significant correlations between HER3 overexpression and decreased survival rate in patients with colorectal, breast, laryngeal, and esophageal carcinomas.^{31–34} The prognostic relevance of HER3 expression has yet to be elucidated in gastric cancer. In our study, we observed HER3-positive expression in 68.7% of patients with gastric cancer, although HER3 protein expression was not shown to be associated with any clinicopathologic factors.

Considering its lack of tyrosine kinase functionality, HER3 has not traditionally been regarded as a therapeutic target.³⁵ However, blockade of HER3 in *in vitro* studies downregulated downstream signaling and caused tumor cell death.³⁶ In breast cancer, the formation of the HER2–HER3 heterodimer is associated with a significantly decreased survival rate in patients, and the prevention of dimerization has already shown clinical benefits.³⁷ Hayashi et al⁹ have previously inferred that coexpression of the HER family members may have synergistic effects on the progression of gastric cancer. Our data identified the EGFR–HER3 and HER2–HER3 dimers as more frequent expression partners, although no significant correlations were found between the coexpression of the HER family members and clinical outcomes. HER3 blocking therapies, as well as anti-EGFR or anti-HER2 treatment, might therefore represent a viable novel therapeutic strategy for gastric cancer.

Conclusion

In conclusion, the current study provides further evidence that EGFR overexpression is significantly associated with overall survival in surgically resected gastric adenocarcinomas,

suggesting that EGFR could be a predictor of response to EGFR-targeted therapy in gastric cancer. Although HER2 or HER3 status are not associated with overall survival in this patient cohort, our data demonstrate the frequent overexpression of HER3 protein in gastric cancer, and suggest that HER3-targeted agents could represent promising therapeutics for improving gastric cancer patient outcomes. Limitations of our study include the relatively small sample size, which may not have enough statistical power. Studies with larger sample sizes are needed to further clarify the relationship between the HER family and patient prognosis.

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Disclosure

The authors affiliated with AstraZeneca are full-time employees and/or have stock in AstraZeneca. The authors report no other conflicts of interest in this work.

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