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***HER-2/neu* Gene Amplification in Relation to Expression of HER2 and HER3 Proteins in Patients With Esophageal Adenocarcinoma**

Harry H. Yoon, MD¹, William R. Sukov, MD², Qian Shi, PhD³, Christopher A. Sattler, BS², Anne E. Wiktor, BS², Robert B. Diasio, MD⁴, Tsung-Teh Wu, MD, PhD², Robert B. Jenkins, MD, PhD², and Frank A. Sinicrope, MD^{1,5}

¹Department of Medical Oncology, Mayo Clinic, Rochester, Minnesota

²Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota

³Department of Health Sciences Research, Mayo Clinic, Rochester, Minnesota

⁴Mayo Clinic Cancer Center, Mayo Clinic, Rochester, Minnesota

⁵Department of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota

Abstract

BACKGROUND—Human epidermal growth factor receptor 2 (HER2) is a therapeutic target in patients with esophageal adenocarcinoma (EAC), with gene amplification used as a selection criterion for treatment, although to the authors' knowledge the concordance between amplification and HER2 protein expression remains undefined in EAC. Furthermore, the association between HER2 and its interacting partner, human epidermal growth factor receptor 3 (HER3), is unknown yet appears to be of potential therapeutic relevance.

METHODS—Patients with untreated EACs (N 5 673) were analyzed for *HER2* amplification and polysomy 17 by fluorescence in situ hybridization in parallel with immunohistochemistry (IHC) (IHC scores of 0–1+, 2+, and 3+). Amplification was defined as *HER2=CEP17* 2. HER3 expression by IHC was analyzed in randomly selected cases (n 5 224). IHC and fluorescence in situ hybridization results were compared using least squares linear regression.

RESULTS—Overall, 17% of the EACs (116 of 673 EACs) were *HER2*-amplified with an amplification frequency that was highest among IHC3+ cases (89%) and declined among IHC2+ cases (13%) and IHC0 to IHC1+ cases (4%). Among *HER2*-amplified cases, the level of amplification increased linearly with HER2 membranous expression (*HER2/CEP17* ratio: 7.9 in IHC3+ and 5.5 in IHC2+ vs 2.8 in IHC0 to IHC1+ [$P<.0001$]), with 14% of amplified tumors demonstrating absent/faint expression (IHC0 to IHC1+). Polysomy 17 was not found to be

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Corresponding author: Harry H. Yoon, MD, Department of Medical Oncology, Mayo Clinic, 200 First St SW, Rochester, MN 55905; Fax: (507) 284-1803; yoon.harry@mayo.edu.

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CONFLICT OF INTEREST DISCLOSURES

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associated with HER2 expression. Cytoplasmic HER3 expression was detected in 87% of tumors (195 of 224 tumors) and was found to be significantly associated with better differentiation ($P < .0001$). Stepwise increases in HER3 expression were associated with higher HER2 expression levels ($P = .0019$).

CONCLUSIONS—Levels of HER2 protein expression and amplification were found to be linearly associated and highly concordant. Among amplified tumors with absent/faint expression, the level of amplification was low. Frequent expression of HER3 suggests its relevance as a therapeutic target, and its significant association with HER2 supports ongoing efforts to inhibit HER2/HER3 in patients with EAC.

Keywords

human epidermal growth factor receptor 2 (HER2); ERBB2; concordance; fluorescence in situ hybridization; HER2 gene amplification; HER2 expression; HER3/ERBB3; esophageal cancer; esophageal adenocarcinoma; esophagogastric cancer

INTRODUCTION

The incidence of adenocarcinomas of the esophagus, gastroesophageal junction (GEJ), and gastric cardia (collectively referred to as EAC) has risen 6-fold in the past 3 years, and continues to increase.¹ The 5-year survival remains < 25%, including those patients with regional disease who undergo surgery. The human epidermal growth factor 2 (HER2/ErbB2) oncoprotein is a member of a family of transmembrane receptor tyrosine kinases (HER1/epidermal growth factor receptor, HER3/ErbB3, and HER4/ErbB4) that regulate cell growth, survival, differentiation, and migration. Homodimerization or heterodimerization among HER receptors is essential for their function and signaling activity. The HER2-HER3 heterodimer is considered the most potent HER pair with respect to strength of interaction, ligand-induced tyrosine phosphorylation, and downstream signaling.⁴ HER3 overexpression has been associated with HER2 overexpression⁵ and with adverse survival in multiple tumor types including gastric cancer,^{6–8} but to the best of our knowledge has not been evaluated in patients with EAC. Given its important role as a signaling hub, HER3 is a candidate target for drug discovery that is being actively investigated.⁹

HER2 is an established target in human breast and GEJ/gastric cancers. A recent phase 3 trial (Trastuzumab for GAstric cancer [ToGA]) of patients with advanced HER2-overexpressing GEJ or gastric adenocarcinoma demonstrated that adding trastuzumab, a HER2-targeted monoclonal antibody, to cytotoxic chemotherapy improved overall survival (OS).¹⁰ In the ToGA trial, the benefit of trastuzumab was limited to tumors that demonstrated strong or intermediate HER2 immunoreactivity as confirmed by gene amplification.^{10–12} Based on this study, trastuzumab is now approved worldwide for the treatment of patients with advanced esophagogastric cancer, and determination of HER2 status has become an integral part of the clinical-pathological workup. HER2 overexpression is usually a direct consequence of gene amplification, although overexpression can occur by other mechanisms.^{4,13} The importance of understanding the association between HER2 expression and amplification is underscored by the finding that no trastuzumab benefit was observed in gene-amplified cases demonstrating only absent/faint expression, which

comprised approximately 25% of the ToGA study population. To our knowledge to date, the concordance between HER2 expression and amplification has been inadequately studied in patients with EAC.

HER2 and other key genes (*P53*, *BRCA1*, and *TOP2A*) reside on chromosome 17, and the effect of excess chromosome 17 (polysomy 17) on HER2 expression and response to HER2-targeted therapy remains unknown.¹⁴ Although there are conflicting data, an increase in copies of chromosome 17 in non-*HER2*-amplified breast cancers has been associated with increased HER2 protein expression,¹⁴ and preliminary data have suggested that polysomy 17 may enhance trastuzumab sensitivity, even in the absence of *HER2* gene amplification.¹⁵ It remains unknown whether polysomy 17 leads to HER2 overexpression in patients with EAC.

We determined the association between HER2 protein expression and the frequency and level of gene amplification and with polysomy 17 in patients with EAC, applying interpretive criteria specific for esophagogastric cancers that are used in clinical practice. We examined the full consecutive series of patients with EAC, which enabled the determination of HER2 test parameters such as false negativity and specificity that are critical to inform the selection of patients for trastuzumab therapy. We also examined HER3 expression and its association with *HER2* amplification and expression. Given the potential effects of chemo(radio)therapy on tumor viability and HER2 expression, we studied patients before the routine use of neoadjuvant therapy.

MATERIALS AND METHODS

Study Cohort

The parent cohort (n = 787) was derived from the Mayo Esophageal Cancer Outcomes Database comprising consecutive patients with newly diagnosed, invasive adenocarcinoma of the esophagus, GEJ, or gastric cardia who underwent surgical resection with negative margins at the Mayo Clinic between 1980 and 1997, as described.¹⁶ Patients with subcardial gastric cancers and those with tumors with only nonadenocarcinoma histology were excluded, as were 9 patients who received neoadjuvant therapy. A total of 703 cases had sufficient tumor for evaluation. Regions of invasive carcinoma were identified using a hematoxylin and eosin-stained slide, and sequential whole-tissue sections were cut from formalin-fixed, paraffin-embedded surgical resection blocks, as previously described.^{17,18} The value of evaluating whole-tissue surgical sections is underscored by recent data indicating a significant false-negative rate for detecting HER2 aberrations in tissue microarrays and/or biopsy specimens.¹⁹ The Institutional Review Board at the Mayo Clinic approved this research and waived specific informed consent.

HER2 Gene and Chromosome 17 Copy Number

HER2 amplification in tumor cells was assessed using a US Food and Drug Administration-approved test (*HER2* and centromere 17 [CEP17] probes: PathVysion probe kit; Abbott Molecular, Des Plaines, Ill) as described.^{17,18} For each case, the number of *HER2* or CEP17 copies was determined, as described.^{17,18} Briefly, with reference to a parallel hematoxylin

and eosin-stained slide that identified regions of invasive carcinoma, 60 representative nuclei from the invasive tumor were scored for each tumor. A specimen with a mean *HER2/CEP17* ratio of ≥ 2.0 was classified as being *HER2* amplified, which is consistent with the definition used in the ToGA trial, in accordance with criteria developed for the classification of *HER2* and CEP17 abnormalities as described.^{17,18,20,21} Chromosome 17 gain was determined using CEP17 signal patterns based on methodology and cutoff values that we previously validated using 2 large independent breast cancer sets.²⁰ Accordingly, polysomy 17 (gain) was defined as 3 CEP17 signals in $> 30\%$ of nuclei, as previously described^{18,20}; this cutoff clearly distinguished chromosome 17 polysomic cancers from cancers without chromosome 17 centromere anomalies.²⁰

HER2 Protein Expression

HER2 expression was assessed using the US Food and Drug Administration-approved HercepTest (Dako, Carpinteria, Calif) as described.^{10,22} Each case was analyzed using criteria specific to upper digestive cancer,^{10,22} and the following data were recorded: 1) the intensity of complete, basolateral, or lateral membrane staining (none, faint, weak to moderate, or strong); and 2) the percentage of cancer cells with that intensity. Strong immunohisto-chemistry (IHC) intensity in $\geq 10\%$ of cancer cells was considered positive (IHC3+), weak to moderate intensity in $\geq 10\%$ of cancer cells was considered equivocal (IHC2-plus;), and faint intensity in $\geq 10\%$ or absent staining was considered negative (IHC1+ or IHC0, respectively). Interpretation of *HER2* IHC and fluorescence in situ hybridization (FISH) was performed in parallel in a blinded manner.

HER3 Protein Expression

HER3 expression was analyzed by IHC in a randomly selected subset of the overall cohort ($n = 224$). Antibody staining was performed using the following procedure after antibody optimization using non-study tumor samples. After deparaffinization in xylene, slides were rehydrated and washed with distilled water. After heat-induced epitope retrieval, endogenous peroxidase activity was quenched using a 3% peroxidase block (Dako S2001; Dako) and a serum-free protein block (Dako X0909; Dako). Slides were incubated with an anti-*HER3* antibody (1:100; rabbit polyclonal C-17, sc285; Santa Cruz Biotechnology, Santa Cruz, Calif.) followed by rinsing and the application of a secondary antibody (Dual Envision Link, Dako K4061; Dako). Slides were subsequently counterstained in modified Schmidt hematoxylin. For each case, the intensity of *HER3* cytoplasmic staining (absent, weak, moderate, or strong) and the percentage of immunopositive tumor cells (as a continuous variable) with that intensity was determined and recorded. Slides were scored with blinding to *HER2* expression/amplification and clinical data.

Statistical Analysis

Median values (with interquartile ranges) for *HER2* and CEP17 variables were determined. The chi-square or Fisher exact test and Wilcoxon rank sum test were used to compare variables (ie, overall test). The Cochran-Armitage test for trend was used to determine whether significant monotonic associations existed between variables. Least squares linear regression was used to determine linearity between IHC groups and the *HER2/CEP17* ratio.

For Cox proportional hazards models, OS and disease-specific survival (DSS) were endpoints; events beyond 5 years were censored. All *P* values were 2-sided. A *P* value < .05 was considered to be statistically significant. Analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc, Cary, NC).

RESULTS

A total of 703 patients with EAC for whom there was sufficient tumor underwent HER2 testing by both IHC and FISH. Although IHC was successful in all cases, the failure rate for FISH was 4% (29 of 703 cases); 1 case had missing FISH data. Accordingly, both IHC and FISH were evaluable in 673 tumors. Table 1 shows key clinicopathologic variables.²³ The median age of the patients was 65 years; the majority of patients were male (88%) and had locally advanced disease (68% with T3-T4 disease and 73% with lymph node-positive disease). By anatomic site, 316 cases were located in the esophagus, 321 were located at the GEJ, and 36 were limited to the gastric cardia. No patient received neoadjuvant or HER2-targeted therapy.

Concordance Between HER2 Expression and Gene Amplification

Using the HER2 protein expression level, 62% of cases demonstrated negative expression (39% were IHC0 and 23% were IHC1+), 25% demonstrated equivocal expression (IHC2+), and 13% demonstrated positive expression (IHC3+).

As shown in Table 2, Table 17% of the cases (116 of 673 cases) demonstrated *HER2* amplification. *HER2* amplification was detected in 89% of IHC3+ cases, 13% of IHC2+ cases, and 4% of IHC0 to IHC1+ cases. As shown in Table 3, using FISH as the reference standard, the positive predictive value of IHC3+ was 89% and the negative predictive value of IHC0 to IHC1+ was 96%. The negative predictive value of an IHC0 or IHC1+ score was similarly high (97% [256 of 264 cases] or 95% [145 of 153 cases], respectively) (Table 3). Importantly, the positive predictive value of IHC2+ expression was 13% (21 of 167 cases). The specificity of IHC3+ expression was 98% (Table 3). The concordance rate between the IHC and FISH results was 74% (501 of 673 cases) when IHC scores of 2+ and 3+ were grouped together. However, when the IHC2+ group (167 tumors) was excluded, the concordance rate increased to 95% (480 of 506 cases).

HER2-Amplified Subgroup: Level of Amplification and Protein Expression

Among *HER2*-amplified tumors, the level of gene amplification increased linearly with protein expression level, and was found to be highest among IHC3+ cases, intermediate in IHC2+ cases, and lowest among IHC0 to IHC1+ cases (median gene/chromosome ratio of 7.9 vs 5.5 vs 2.8, respectively; $P < .0001$ for linearity) (Fig. 1). The level of gene amplification was similar between IHC0 versus IHC1+ tumors (median gene/chromosome ratio of 2.8 vs 2.9; $P = .92$), but differed significantly between IHC0 to IHC1+ versus IHC2+ cases ($P = .0070$), and between IHC2+ versus IHC3+ cases ($P = .0060$). Accordingly, the number of *HER2* gene copies increased with HER2 expression levels ($P < .0001$), although the number of chromosome 17 copies was similar ($P = .14$) (Fig. 1).

HER2 Expression and Polysomy 17

Overall, 73% of EACs (491 of 673 EACs) demonstrated polysomy 17. The frequency of polysomy 17 was higher in non-*HER2*-amplified cases compared with *HER2*-amplified cases (75% [417 of 556 cases] vs 64% [74 of 115 cases]; $P = .024$), as reported.¹⁸ However, polysomy 17 rates did not differ by *HER2* protein expression overall ($P = .33$) or among non-*HER2*-amplified cases (data not shown).

HER2 and Clinicopathologic Characteristics

As shown in Table 1, *HER2* amplification and expression were each found to be significantly associated with well to moderate (vs poor) tumor differentiation ($P < .0001$ for both). Fewer regional lymph node metastases were present in cases with *HER2* amplification (vs those without) ($P = .0034$) and in cases with *HER2* overexpression (vs no overexpression) ($P = .063$).

HER3 Expression

HER3 immunoreactivity was detected in 87% of randomly selected EACs (195 of 224 EACs), with 8%, 40%, and 40% of the cases, respectively, demonstrating strong, moderate, and weak expression, and expression was absent in 13% of cases (Fig. 2). Among EACs showing weak or moderate *HER3* expression, 99% of tumors (176 of 178 tumors) demonstrated expression in 10% of tumor cells, with a relatively even distribution noted across quartiles of tumor cell percentage (ie, 10%–25%, 26%–50%, 51%–75%, and > 75%) generated from continuous data. Among EACs demonstrating strong *HER3* expression, 82% of cases (14 of 17 EACs) demonstrated expression in 10% of tumor cells, with the majority showing expression in 26% to 50% of tumor cells. As shown in Table 1, *HER3* expression was more common in tumors with well to moderate versus poor differentiation (96% vs 77%; $P < .0001$).

Association Between *HER2* and *HER3*

HER2 and *HER3* expression was found to be positively associated (Table 4) (Fig. 3). Specifically, the frequency of *HER2* expression was higher in tumors demonstrating any *HER3* expression (ie, weak, moderate, or strong) compared with tumors lacking *HER3* expression (P value for trend, .0019; overall $P = .0089$) (Table 4). Moreover, a positive stepwise gradient was observed between *HER2* and *HER3* expression levels (overall $P = .0139$) (Fig. 3). As shown in Figure 3, *HER2* IHC3+ expression was observed in 24% of tumors demonstrating strong *HER3* expression, but *HER2* IHC3+ expression was not detected in any tumors that lacked *HER3* expression. Likewise, *HER2* was absent (*HER2* IHC0 expression) in 24% of tumors with strong *HER3* expression compared with 83% of tumors lacking *HER3* expression (Fig. 3).

The frequency of any *HER3* expression was not found to be significantly higher in *HER2*-amplified versus non-*HER2*-amplified tumors (93% vs 86%, respectively; $P = .235$).

Association of HER2 and HER3 Expression With Prognosis

The median follow-up for vital status for surviving patients was 12.6 years. HER2 positivity (defined as IHC3+ or IHC2+ with gene amplification) was found to be significantly associated with favorable OS (hazards ratio [HR], 0.76; 95% confidence interval [95% CI], 0.59–0.96 [$P=.024$]) and with a trend toward favorable DSS (HR, 0.79; $P=.066$), but the associations were not found to be statistically significant after adjustment for pathologic tumor stage (Table 5²³) (Fig. 4), as previously reported.¹⁷ Similarly, HER3 expression (any vs absent) was not prognostic for either OS (univariate HR, 0.66; 95% CI, 0.43–1.03 [$P=.069$]) or DSS (HR, 0.72; 95% CI, 0.45–1.15 [$P=.16$]) (Table 5) (Fig. 4).

DISCUSSION

In the current study, we determined the frequency and level of *HER2* gene amplification (defined as a *HER2* / *CEP17* ratio ≥ 2) and chromosome 17 copy number in relation to HER2 protein expression in a consecutive series of surgically resected EACs. We found highly concordant results for HER2 expression and gene amplification at the extremes of IHC scores (IHC0-IHC1+ or IHC3+), but not in equivocal (IHC2+) cases, which had a 13% amplification rate. A negative IHC score (IHC0-IHC1+) was found to accurately predict a lack of *HER2* amplification in 96% of tumors, as has been shown in patients with breast cancer.^{24,25} A HER2 score of IHC3+ expression was highly specific (98%) for gene amplification. Restricting analysis to *HER2*-amplified cases demonstrated a positive linear association between the level of amplification and protein expression. It is important to note that the level of amplification in IHC0 to IHC1+ tumors, despite being gene amplified, was low (median gene/chromosome ratio, 2.8) compared with tumors with IHC2+ (median gene/chromosome ratio, 5.5) or IHC3+ (median gene/chromosome ratio, 7.9) HER2 expression. The clinical relevance of this finding derives from the ToGA study, in which patients with *HER2*-amplified IHC0 to IHC1+ tumors did not benefit from treatment with trastuzumab, in contrast to those with *HER2*-amplified IHC2+ or IHC3+ tumors, who experienced a relative risk reduction for death of 22% or 34%, respectively.^{10,26} Our finding of low-level amplification in amplified IHC0 to IHC1+ cases provides a biologic rationale for the reported lack of benefit noted with trastuzumab. The data from the current study can assist in patient selection for HER2-targeted therapy by excluding patients with IHC0 to IHC1+ tumors and supporting FISH analysis in patients with IHC2+ tumors.

We found that *HER2* amplification and overexpression were each associated with less aggressive tumor characteristics, including favorable tumor differentiation, and were not prognostic, as was previously reported by ourselves^{17,18} and in clinical trial cohorts of patients with gastroesophageal cancer.^{27–31} We found that polysomy 17 was significantly more common in non-*HER2*-amplified compared with *HER2*-amplified tumors, but was not associated with HER2 protein expression. This finding suggests that extra copies of the *HER2* gene due to polysomy 17 do not lead to HER2 overexpression.

HER3 was overexpressed in the majority of cases of EAC. To our knowledge, these data represent the first description of HER3 expression in human EACs. HER3 expression has been frequently detected in gastric cancers,^{7,32} in which it has not been found to be associated with numeric alterations of the *HER3* gene or chromosome 12.⁷ We found that

HER3 expression was significantly more common in EACs with well to moderate versus poor tumor differentiation and, similar to HER2, was not prognostic. HER3-expressing tumors demonstrated a higher frequency of HER2 expression. HER2 forms heterodimers with HER3 and epidermal growth factor receptor, with each independently implicated as a key coreceptor that drives *HER2*-amplified breast cancer.^{33,34} Data have indicated that HER3 is as critical as HER2 to maintaining cell proliferation in breast cancer cells, and a HER2 antibody that inhibits HER3 signaling by blocking ligand-induced heterodimerization can induce tumor regression with effects that are distinct from those of trastuzumab.³³ These and other data³⁵ have suggested that blocking HER3 in addition to HER2 may augment therapeutic efficacy by inhibition of HER2/HER3 signaling. While this hypothesis awaits further study in patients with esophagogastric cancer, our finding of frequent HER3 expression and its coexpression with HER2 suggests that dual targeting may be a clinically relevant strategy in this malignancy and it is currently being pursued in ongoing clinical trials.

To our knowledge, the current study is the largest to date to examine HER2 by both IHC and FISH in whole-tissue sections of esophageal or gastric cancer. Compared with our prior report that examined HER2 expression and prognosis,¹⁷ the current study analyzed gene amplification in the full study cohort to enable a more robust estimation of HER2 test concordance and examined the association between HER2 and HER3. It is important to examine EACs separately from subcardial gastric cancer because these diseases are different with regard to HER2 frequency and epidemiology.^{10,17,36,37} The *HER2* amplification rate observed in the current study is comparable to those reported in patients with breast carcinoma. An important limitation of the current study is its retrospective nature and the finding that response data were not available to correlate with HER2 or HER3 expression levels, although trial data in which this question can be addressed are limited. In addition, the current study cohort was limited to patients with resectable disease, and it is possible that these findings may not be generalizable to those with metastatic disease.

HER2 protein expression and gene amplification were found to be highly concordant and, among amplified cases, the level of amplification increased linearly with protein expression. Equivocal (IHC2+) HER2 expression, which occurs in approximately 25% of patients, warrants analysis by FISH to identify those patients with gene amplification who may benefit from treatment with trastuzumab. We found frequent expression of HER3 among patients with EAC and a significant association with HER2 expression. These data suggest the clinical relevance of HER3 in the treatment of patients with EAC and support ongoing efforts to therapeutically target HER3 and its interaction with HER2.

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Figure 1.

Level of human epidermal growth factor receptor 2 (*HER2*) gene amplification in relation to protein expression is shown among *HER2*-amplified cases (n=116). For each cytogenetic abnormality, the mean measurements per nucleus were determined for each case, and the median values (with interquartile ranges) across patients are shown for (A) the ratio between the *HER2* gene copy number and chromosome 17 copy number ($P < .0001$ for linearity), (B) *HER2* gene copy number, and (C) chromosome 17 copy number. NS indicates not significant.

Figure 2. Representative images of human epidermal growth factor receptor 3 (HER3) protein expression by immunohistochemistry in esophageal adenocarcinomas are shown demonstrating (A) strong, (B) moderate, (C) weak, and (D) absent expression (magnification $\times 400$).

Figure 3.

Association between human epidermal growth factor receptor 3 (HER3) and human epidermal growth factor receptor 2 (HER2) protein expression is shown in randomly selected cases (n = 224). HER3 status is shown in relation to HER2 scores (immunohistochemistry [IHC] 0 indicates absent; IHC 1+, faint in 10% of cancer cells; IHC 2+, weak to moderate in 10% of cancer cells; and IHC 3+, strong in 10% of cancer cells). Histogram bars represent the percentage of cases.

Figure 4.

Prognostic impact of human epidermal growth factor receptor 2 (HER2) and human epidermal growth factor receptor 3 (HER3) is shown. Kaplan-Meier survival curves are shown for (A and B) HER3 expression (any [n=29] vs absent [n=195]) and (C and D) HER2 positivity (immunohistochemistry [IHC]3+ or IHC2+ with *HER2* gene amplification [n=119] vs IHC2+ without amplification or IHC0 to IHC1+ [n=589]) with HER2 analysis including cases with IHC data alone or with concurrent amplification data. Endpoints were (A and C) disease-specific survival and (B and D) overall survival. Hazards ratios (HRs) and

95% confidence intervals (95% CIs) from univariate Cox proportional hazards models are shown.

HER2 and HER3 Protein Expression and *HER2* Gene Amplification in Relation to Clinicopathologic Characteristics in a Patient Cohort With Resected Esophageal Adenocarcinoma

TABLE 1

Variable	HER2 Protein Expression N = 673				P	HER2 Gene Amplification N = 673		P	HER3 Protein Expression n = 224 ^a		
	Total N = 673 No. (%)	0 or 1 + (n = 417) No. (%)	2 + (n = 167) No. (%)	3 + (n = 89) No. (%)		Negative (n = 557) No. (%)	Positive (n = 116) No. (%)		Absent (n = 29) No. (%)	Any ^b (n = 195) No. (%)	P
Median age, y	64.8	65.3	63.3	63.8	.273	65.0	63.7	.193	66.6	63.9	.343
Sex											
Male	595 (88)	369 (62)	149(25)	77(13)	.810	494 (83)	101 (17)	.620	26(13)	171 (87)	.762
Female	78(12)	48 (61)	18(23)	12(15)		63(81)	15(19)		3(11)	24 (89)	
Depth of invasion											
T1-2	217(32)	130(60)	51 (24)	36(16)	.217	169(78)	48 (22)	.024	7(10)	62 (90)	.537
T3-4	452 (68)	283 (63)	116(26)	53(12)		384 (85)	68(15)		20(13)	133(87)	
Missing data	4	4	0	0		4	0		2	0	
Median no. of LN metastases	2.0	2.0	2.0	1.0	.063	2.0	1.0	.0034	2.0	2.0	.701
Differentiation											
Well to moderate	397 (59)	209 (53)	116(29)	72 (18)	<.0001	305 (77)	92 (23)	<.0001	5(4)	121 (96)	<.0001
Poor	270(41)	205 (76)	49(18)	16(6)		249 (92)	21 (8)		22 (23)	73 (77)	
Missing	6	3	2	1		3	3		2	1	
Stage ^c											
I-II	235 (35)	140(60)	56 (24)	39(16)	.174	185(79)	50 (21)	.032	6(8)	72 (92)	.197
III	428 (65)	270 (63)	109(26)	49(11)		365 (85)	63(15)		19(13)	122 (87)	
Missing data	10	7	2	1		7	3		4	1	

Abbreviations: HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; LN, lymph node.

^aRandomly selected cases.

^bWeak, moderate, or strong HER3 expression.

^cPathologic tumor stage according to the American Joint Committee on Cancer, which incorporates histologic differentiation.²³

TABLE 2Frequency of *HER2* Gene Amplification in Relation to HER2 Protein Expression

HER2 Protein Expression (Immunohistochemistry)	No. of Patients (%)		
	<u>HER2 Gene Amplification(FISH)</u>		
	Total N = 673	Not Amplified n = 557	Amplified n = 116
Positive (3+)	89 (13)	10 (11)	79 (89)
Equivocal (2+)	167 (25)	146 (87)	21 (13)
Negative (0 or 1+)	417 (62)	401 (96)	16 (4)

Abbreviation: FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2.

TABLE 3Sensitivity, Specificity, and Predictive Values of HER2 Protein Expression for *HER2* Gene Amplification

Parameter	Numerator/ Denominator	%
Concordance		
All cases (IHC2+ and IHC3+ grouped together)	501/673	74%
Excluding IHC2+ cases	480/506	95%
Sensitivity (including IHC2+ and IHC3+)	100/116	86%
Specificity (IHC3+ only)	547/557	98%
Predictive value		
Positive (IHC3+ only)	79/89	89%
Positive (IHC2+ only)	21/167	13%
Negative (IHC0 or IHC1+)	401/417	96%

Abbreviation: HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

TABLE 4

Positive Association Between HER3 and HER2 Immunohistochemical Protein Expression

HER2 Expression N = 224	HER3 Expression % (No.)	
	Any (n = 195)	
	Absent (n = 29)	Weak, Moderate, or Strong
IHC0 (n = 123)	83% (24)	51% (99)
IHC1+ (n = 36)	7% (2)	17% (34)
IHC2+ (n = 39)	10% (3)	19% (36)
IHC3+ (n = 26)	0	13% (26)
<i>P</i> for trend	Reference	.0019
<i>P</i> overall		.0089

Abbreviation: HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; IHC, immunohistochemistry.

TABLE 5
Multivariable Cox Proportional Hazards Models Showing the Association of HER3 or HER2 With Patient Survival^a

Variable	Disease-Specific Survival			Overall Survival		
	HR	95% CI	P	HR	95% CI	P
Model 1						
HER3 any	0.87	0.52–1.44	.58	.76	0.47–1.22	.25
HER3 absent		Reference			Reference	
Model 2						
HER2-positive (IHC3+ or IHC2+/FISHpositive)	0.89	0.69–1.14	.35	.84	0.66–1.08	.17
HER2-negative (IHC0-1+ or IHC2+/FISHnegative)		Reference			Reference	

95% CI, 95% confidence interval; FISH; fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; HR, hazards ratio; IHC, immunohistochemistry.

^aHRs and 95% CIs were adjusted for American Joint Committee on Cancer pathologic tumor stage.^{2,3}