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HER2 amplification, overexpression and score criteria in esophageal adenocarcinoma

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The *HER2* oncogene was recently reported to be amplified and overexpressed in esophageal adenocarcinoma. However, the relationship of *HER2* amplification in esophageal adenocarcinoma with prognosis has not been well defined. The scoring systems for clinically evaluating HER2 in esophageal adenocarcinoma are not established. The aims of the study were to establish a HER2 scoring system and comprehensively investigate *HER2* amplification and overexpression in esophageal adenocarcinoma and its precursor lesion. Using a tissue microarray, containing 116 cases of esophageal adenocarcinoma, 34 cases of Barrett's esophagus, 18 cases of low-grade dysplasia and 15 cases of high-grade dysplasia, *HER2* amplification and overexpression were analyzed by HercepTest and chromogenic *in situ* hybridization methods. The amplification frequency in an independent series of 116 esophageal adenocarcinoma samples was also analyzed using Affymetrix SNP 6.0 microarrays. In our studies, we have found that *HER2* amplification does not associate with poor prognosis in total 232 esophageal adenocarcinoma patients by chromogenic *in situ* hybridization and high-density microarrays. We further confirm the similar frequency of *HER2* amplification by chromogenic *in situ* hybridization (18%; 21 out of 116) and SNP 6.0 microarrays (16%, 19 out of 116) in esophageal adenocarcinoma. HER2 protein overexpression was observed in 12% (14 out of 116) of esophageal adenocarcinoma and 7% (1 out of 15) of high-grade dysplasia. No *HER2* amplification or overexpression was identified in Barrett's esophagus or low-grade dysplasia. All HER2 protein overexpression cases showed *HER2* gene amplification. Gene amplification was found to be more frequent by chromogenic *in situ* hybridization than protein overexpression in esophageal adenocarcinoma (18 *vs* 12%). A modified two-step model for esophageal adenocarcinoma HER2 testing is recommended for clinical esophageal adenocarcinoma HER2 trial.
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Esophageal adenocarcinoma is thought to develop as a result of gastroesophageal reflux, which initiates a metaplastic change in the lower esophageal epithelium. Accumulation of genetic and epigenetic changes then results in progression to dysplasia and cancer in some individuals.[1,2] The incidence of esophageal adenocarcinoma has increased rapidly in the last three decades and the prognosis is usually very poor with 5-year survival rates ranging from 14–22%.[3–6] The surgical treatment of esophageal adenocarcinoma can offer cure for some, however, many patients have locally advanced or disseminated disease at presentation and require systemic therapy. Current chemotherapy regimens provide only minimal survival benefit, predominantly when used in combination with surgery or radiation. Identification of genetic alterations in esophageal adenocarcinoma that offer potential for biologically targeted treatment is one of the best hopes to improve the selectivity of therapy and enhance patient survival.

The *HER2* (c-erbB2) gene, a proto-oncogene, is located on chromosome 17q11.2-12 and encodes

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epidermal growth factor receptor 2 (HER2), a transmembrane glycoprotein receptor $p185^{HER2}$, which is targeted by the humanized monoclonal antibody trastuzumab (Herceptin).[7] *HER2* is amplified and overexpressed in ~25% of breast cancer patients and is associated with an aggressive clinical course and poor prognosis.[8] Trastuzumab treatment in combination with chemotherapy for breast cancer patients with *HER2* amplification/overexpression has shown a significant clinical efficacy in both the metastatic and adjuvant settings.[9] In esophageal adenocarcinoma, *HER2* overexpression and amplification has been reported at frequencies similar to those observed in breast cancer.[8,10–16] In esophageal adenocarcinoma, however, the relationship between *HER2* amplification and prognosis is controversial.[12,17] Recent studies have shown that anti-HER2 treatment enhanced radiosensitivity of esophageal cancer cell lines with *HER2* overexpression.[18] In esophageal adenocarcinoma, although the initial results from 19 esophageal adenocarcinoma patients did not show significant improvement of patient survival,[19] ToGA clinical trials in patients with gastric adenocarcinoma (trial *vs* control: 236 *vs* 243 patients) and gastroesophageal junction adenocarcinoma (trial *vs* control: 58 *vs* 48 patients) have shown a significant survival benefit for patients treated with a combination of Trastuzumab and standard chemotherapy.[20,21] The interest for treatment of esophageal adenocarcinoma therefore remains high.

The guidelines for detection and interpretation of *HER2* amplification/overexpression in breast cancer have been well established and anti-HER2 targeted treatment is routinely considered in the management of these patients.[22] Recently, Hofmann *et al*[23] established a modified HER2 scoring system in gastric cancer to identify suitable patients for enrollment in clinical trastuzumab trial. They found an incomplete moderate to strong basal/lateral membranous HER2 staining pattern and higher rate of tumor heterogeneity in gastric adenocarcinoma compared with breast cancer. However, the clinical assessment of *HER2* amplification and overexpression with immunohistochemistry and chromogenic *in situ* hybridization in esophageal adenocarcinoma is not well defined. In addition, the data on the frequency of *HER2* amplification and overexpression in esophageal adenocarcinoma and its precursor lesions including columnar cell metaplasia, Barrett's esophagus and dysplasia is very limited, particularly in US populations. Therefore, the aims of this study are (1) to comprehensively investigate the frequency of *HER2* overexpression and amplification in esophageal adenocarcinoma and precursor lesion using HER2 immunohistochemistry, chromogenic *in situ* hybridization and SNP 6.0 microarray, (2) to establish clinical methods to assess *HER2* amplification and overexpression, (3) to determine whether *HER2* amplification is a prognostic marker for esophageal adenocarcinoma patients by chromogenic *in situ* hybridization and high definition microarray in separate large clinical specimen and (4) to determine the relationship of *HER2* amplification with clinical factors including sex, age, lymph node metastasis and tumor stage.

Materials and methods

Construction of Tissue Microarray

Tissue microarrays, containing 38 cases of Barrett's esophagus, 81 cases of columnar cell metaplasia, 86 cases of squamous epithelium, 18 cases of low-grade dysplasia, 15 cases of high-grade dysplasia, and 116 cases of esophageal adenocarcinoma, were constructed from the representative areas of formalin-fixed specimens collected between 1997–2005 in the Department of Pathology and Laboratory Medicine, University of Rochester Medical Center/Strong Memorial Hospital, Rochester, New York. The 5-μ sections were cut from tissue microarrays and were stained with H&E to confirm the presence of the expected tissue histology within each tissue core. Additional sections were cut for immunohistochemistry and chromogenic *in situ* hybridization analysis.

Patients for Tissue Microarrays

All the 116 patients with esophageal adenocarcinoma used for the tissue microarray construction were treated with esophagectomy in Strong Memorial Hospital/University of Rochester between 1997 and 2005. These patients included 104 males (90%) and 12 females (10%). The patient age ranged from 34 to 85 years with a mean of 65 years. The follow-up period after esophagectomy ranged from 0.03 to 142 months with a mean of 39 months.

Patients for Affymetrix SNP 6.0 Analysis

Frozen tumors were obtained from 116 patients undergoing esophagectomy at the University of Pittsburgh Medical Center, Pittsburgh, PA between 2002 and 2008. Patient age ranged from 43–88 and the cohort consisted of 95 males and 21 females. Final pathologic stages were stage I (28), stage II (31), stage III (49) and stage IV (7). All tumor specimens were evaluated by a pathologist and were determined to be >70% tumor cell representation. Further details of this patient cohort and comprehensive genomic analysis of these tumors is to be published elsewhere.

In both institutes, all research was performed under protocols approved at both participating institutions.

Affymetrix SNP 6.0 Analysis

Genomic DNA was isolated using the QiaAmp DNA Mini Kit (Qiagen, CA) and 600 ng was used for

labeling and array hybridization at the SUNY Upstate Medical University microarray core facility (Syracuse, NY) using kits and protocols provided by Affymetrix. Array data quality was assessed using Affymetrix Genotyping Console 3.0 and all further data analysis was performed using Nexus 5.0 Copy Number Analysis software (Biodiscovery, CA).

Immunohistochemistry

Tissue sections from the tissue microarray were deparaffinized, rehydrated through graded alcohols, and washed with phosphate buffered saline. Antigen retrieval for HER2 was performed by heating sections in 99 °C water bath for 40 min. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, ready-to-use mouse monoclonal antibody anti-HER2 (DAKO, CA) was incubated at room temperature for 30 min. The secondary antibody (Flex HRP) was allowed to incubate for 30 min. After washing, sections were incubated with Flex DAB chromogen for 10 min and counterstained with Flex hematoxylin for 5 min. A breast carcinoma with known *HER2* overexpression served as positive control. Negative control was performed by replacing anti-HER2 antibody with normal serum.

Chromogenic *In Situ* Hybridization

HER2 chromogenic *in situ* hybridization was performed according to the manufacturer's protocol (DAKO DuoCISH kit, DAKO, CA) and stained in the Dako Autostainer. Briefly, tissue microarray sections were deparaffined in xylene, rehydrated through graded alcohols and washed in diluted wash buffer for 3 min. Endogenous peroxidase was blocked by peroxidase block solution for 5 min. Sections were subsequently incubated with chromogenic *in situ* hybridization antibody mix for 30 min. After washing, sections were incubated with red chromogen solution and blue chromogen solution for 10 min, respectively. Sections were counterstained with hematoxylin, dried and mounted. A breast carcinoma and esophageal adenocarcinoma with known *HER2* amplification was used as positive control. Negative control was performed by replacing *HER2* probe by normal serum.

Scoring of Immunohistochemistry and Chromogenic *In Situ* Hybridization

All sections were reviewed independently by ZZ and YH blinded to all clinical and pathologic information. Discordant cases were reviewed by DH and a final consensus was reached. For HER2 immunohistochemical stain, percentage (0-100%) of positive cells was determined. Gastric HER2 scoring criteria were used for evaluation of these samples. The intensity of HER2 staining was graded as 0, 1+, 2+, or 3+. HER2 protein was considered overexpressed if 10% or more of cells stained with a moderate to strong intensity and showed either complete or a basal/lateral membrane staining pattern (Figure 3, Table 4). The luminal part of well to moderately differentiated tumor glands often show negative stain. This pattern is also observed in gastric adenocarcinoma.[23] For *HER2* chromogenic *in situ* hybridization, the hybridization signals were counted in 50 nuclei per tissue core (Figure 2). All overlapping nuclei were excluded, only nuclei with a distinct nuclear border were evaluated. The ratio between *HER2* and chromosome 17 centromere copy numbers was calculated. The *HER2* gene was considered as amplification when the ratio of gene-specific *HER2*: centromere probe signals (CEP17) was ≥ 2.0.

Statistical analysis

Kaplan–Meier survival estimator and logrank test was used to analyze the patient survival between *HER2* amplified group and non-*HER2* amplified group. Both a univariate model with *HER2* as the soul explanatory variable and a multivariate model with several other clinical covariate, including gender, age, lymph node metastasis and tumor stage, were used. In addition, Fisher's exact test and χ^2-test were used to compare *HER2* positivity rate between esophageal adenocarcinoma, high- and low-grade dysplasia, columnar cell metaplasia and squamous epithelium subpopulations. *P*-value less than 0.05 is considered statistically significant.

Results

Defining the *HER2/ERBB2* Amplicon in Esophageal Adenocarcinoma With High-Density Microarrays

Analysis of 116 esophageal adenocarcinoma specimens using high-density microarrays revealed amplification in 16% (19 out of 116). In 9% (10 out of 116), amplification was considered high level (log2 ratio >0.6). The minimal region of amplification spans 68 kb (Figure 1) and contains only three genes (ERBB2, C17orf37 and GRB7). In this cohort study, the median overall survival of *HER2* amplification is 21 months and non-*HER2* amplification is 25 months. No association was found with *HER2* amplification and either disease free survival ($P = 0.709$) or overall survival ($P = 0.27$).

Chromogenic *In Situ* Hybridization Analysis

The *HER2* chromogenic *in situ* hybridization results are summarized in Table 1. No *HER2* gene amplification was identified in esophageal squamous epithelium ($n = 86$), columnar cell metaplasia ($n = 81$), Barrett's esophagus ($n = 34$) and low-grade

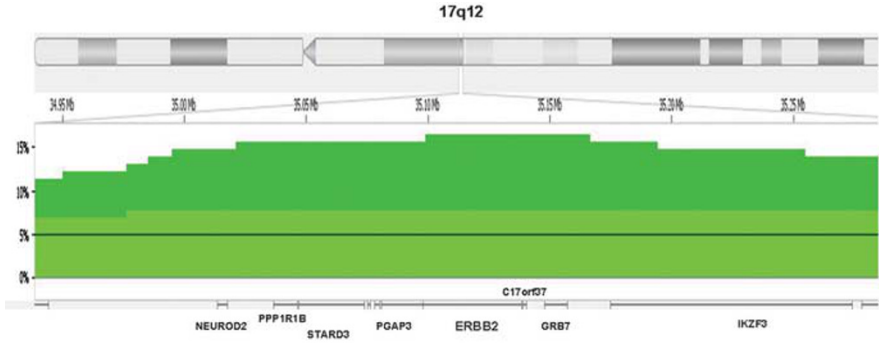

Figure 1 Frequency histogram showing amplification of the *ERBB2* (*HER2*) locus at chromosome 17q12 in 116 esophageal adenocarcinoma samples. The minimal amplicon (peak) spans 68 kb and contains only three genes, *ERBB2*, *C17orf37* and *GRB7*. This locus is amplified in 19 out of 116 (16%) cases in this patient cohort.

Table 1 Results of immunohistochemistry for HER2 oncoprotein overexpression and chromogenic *in situ* hybridization for HER2 gene amplification in esophageal squamous epithelium, columnar cell metaplasia, Barrett's esophagus, dysplasia and adenocarcinoma.

		HER2 overexpression		*HER2 amplification*	
Tissue	n	n	%	n	%
Squamous epithelium	86	0	0	0	0
Columnar cell metaplasia	81	0	0	0	0
Barrett's esophagus	34	0	0	0	0
Low-grade dysplasia	18	0	0	0	0
High-grade dysplasia	15	1	7	1	7
Esophageal adenocarcinoma	116	14[a]	12	21	18

[a]14 cases including 11 cases with positive HER2 immunohistochemical stain from tissue microarray and three cases from whole slides.

dysplasia ($n=18$). One of 15 cases of high-grade dysplasia (7%) showed *HER2* gene amplification (Table 1) and 21 of 116 cases of esophageal adenocarcinoma (18.10%) showed *HER2* gene amplification (Table 1, Figure 2). Esophageal adenocarcinoma showed statistically significant higher *HER2* gene amplification compared with the low-grade dysplasia, Barrett's esophagus, columnar cell metaplasia, and squamous epithelium groups ($P<0.05$), but not when compared with high-grade dysplasia group ($P>0.05$).

Immunohistochemical Analysis

The HER2 immunohistochemical staining results are summarized in Table 1 and Figure 3. No HER2 protein overexpression was identified in esophageal squamous epithelium ($n=86$), columnar cell metaplasia ($n=81$), Barrett's esophagus ($n=34$) and low-grade dysplasia ($n=18$). One of 15 cases of high-grade dysplasia (7%) showed HER2 overexpression (Table 1). Eleven of 116 cases of esophageal adenocarcinoma (10%) showed HER2 overexpression from tissue microarray (Table 1, Figure 3). The whole slides from 10 cases with *HER2* amplification and non-HER2 overexpression were used to investigate the heterogeneity of HER2 overexpression. Three cases in whole slides showed focal positive immunohistochemical stain (2+). Total 14 of 116 cases of esophageal adenocarcinoma (12%) showed HER2 overexpression. esophageal adenocarcinoma showed statistically significant higher HER2 protein overexpression compared with the low grade dysplasia, Barrett's esophagus, columnar cell metaplasia, and squamous epithelium groups ($P<0.05$), but not when compared with high-grade dysplasia group ($P>0.05$).

Association Between Immunohistochemistry and Chromogenic *In Situ* Hybridization Analyses

The correlation between HER2 protein overexpression by immunohistochemical stain and *HER2* gene amplification by chromogenic *in situ* hybridization in 116 cases of esophageal adenocarcinoma is summarized in Table 2. All 14 cases of esophageal adenocarcinoma with HER2 protein overexpression identified by immunohistochemical stain also showed *HER2* gene amplification by chromogenic *in situ* hybridization. Ten cases with *HER2* gene amplification identified by chromogenic *in situ* hybridization showed HER2 protein overexpression in three cases with whole slides immunohistochemical stain (Table 2). No HER2 expression showed in the other seven cases. Total 95 cases (82%) of esophageal adenocarcinoma showed neither HER2 protein overexpression nor *HER2* gene amplification. Concordance between immunohistochemical stain and chromogenic *in situ* hybridization was 76%.

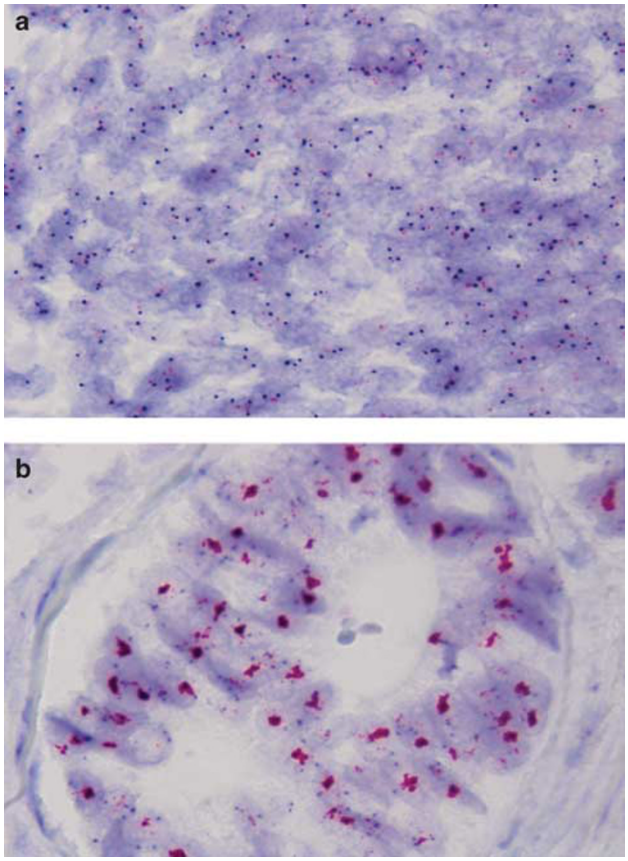

Figure 2 Chromogenic *in situ* hybridization showing *HER2* gene normal (**a**) and amplification (**b**) in esophageal adenocarcinoma ($\times 1000$). The *HER2* gene was considered as amplification when the ratio of gene-specific (*HER2*): centromere probe signals (CEP17) was ≥ 2.0 (red signals: *HER2*; blue signals: CEP17).

Association of HER2 Amplification with Survival and Other Clinical Factors

The median survival after esophagectomy between *HER2* amplification group and no *HER2* amplification group is 25 and 23 months, respectively, by chromogenic *in situ* hybridization. A survival analysis was based on Cox regression and log-rank test showed that there is no statistical significance in overall survival between the two groups ($P=0.19$, Figure 4). When this regression was refit by using one of the following clinical covariate: age, gender, the stage, and metastatic lymph nodes of the tumor, instead of *HER2*, we found that age ($P=0.064$) and gender ($P=0.448$) were not significantly associated with patient survival, but the stage ($P<0.001$) and the number of metastatic lymph nodes of the tumor ($P<0.001$) have strong association with patient survival.

In addition, we studied the association between *HER2* amplification and these clinical factors (Table 3). Of 116 esophageal adenocarcinoma patients, 21 had *HER2* amplification. Nineteen were male, and two female (M:F ratio, 10:1), with a mean age of 63 years (range, 51 to 74 years). The remaining patients (85 males and 10 females (M:F ratio, 9:1), mean age 85 years, (34–85 years)) had no amplification. A Fisher's exact test shows that there is no significant association between *HER2* and gender ($P=1.0$), age ($P=0.188$), the stage ($P=0.325$), and the number of metastatic lymph nodes ($P=0.234$). However, the frequency of HER2 amplification was found to be significantly higher ($P=0.004$) in moderately differentiated tumors (13/22) compared with poor or well-differentiated tumors (1/6 and 7/61 respectively).

Discussion

In the present study, we provide evidence that *HER2* amplification does not associate with poor prognosis in total 232 esophageal adenocarcinoma patients by chromogenic *in situ* hybridization (116 patients) and high-density microarrays (116 patients). We further confirm the frequency of *HER2* amplification and overexpression in esophageal adenocarcinoma and high-grade dysplasia (see Table 1) by various methods including high-density microarrays, chromogenic *in situ* hybridization and immunohistochemistry. In addition, we recommend a modified two-step model for clinical HER2 study in esophageal adenocarcinoma patients, similar to what has been proposed for the HER2 evaluation of gastric adenocarcinomas.[23]

Data on the association between *HER2* amplification and survival duration in esophageal adenocarcinoma are limited and conflicting. Brien *et al*[17] found that patients with *HER2* amplification ($n=11$) had shorter survival durations than did patients without amplification ($n=43$). However, Reichelt *et al*[12] found no survival difference between the *HER2* amplification ($n=16$) and no *HER2* amplification groups ($n=90$)($P=0.953$). In addition, Rauser *et al*[24] found that *HER2* gene amplification was associated with increased disease-specific mortality on three-dimensional fluorescence *in situ* hybridization (FISH) analysis in thick slides (16 μm), but not on FISH and immunohistochemical analyses in thin (4 μm) sections. Our results indicate no association of *HER2* amplification with patient survival in a large cohort studies (total 232 patients) by both chromogenic *in situ* hybridization and high-density microarrays.

In esophageal adenocarcinoma, recently Reichelt *et al*[12] found that 15% (16 out of 110) of tumors had *HER2* gene amplification with FISH. Similarly, Brien *et al*. showed that 19% (12 out of 63) of esophageal adenocarcinomas had *HER2* gene amplification.[17] In addition, with three-dimentional FISH method in thick slides (16 μm, $n=124$), Rauser *et al*[24] found that *HER2* amplification was 10.5% in high-level amplification (≥ 6.0 signals) and 60% in low-level copy number change (≥ 2.5–4.0 signals). However, in thin slides (4 μm, $n=123$), *HER2* amplification was found in 9% in high-level

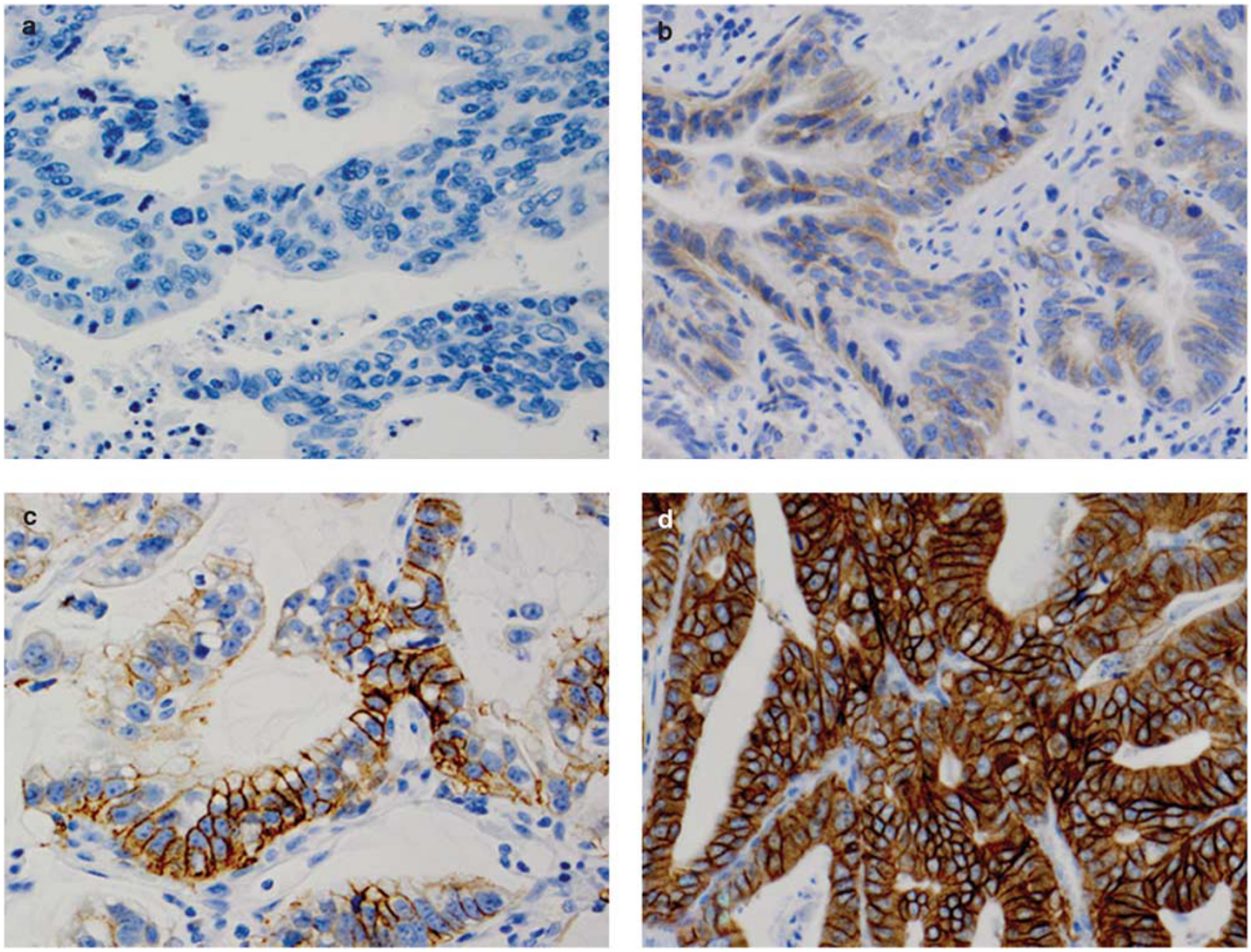

Figure 3 HER2 immunohistochemical staining showing 0 immunostaining ((**a**) ×400), 1+ immunostaining ((**b**) ×400), 2+ immunostaining ((**c**) ×400) and 3+ immunostaining ((**d**) ×400) in esophageal adenocarcinoma. Both 2+ and 3+ uniform staining are considered as HER2 protein overexpression. Incomplete membranous 'U' shape stain is presented in **c**.

Table 2 Correlation between immunohistochemistry and chromogenic *in situ* hybridization in esophageal adenocarcinoma

	Immunohistochemistry score				
	3+	*2+*	*1+*	*0*	*Total*
Chromogenic *in situ* hybridization+	5	9[a]	3	4	21
Chromogenic *in situ* hybridization−	0	0	6	89	95
Total	5	9	9	93	116

[a]Including three cases on whole slides with 2+ immunohistochemical staining.

amplification (≥6.0 signals) and 6% in low-level copy number change (≥2.5–4.0 signals). In the current study, we found that *HER2* amplification was 18% (21 out of 116) detected by chromogenic *in situ* hybridization and 16.4% (19 out of 116) by high definition microarray in cases of esophageal adenocarcinoma. In addition we found no evidence of *HER2* amplification in low-grad dysplasia, Barrett's esophagus, columnar cell metaplasia or normal esophageal squamous epithelium. Thus, the frequency of *HER2* amplification in esophageal adenocarcinoma appears to be consistent between studies with a range of 15–19% and this event appears not to occur before the development of high-grade dysplasia. However, there is a huge difference between traditional FISH in thin section (6%) and three-dimensional FISH in thick section (60%) to detect the low-level *HER2* amplification. They considered that the tumor cell nuclei were truncated due to standardized thin tissue sectioning. Therefore, three dimension FISH need to be further evaluated to help better understand any prognostic significance.

With previous gene amplification with PCR and microarray analysis, the 17q gain was found in various percentage from 21–33% in Albrecht *et al*[25] ($n=18$), van Dekken *et al*[26] ($n=28$) and Miller *et al*[27] ($n=87$). In current studies with high definition microarray analysis, 16.4% (19 out of 116) had *HER2* amplification detected. The incidences of

amplification defined by high-density microarrays in our study is close to reported FISH and chromogenic *in situ* hybridization results, but lower than those of previous studies.

The guidelines for detection and interpretation of *HER2* amplification/overexpression in breast cancer have been well established and anti-HER2 targeted treatment is routinely considered in the management of these patients.[22,28–30] Recently, HER2 scoring for gastric cancer has been modified due to HER2 stain pattern and heterogeneity,[17] which helps to improve the concordance between immunohistochemical stain and FISH and will help select the most appropriate HER2 positive patients for clinical trials with a combination of Trastuzumab and standard chemotherapy. The guideline for HER2 scoring in esophageal adenocarcinoma is critical for enrolling the patient for clinical trial. In our study, all HER2 with 2+ and 3+ expression cases identified with immunohistochemical stain showed *HER2* amplification with chromogenic *in situ* hybridization. The result is similar to the results of Reichelt's study from Germany,[12] but it does not agree to the gastric carcinoma studies.[23] With 2+ expression of HER2, only 36% of immunohistochemical stain 2+ cases were also FISH positive in gastric carcinoma. There are many causes of disagreement including immunohistochemical stain reactivity of esophageal adenocarcinoma, antibody, immunohistochemical methods, tissue fixation condition and etc. Actually, the HercepTest kit (DACO) was used in both studies. In addition, our studies were repeated several times in different immunohistochemical methods for the best results. In our study, modified two-step model is recommended for the guideline of clinical HER2 study in esophageal adenocarcinoma. In this model, immunohistochemical stain is first used to detect HER2 overexpression with 2+ and 3+ intensity and >10% of positive cells (see Table 4). If HER2 protein is overexpressed, *HER2* DNA amplification can be assumed, and chromogenic *in situ* hybridization analysis may not be needed. If HER2 protein is not overexpressed, chromogenic *in situ* hybridization or FISH analysis should be performed to rule out gene amplification. The *HER2* gene was considered as amplification when the ratio of gene-specific:centromere probe signals was ≥2.0 in our study, which is also the criteria used by Hofmann *et al*.[23] The definition for HER2 overexpression should be 2+ and 3+ instead of 3+ only in breast and gastric cancer as all 2+ expression cases in esophageal adenocarcinoma showed *HER2* amplification by chromogenic *in situ* hybridization. The negative overexpression of HER2 in esophageal adenocarcinoma also need to do chromogenic *in situ* hybridization as 7 of 102 patients show positive for chromogenic *in situ*

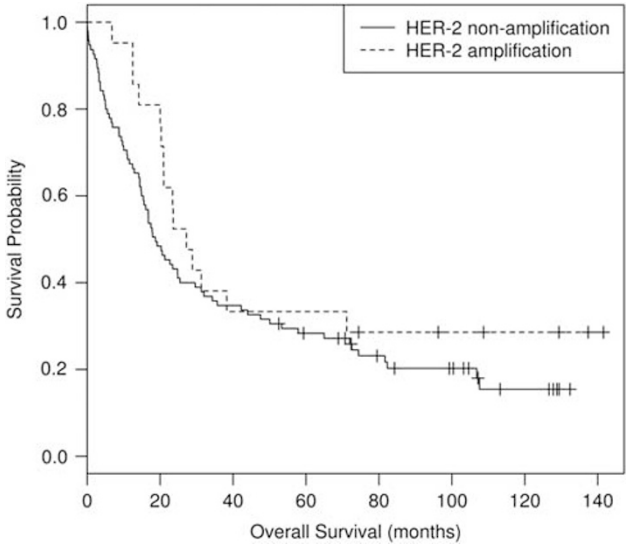

Figure 4 Kaplan–Meier analysis showing that there was no survival difference between *HER2* amplification group ($n=21$) and non-*HER2* amplification group ($n=95$) ($P=0.19$). The median survival after esophagectomy between *HER2* amplification group and no *HER2* amplification group is 25 and 23 months, respectively.

Table 3 Association o*f HER2* amplified group and *non-HER2* amplified group with multiple clinical factors

	HER2 amplified		*HER2 non-amplified*		P *value*
Age	63 (51–74)		65 (34–85)		0.188
Gender	Male	Female	Male	Female	1.0
	19	2	85	10	
	POS	NEG	POS	NEG	
Lymph node metastasis	13	8	69	26	0.234
pStaging					0.325
I	3		10		
II	8		25		
III	10		60		
Median survival (months)	25 (7–71)		23 (0.03–108)		0.19
Differentiation					0.004
Poor	7		61		
Moderate	13		22		
Well	1		6		

Table 4 Score criteria of HER2 immunohistochemical stain and FISH/chromogenic *in situ* hybridization for esophageal adenocarcinoma

HER2 immunohistochemistry features	*Score*
No reactivity or very faint membranous stain in $<10\%$ of cells	0
Faint membranous stain in $>10\%$ of cells	1+
Weak to moderate complete or baso/lateral membranous stain in $>10\%$ of tumor cells	2+/positive
Strong complete or basal/lateral membranous stain in $>10\%$ of tumor cells	3+/positive
HER2 FISH/chromogenic in situ hybridization test	
Ratio of average *HER2*/CEP17 $\geqslant 2.0$	Positive

Abbreviation: FISH: florescence *in situ* hybridization.

hybridization test with negative for immunohistochemical HER2 overexpression (1+ or 0). In addition, the 'U' shaped stain pattern in esophageal adenocarcinoma without complete membrane is the common pattern in well differentiated esophageal adenocarcinoma, which is considered as positive immunohistochemical stain. The heterogeneity of HER2 overexpression in gastric adenocarcinoma is higher than that in breast cancer.[23] We also found that the heterogeneity in esophageal adenocarcinoma is in our preliminary data in 10 chromogenic *in situ* hybridization positive and immunohistochemical negative cases. There is a potential problem to evaluate HER2 overexpression of the biopsy specimen in clinical practice. The heterogeneity will be further studied in following research. For gastric biopsy, Hofmann *et al*[23] suggested that immunohistochemical intensity 3+ or FISH positive gastric adenocarcinoma should be counted as positive even in less than 10% cells. However, comparison of the treatment response from patients with less than 10% HER2 overexpression or amplification *vs* more than 10% should be studied. At present, we recommended that multiple biopsies of esophageal adenocarcinoma and gastric adenocarcinoma should be tested for HER2 amplification and overexpression. The criteria for gastric biopsy can be used for esophageal biopsy.

For our study, we used chromogenic *in situ* hybridization instead of FISH because of the increased stability of the chromogenic *in situ* hybridization labeled slides and the ease of interpretation of results, which does not require a fluorescent microscope and dark room. Furthermore the use of a light microscope allows us to see both signals and morphology simultaneously. Our experience and that of other groups showed that chromogenic *in situ* hybridization results are equivalent to FISH and that chromogenic *in situ* hybridization is a reliable method for detecting *HER2* genomic abnormalities.[31,32] Although both have similar turnaround time and cost in the laboratory, the DNA-specific probes for chromogenic *in situ* hybridization are 25–50% cheaper than those for FISH.[31] Because of the disadvantages of FISH, chromogenic *in situ* hybridization has shown a great potential to replace FISH for detecting *HER2* gene amplificaton.[32] In addition, our results showed that chromogenic *in situ* hybridization is more sensitive than immunohistochemical stain to detect HER2 abnormalities. This may be because DNA is more stable than protein during the specimen processing. However, the amplification without overexpression in some tumors also was reported.[12]

In summary, our study showed that HER2 is overexpressed at the protein level and amplified at the DNA level in a subset of esophageal adenocarcinoma and high-grade dysplasia. We observed neither HER2 protein overexpression nor DNA amplification in normal esophageal squamous epithelium, columnar cell metaplasia, Barrett's esophagus or low-grade dysplasia. A modified two-step model for the evaluation of the HER2 status in esophageal adenocarcinoma is suggested by our studies. Our results showed that *HER2* amplification is not a prognostic predictor for esophageal adenocarcinoma. However, ToGA clinical trial for gastric and GE junctional adenocarcinoma with significant improving patient's survival suggests that the patients with HER2 positive esophageal adenocarcinoma would be potential candidates for anti-HER2 targeted treatment.

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Disclosure/conflict of Interest

The authors declare no conflict of interest.

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