Med Oncol (2012) 29:933–940
DOI 10.1007/s12032-011-9850-y

ORIGINAL PAPER

Analysis of *HER2* gene amplification and protein expression in esophageal squamous cell carcinoma

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Received: 22 January 2011 / Accepted: 27 January 2011 / Published online: 13 February 2011

Abstract The *HER2* gene, which is located on chromosomes 17, is a therapeutic target for cancer. Amplification of *HER2* has been described in several tumor types. However, few studies of *HER2* gene amplification and protein expression in esophageal carcinoma have been conducted. This study was to investigate the relationship between the expression of *HER2*/neu and the clinical characteristics, including survival rate, of esophageal squamous carcinoma. The clinical data of 145 patients admitted in Renmin Hospital of Wuhan University, from 2000 to 2005, were reviewed. The *HER2* protein expression and gene status in 145 esophageal carcinomas were evaluated using immunohistochemistry and fluorescence in situ hybridization. The survival rate was calculated by the Kaplan–Meier method and the log-rank test using SPSS13.0 software. Compared to normal esophageal epithelium (23/95, 24.2%), *HER2* protein was overexpressed in most esophageal squamous carcinoma tissues (60/145, 41.4%), of which 45 (31.0%) were 2+ and 15 (10.4%) were 3+, *HER2* overexpression associated significantly with *HER2* gene amplification. There is a correlation between the overexpression of *HER2* and the differentiation of the carcinoma, the *HER2* gene amplification and the differentiation of the carcinoma and the tumor stage. According to univariate analysis, there was a significant difference in survival rates when cases with and without *HER-2/neu* overexpression or amplification were compared. *HER-2/neu* amplification/overexpression may be used as an independent prognostic factor in patients with esophageal squamous cancer, and patients with *HER-2/neu* amplification/overexpression might be potential candidates for new adjuvant therapies that involve the use of humanized monoclonal antibodies.

Keywords Esophageal squamous carcinoma · *HER2* · Immunohistochemistry · Fluorescence in situ hybridization

Introduction

Esophageal carcinoma remains the 8th leading cause of malignancy-related deaths worldwide [1]. Squamous cell carcinoma is the most frequent histological type of cancer of the esophagus (ESCC) [2]. It is usually associated with a poor prognosis because it is often at an advanced stage when diagnosed and there is a high frequency of lymph node metastases. Treatment for esophageal carcinoma remains a challenge for physicians. Recently, targeted therapy has been applied to esophageal carcinoma, which may open new avenues for cancer treatment. Current targeted therapy depends on the evaluation of the status of target genes [3, 4].

A member of the epidermal growth factor receptor (EGFR) family, c-erbB-2 (*HER2*), has received much attention because it is the therapeutic target in several tumors. Overall, 15–25% of patients with breast cancer [5–7] have been found to be *HER2* 2+/3+, which is associated with an unfavorable prognosis, especially in patients with

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lymph node metastases. In addition, there is accruing evidence to indicate that *HER2* is an important predictive factor of response to chemotherapy and hormonal therapy in breast cancer [8, 9].

A number of studies have analyzed *HER2* overexpression in esophageal carcinoma. A recent study has reported 30.3% of patients with esophageal squamous cell carcinomas had overexpression of *HER2* [10]. The clinical significance of *HER2* gene amplification and protein overexpression is not yet fully understood. In some studies, *HER2* appears to be an important prognostic factor in ESCC [11, 12]. However, the literature is conflicting in this respect, and not all studies have shown an association between *HER2* overexpression and poor prognosis [13–15].

The objectives of this study were (1) to determine the frequency of *HER-2/neu* amplification and overexpression in ESCC, (2) to clarify whether the same mechanisms of gene amplification and protein overexpression function in ESCC as in breast cancer, and (3) to investigate the relationship between *HER-2/neu* amplification/overexpression and the clinicopathological characteristics of tumors, including survival rates. This study was conducted with a view toward the future introduction of Herceptin therapy for the treatment of patients with ESCC.

Methods

Tumor tissue collection and human subjects approval

The specimens were selected from archive paraffin embedded blocks in Renmin Hospital of Wuhan University by two pathologists. A total of 312 Chinese patients with esophageal carcinoma who underwent surgery at the Department of Surgery, Renmin Hospital of Wuhan University, during the period of 2000–2005, were eligible. Only those patients whose clinical data (including diagnosis, age, sex, address, and disease history) were intact were included; 145 Chinese patients with ESCC were finally selected in this study. None of the patients had undergone preoperative radiation or chemotherapy. Ninety-five cases of normal esophagal tissues were cut from the distal esophagus of the same patients.

For all patients, we reviewed age, gender, tumor size, histological grade, extent of infiltration, lymphatic invasion, and evolution of disease. The clinicopathological data are summarized in Table 1. The age of the 145 patients selected for this study ranged from 34 to 81 years, with a mean of 59.2 years.

The follow-up time ranged from 0 to 120 months with an average of 47.9 months. The causes of death were ascertained from medical records or autopsy, if performed. Patients who had died within 4 weeks of radical surgery were excluded from our analyses. Deaths due to other causes resulted in censored observations beginning at time of death. The institutional review board at the Renmin Hospital of Wuhan University approved this study, and informed consent was obtained from all patients. Each specimen was routinely fixed in 10% formalin and embedded in paraffin. Before inclusion in the study, each specimen was verified by a histopathologist.

Immunohistochemistry

All esophageal tumor and normal esophageal epithelium specimens were fixed in 10% buffered formalin and embedded in paraffin according to standard procedures. Serial sections (4 μm thickness) placed on positively charged slides (Menzel-Glasser, German) were used for hematoxylin and eosin staining, immunohistochemistry, and FISH detection of *HER2*.

Immunohistochemistry for *HER2* was performed using the Hercep Test kit (DakoCytomation, Denmark), according to the manufacturer's instructions. Antibody binding was visualized by the EnVison detection kit (DakoCytomation, Denmark).

Immunohistochemical (IHC) staining was scored by two pathologists and evaluated following the criteria recommended by the manufacturer: no staining, or weak staining in fewer than 10% of the tumor cells (0); weak staining in part of the membrane in more than 10% of the tumor cells (1+); complete staining of the membrane with weak or moderate intensity in more than 10% of the neoplastic cells (2+); and strong staining in more than 10% (3+). *HER-2*/neu protein overexpression was defined as either negative (score 0 and 1+) or positive (score 2+ and 3+).This cutoff point was predicted on the results of previous breast cancer studies. Interpretations were made independently by two pathologists, who had been blinded to each other's findings, and to the results of the other assays. We used paraffin slides of invasive breast carcinoma as a positive control.

Fluorescence in situ hybridization

HER-2/neu amplification was analyzed using FISH *HER2* PharmDx (Dako, Denmark),which contains both fluorescently labeled *HER-2*/neu gene and chromosome 17 centromere probes. In brief, the sections were incubated at 56°C overnight and deparaffinized by washing in xylene, ethanol, and distilled water. After incubation in 0.2 M HCl at room temperature for 20 min, they were heat-treated in citrate buffer (2 × SSC, pH 6.0) at 80°C for 1–1.5 h. They were then digested with pepsin at room temperature for 8–14 min, rinsed in 2 × SSC at room temperature for 2 min and dehydrated in graded ethanol (75, 80, and 100%) for 2 min. After the *HER2*/CEN17 probe mix was applied

Table 1 Correlation between the HER2 protein expression, amplification and clinicopathologic feature in 145 esophageal carcinomas

Variable	HER2 protein expression			*P* value	HER2 amplification			*P* value
	Negative	Positive	Total		No amplification	Amplification	Total	
Age								
<55	38 (56.7)	29 (43.3)	67	0.666	57 (85.1)	10 (14.9)	67	0.625
≥55	47 (60.3)	31 (39.7)	78		64 (82.1)	14 (17.9)	78	
Gender								
Female	18 (66.7)	9 (33.3)	27	0.393	22 (81.5)	5 (18.5)	27	0.776
Male	67 (56.8)	51 (43.2)	118		99 (83.9)	19 (16.1)	118	
Tumor size (cm)								
$S < 3$	8 (61.5)	5 (38.5)	13	0.693	10 (76.9)	3 (23.1)	13	0.440
$3 \leq S < 5$	37 (56.9)	28 (43.1)	65		57 (87.7)	8 (12.3)	65	
$S \geq 5$	43 (64.2)	24 (35.8)	67		54 (80.6)	13 (19.4)	67	
Histological grade								
G_1-well	23 (76.7)	7 (23.3)	30	0.017	27 (90.0)	3 (10.0)	30	0.010
G_2-moderate	52 (57.1)	39 (42.9)	91		79 (86.8)	12 (13.2)	91	
G_3-poor	10 (41.7)	14 (58.3)	24		15 (62.5)	9 (37.5)	24	
Infiltration level								
T_1-mucous layer	5 (71.4)	2 (28.6)	7	0.157	6 (85.7)	1 (14.3)	7	0.694
T_2-muscular layer	23 (74.2)	8 (25.8)	31		28 (90.3)	3 (9.7)	31	
T_3-Serosa layer	55 (53.9)	47 (46.1)	102		83 (81.4)	19 (18.6)	102	
T_4-peripheral tissue	2 (40.0)	3 (60.0)	5		4 (80.0)	1 (20.0)	5	
Lymphatic invasion								
No	73 (61.9)	45 (38.1)	118	0.129	101 (85.6)	17 (14.4)	118	0.158
Yes	12 (44.4)	15 (55.6)	27		20 (74.1)	7 (25.9)	27	
Distant metastasis								
No	83 (60.1)	55 (39.9)	138	0.126	117 (84.8)	21 (15.2)	138	0.089
Yes	2 (28.6)	5 (71.4)	7		4 (57.1)	3 (42.9)	7	
Stage								
I	4 (80.0)	1 (20.0)	5	0.070	4 (80.0)	1 (20.0)	5	0.001
II	44 (66.7)	22 (33.3)	66		60 (90.9)	6 (9.1)	66	
III	25 (56.8)	19 (43.2)	44		39 (88.6)	5 (11.4)	44	
IV	12 (40.0)	18 (60.0)	30		18 (60.0)	12 (16.6)	30	

to the dry slides, the tissue area was cover slipped and sealed with rubber cement. The slides were then incubated in hybridizer (Hybridizer Instrument for in situ hybridization, DAKO, Denmark) for denaturation at 82°C for 5 min and hybridization at 45°C for about 18 h. Post-hybridization washes were performed in urea/0.1 × SSX at 45°C for 30 min and in 2 × SSC at room temperature for 2 min. The slides were dehydrated in graded ethanol, and after application of 15 μL of mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI), the tissue area was cover slipped.

FISH analyses were performed according to the *HER2* FISH PharmDx (Dako, Denmark) criteria. In each case, 100 non-overlapped, intact interphase tumor nuclei identified by DAPI staining were evaluated, and *HER2* gene (red signal) and CEN17 (green signal) copy numbers in each nucleus were assessed. The cases were considered to be amplified when the average copy number ratio, *HER2*/CEN17, was ≥2.0 in all nuclei evaluated or when the *HER2* signals formed a tight gene cluster. Among the cases in which *HER2* gene was not amplified, samples showing more than four copies of the *HER2* gene and more than four CEN17 in more than 10% of the tumor cells were considered to be polysomic for chromosome 17.

Statistical analyses

All statistical analyses were performed using SPSS for Windows 13.0, SPSS Inc. Categorical variables were compared by the Pearson Chi-square test or Fisher's exact test, depending on the expected values found in the contingency table. The overall survival rates were calculated

using the Kaplan–Meier method, and the curves were compared by the log-rank test. In all statistical tests, the alpha error was set at 5%. The survival period was calculated from the date of hospital admission to death or the date of last follow-up.

Results

HER-2/neu immunohistochemistry

The *HER2* protein was overexpressed in most esophageal carcinoma tissues (60/145, 41.4%), of which 45 (31.0%) were 2+ and 15 (10.4%) were 3+ (Fig. 1), compared to normal esophageal epithelium (23/95, 24.2%) (Table 2). Statistical analysis revealed an association between the expression of *HER2* and the differentiation of the carcinoma (Table 1).

HER-2/neu amplification

The same cases evaluated by immunohistochemistry were also examined using FISH. Gene amplification was found in 24 (16.6%) cases; 121 (83.4%) showed no amplification (Fig. 2). The *HER2* amplification cases included all fifteen of the *HER2* 3+ cases, six of the *HER2* 2+ cases, two of the *HER2* 1+ case and one of the *HER2* 0 cases by immunohistochemistry. *HER2* amplification was associated with the differentiation of the carcinoma and the tumor stage (Table 1). Statistical analysis revealed an association between *HER2* overexpression and *HER2* amplification ($P < 0.0001$) (Table 3). 13.1% (19/145)of the patients showed chromosome 17 polysomy. Two of the 19 patients with chromosome 17 polysomy showed *HER2* amplification. There was no association between chromosome 17 polysomy and *HER2* amplification. Four patients with *HER2* overexpression showed polysomy of chromosome 17, two (50%) were 3+ and two (25%) were 2+. However, there was no significant association between chromosome 17 polysomy and *HER2* overexpression (Table 4).

Survival analysis

Survival analysis was performed on 145 patients who had survived for more than 4 weeks after surgery. The survival curves, according to *HER-2/neu* amplification and overexpression, are shown in Figs. 3 and 4. Tumors associated

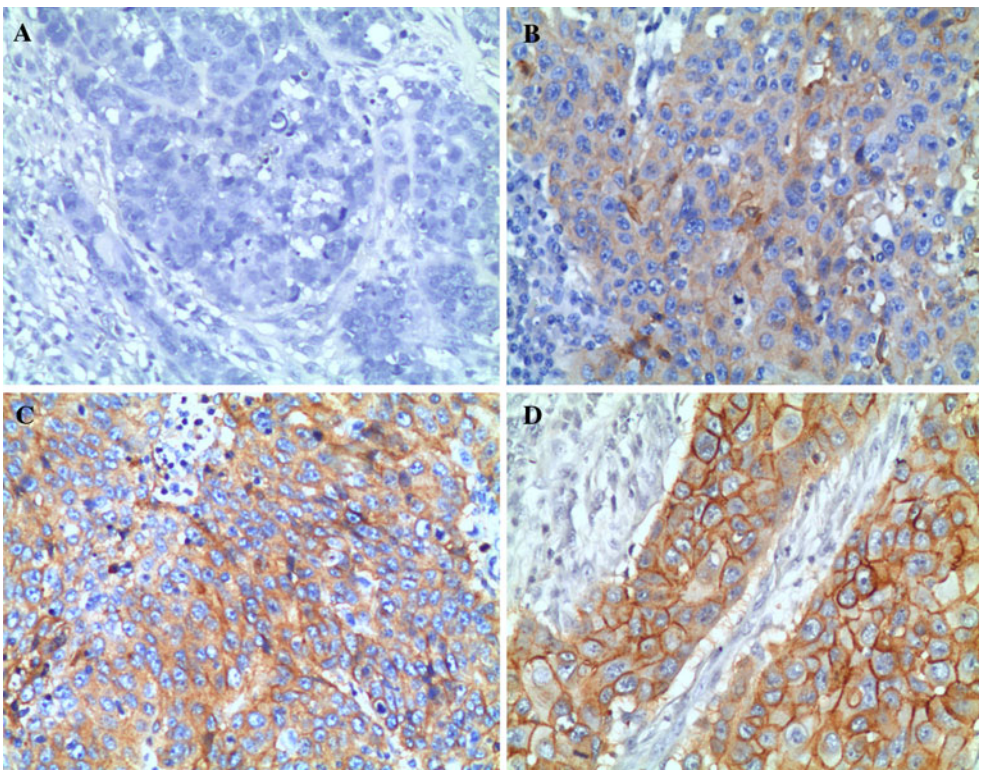

Fig. 1 Immunohistochemistry showing HER2 membrane staining in ESCC. **a** sample negative for expression of this protein, 400×; **b** negative case 1+, 400×; **c** positive case 2+, 400×and **d** positive cases 3+, 400×

Table 2 Expression of HER2 protein in normal esophageal epithelium and esophageal carcinoma

Variable	HER2 expression			*P* value
	0/1+	2+	3+	
Normal esophageal epithelium	72 (75.8%)	15 (15.8%)	8 (8.4%)	0.017
Esophageal carcinoma	85 (58.6%)	45 (31.0%)	15 (10.4%)	

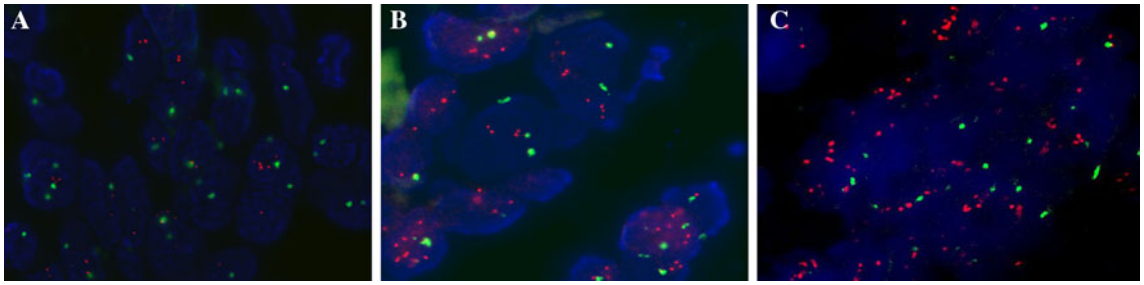

Fig. 2 Analysis of HER-2/neu gene amplification in ESCC. **a** sample with no amplification; **b** case considered amplified. In detail, nucleus shows ratio ≥2 and **c** gene amplification showing cluster pattern 1,000×

Table 3 Relationship between HER2 amplification and HER2 protein expression

HER2 IHC	HER2 FISH		Total
	Negative	Positive	
Negative	82	3	85
Positive	39	21	60
Total number	121	24	145
P value	<0.0001		

Positive: HER2 amplification, Negative: No HER2 amplication

Table 4 Relationship between chromosome 17 copy number and HER2 amplification/HER2 protein expression

Chromosome 17 Copy number	HER2 FISH		HER2 IHC	
	Negative	Positive	Negative	Positive
Non-polysomy	104	22	70	56
Polysome	17	2	15	4
Total	121	24	85	60
P value	0.740*	0.079#		

*Association between chromosome 17 polysomy and HER2 amplification

Association between chromosome 17 polysomy and HER2 protein expression

HER2 FISH Positive: HER2 amplification, HER2 FISH Negative: No HER2 amplification

HER2 IHC Positive: IHC staining level of 3+, 2+, HER2 Negative: IHC staining level of 0.1+

with *HER-2/neu* amplification exhibited poor mean survival rates (28.0 vs. 50.7 months). Tumors associated with HER-2/neu overexpression also exhibited poor mean survival rates (38.3 vs. 52.8 months).

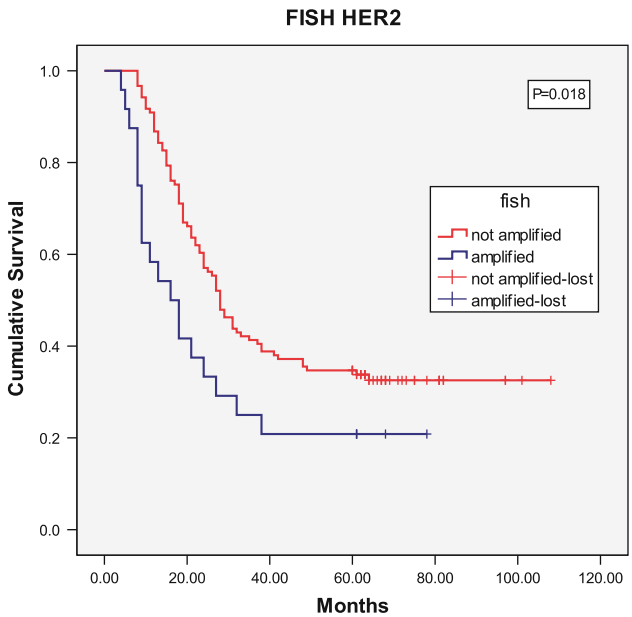

Fig. 3 Kaplan–Meier plot for overall survival in 145 patients with ESCC according to detection of *HER-2/neu* amplification

Discussion

HER2 is a transmembrane receptor with an intracellular domain with tyrosine kinase activity [16]. If *HER2* is overexpressed in the malignant cell, there is a subsequent signaling from the receptor, resulting in increased cell proliferation and mitosis, ultimately causing tumor progression and metastasis [17].

In this study, we demonstrated that *HER2* is overexpressed and amplified in ESCC; similar results were observed in other researches [1, 10–13, 18–22]. Therefore,

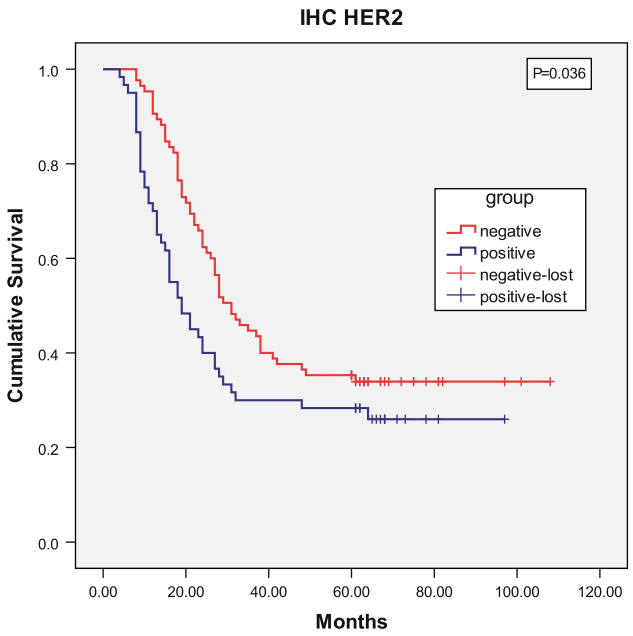

Fig. 4 Kaplan–Meier plot for overall survival in 145 patients with ESCC according to detection of HER-2/neu overexpression

it is reasonable to focus on *HER2* overexpression and/or gene amplification when developing therapeutic strategies to target carcinomas.

Immunohistochemical analysis showed that 41.4% of the cases were positive for expression of *HER2*. The few studies reporting *HER2* expression in ESCC show discrepant frequencies ranging from 0 to 64% [18–22]. This variability may have resulted from differences in immunohistochemical protocols, different antibody sources used by the different authors, or different criteria for evaluating expression.

Previous studies to determine whether *HER2* expression was associated with the clinicopathologic characteristics of ESCC have been controversial. We found there is significant association between *HER2* expression and the clinicopathological findings, such as histological grade and tumor stage.

HER2 overexpression is associated with lower rates of survival. These findings are consistent with previous reports [19, 23].

The reported frequencies of *HER-2*/neu amplification in ESCC vary from 2 to 19.1% in the patients [10–15]. This variability may have resulted from differences in tissue preparation, probes, and the methods used to evaluate the alterations. Our study demonstrated 16.6% of cases showed gene amplification.

Mimura et al. (2005) reported that all 3+ cases, 50% of 2+ cases showed gene amplification [11]. Another study showed a significant association between gene amplification and protein overexpression in 70% of 3+ and in 30% of 2+ amplified cases [12]. In contrast to these results, Sunpaweravong et al. (2005) found no significant association between gene amplification and immunohistochemical expression; the one case positive for amplification did not overexpress c-erbB-2 [13]. Our date agree that most c-erbB-2 overexpression is caused by gene amplification [24].

Chromosome 17 harbors a number of important oncogenes and tumor suppressor genes, including *HER2*, TOP2A, DARPP32, p53, and BRCA1 [25]. We found chromosome 17 polysomy did not correlate with *HER2* amplification or with *HER2* overexpression. Our results suggest that increased *HER2* gene dosage resulting from gene amplification is the most important determinant for *HER2* overexpression, whereas any influence resulting from chromosome 17 polysomy alone is unlikely to play a significant role in *HER2* gene overexpression at the transcriptional level. A similar finding was described previously in breast cancer [26].

FISH and IHC are two methods which have been used widely in clinical laboratories. These methods have both proven sensitive and specific in the laboratory. Compared to FISH, IHC is less time-consuming, less expensive, much easier to perform and requires minimal instrumentation. However, IHC methods can potentially be affected by a host of variables, including tissue fixation, processing, choice of primary antibodies, detection systems, and methods of antigen retrieval [27]. Furthermore, the interpretation for IHC may vary among observers, since the suggested scoring system for IHC is subjective. These factors, in addition to small study sample sizes, may also account for the variable rates of *HER-2*/neu immunoreactivity, as well as the conflicting reports suggesting the association of *HER-2/neu* with adverse clinical outcomes. FISH is currently regarded as the "gold standard" for the detection of *HER-2/neu* amplification: it is associated with both high sensitivity (96.5%) and high specificity (100%) [28]. FISH can be conducted with small tumor samples. Both formalin-fixed and paraffin-embedded tissue samples can be used since tissue preparation having little or no effect on the testing. It also allows for the direct visualization of gene amplification in the nuclei and provides an objective count of genes and chromosomes on a cell-by-cell basis. The disadvantage for FISH is that it requires a fluorescence microscope and special training in order to interpret the results. It also may prove quite difficult to visualize the morphological features of the tumor cells and, also, to separate in situ from invasive carcinoma when evaluating the amplification products via fluorescence. In addition, fluorescence fades quickly and does not create a permanent record [29].

Therefore, we think that c-erbB-2 expression must be evaluated initially by immunohistochemistry and, if the

results are not conclusive, FISH should be performed. Such a practice has been standard procedure to assist in making therapeutic decisions in patients with breast and lung cancer [30–32].

With respect to overall survival rate, our data are in agreement with the findings of Mimura et al. (2005), showing significant differences in survival rates in cases with gene amplification and *HER2* overexpression[11]. The significant association between *HER-2/neu* amplification and lower survival rate indicates a role for analyzing the alteration analysis in ESCC prognosis. Further studies with more cases are necessary for a better understanding of the influence of this gene on ESCC progression.

Antibody-based therapy with trastuzumab (Herceptin) is used clinically in *HER-2*–positive breast cancer [33–35]. Trastuzumab is most effective in patients with *HER-2*–positive breast cancer when used as adjuvant therapy [36]. Similarly, patients with ESCC having *HER-2/neu* gene amplification might also benefit from treatment with Trastuzumab, since *HER-2/neu* amplification indicates a group of cases in which this type of treatment could improve the prognosis [11, 37]. A recent study indicated that *HER2*-targeted therapy with trastuzumab (Herceptin) shows a significant primary tumor growth reduction as well as a reduction of lymph node metastases in an orthotopic mouse model of metastatic esophageal carcinoma. These preclinical results suggest a role for *HER2*-targeted antibody-based treatment of *HER-2*–overexpressing esophageal carcinoma. The results suggest, in particular, trastuzumab treatment in the adjuvant setting to prevent lymph node metastasis after primary tumor resection [38].

Conclusion

Our results indicate that *HER-2/neu* amplification/overexpression may constitute an independent prognostic factor in patients with esophageal squamous cancer and that patients exhibiting *HER-2/neu* amplification/overexpression might be potential candidates for new adjuvant therapies that involve the use of humanized monoclonal antibodies. Further studies with more cases and including additional techniques are necessary to verify other molecular alterations involved in tumor progression, which will contribute to the development of new therapies for ESCC.

Acknowledgments The authors thank Mr. Guo-sheng Xiong and Mis. Hong Hu for providing technical assistance, Dr. Ya-bing Huang and Dr. Lin Liu for assistance in standardizing the FISH protocol, Dr. Xiao-fan Li for revising this article.

Conflict of interests The authors declare that they have no conflict of interests.

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