

Epidermal growth factor receptor expression and gene copy number analysis in gastric carcinoma samples from Chinese patients

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Abstract. Epidermal growth factor receptor (EGFR) expression and gene copy number have been observed to be associated with a positive clinical response to EGFR inhibitors. The present study aimed to evaluate EGFR expression and gene copy number in samples of gastric carcinoma (GC) from Chinese patients. EGFR expression and gene copy number were detected using immunohistochemistry and fluorescence *in situ* hybridization, in tissue array slides containing 150 individual samples of GC tissue. The association between EGFR status, clinicopathological features and overall patient survival was analyzed. Out of the 150 cases of GC evaluated, 63 (42.00%) demonstrated weak EGFR expression and 20 (13.33%) demonstrated EGFR overexpression. EGFR expression was observed to be associated with tumor location ($P<0.05$). Out of 104 cases of GC, which produced a clear FISH signal, 6 (5.77%) exhibited EGFR gene amplification and 5 (4.80%) exhibited balanced polysomy. Patients exhibiting GC, who demonstrated weak EGFR expression, EGFR overexpression or increased EGFR gene copy number, possessed an unfavorable prognosis. Multivariate analysis revealed that EGFR expression, tumor/node/metastasis stage and tumor location were potential independent unfavorable prognostic factors for GC patients. In conclusion, EGFR overexpression, gene amplification and polysomy were observed in GC patients and were associated with an unfavorable prognosis. Evaluation of EGFR status may therefore facilitate the identification of a subset of GC patients sensitive to treatment with EGFR-targeted therapies.

Introduction

Gastric carcinoma (GC) is the second most common tumor worldwide (1). The highest mortality rates for GC have been reported in East Asia, including Japan, Korea and China (2). Currently, typical treatments for GC comprise surgery and chemotherapy, however recurrence frequently occurs, particularly with advanced stage GC (3). Currently, the majority of chemotherapy regimens are only able to achieve a low clinical complete response rate, and are not capable of improving overall survival rates (4). Therefore, the development of novel therapies for the treatment of GC is urgently required.

Epidermal growth factor receptor (EGFR/ErbB1) is a member of the ErbB family of receptor tyrosine kinases. The EGFR gene is located on the short arm of human chromosome 7 and produces a 170 kDa transmembrane glycoprotein (5). When EGFR binds certain ligands, including epidermal growth factor or transforming growth factor- α , it is capable of activating a number of intracellular signaling cascades, for example, the RAS/mitogen activated protein kinase, phosphatidylinositol-3-kinase and signal transducer and activator of transcription-3 signal transduction pathways (6,7). These pathways regulate cell proliferation, migration, adhesion, differentiation and survival (7,8).

Overexpression and/or increased activity of EGFR may be detected in a number of human tumors and is frequently associated with aggressive tumor behaviors and poor prognosis (7,9). Therefore, EGFR is considered to be a significant therapeutic target for the treatment of human cancer. EGFR-targeting drugs have been developed and approved for use in the treatment of patients exhibiting EGFR-expressing non-small cell lung cancer (NSCLC) and colorectal carcinoma (CRC) (9,10). Cetuximab and panitumumab are EGFR-binding monoclonal antibodies (mAbs), which are currently approved for use in the treatment of CRC (11). Gefitinib and erlotinib are EGFR tyrosine kinase inhibitors that are approved for use in the treatment of NSCLC (12).

In patients exhibiting advanced NSCLC, a positive response following treatment with tyrosine kinase inhibitor gefitinib was correlated with increased EGFR gene copy number and protein expression (13). Certain studies have identified overexpression of EGFR as a potential prognostic indicator for GC (14,15). A

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small number of phase II and III clinical trials, in which GC was treated with cetuximab, have been performed, however ambiguous results were obtained (16,17). It has been reported that the alteration of EGFR expression in GC may affect the sensitivity of EGFR-targeted therapies (18).

The incidence of EGFR overexpression and abnormalities in the EGFR gene may vary markedly across ethnicities (19). A small number of studies concerning EGFR status in Chinese GC patients have been published. The present study systemically evaluated EGFR protein expression and gene copy number in 150 samples of GC from Chinese patients. The associations between EGFR status, clinicopathological parameters and treatment outcomes were retrospectively analyzed. The present study may aid in the investigation of the viability of EGFR-targeting therapies as a potential treatment for GC in Chinese patients.

Patients and methods

Case selection and clinicopathological features. Patients pathologically diagnosed with gastric adenocarcinoma between April 2005 and June 2007 at the Second Affiliated Hospital of Dalian Medical University (Dalian, China) were selected for the current study. The current study was approved by the Institutional Review Board of Dalian Medical University. All participants signed a consent form prior to the commencement of surgical procedures and initiation of the study. Pathological specimens collected from the primary surgery were routinely fixed in formalin (Kan Nai Xin Zhongshan Biotechnology Co., Ltd., Zhongshan, China) and embedded in paraffin (Shanghai Hualing Health Machinery Plant, Shanghai, China). Each slide was re-evaluated by a pathologist, with no knowledge of the patient's pathological diagnosis, prior to the performance of experiments. Clinicopathological parameters were noted, including gender, age, tumor/node/metastasis (TNM) and pathological stages, depth of invasion, the presence of lymph node or distant metastasis and tumor location. Patient characteristics and details of each sample are listed in Table I.

Survival times were calculated from the initial surgery, and were considered censored for patients who were alive at the final follow-up or who succumbed with no evidence of GC recurrence. Clinical outcome was determined from the date of surgery until mortality, or 31 November 2013, which resulted in a follow-up period of 1-104 months (mean, 49 months). A total of 189 GC cases were included at the initiation of the present study, however 39 cases were lost to follow-up. Patients (150 cases) who possessed complete prognosis data were included in the analysis.

Tissue array method. An expert pathologist evaluated the hematoxylin and eosin-stained (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) slides in order to ensure that the tissue-containing tumor cells were studied. Core tissue biopsy specimens (diameter, 2 mm) were obtained from individual paraffin-embedded GC samples and arranged in recipient paraffin blocks. In order to account for tumor heterogeneity, two separate core samples per tumor were obtained. Non-neoplastic gastric mucosa specimens, which were obtained from adjacent normal tissue, were included in each of the array blocks; in total, 40 specimens were included.

The tissue array blocks contained up to 30 cores, meaning that 12 array blocks were formed from the 150 cases.

Immunohistochemistry (IHC) and interpretation of immunohistochemical results. Immunohistochemical staining of samples was performed using rabbit polyclonal IgG against EGFR (anti-EGFR; 1:50; sc03; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and avidin-biotin-peroxidase techniques (VECTASTAIN® Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA). Paraffin-embedded tissue sections (4 μ m) were deparaffinized and rehydrated. Endogenous peroxidase activity was ablated using 2% H₂O₂-methanol (Tianjin Kermel Chemical Reagent Co., Ltd., Tianjin, China). Antigen retrieval was performed by microwave heating of slides in 10 mmol/l citrate buffer (Zhengzhou Cengfeng Chemical Products Co., Ltd., Zhengzhou, China) at pH 6.0, and 5% normal sheep serum (Zhongshan Jinqiao Biotechnology Co., Inc., Beijing, China) was added to suppress nonspecific protein binding. Tissue sections were incubated at 37°C for 1 h, and subsequently incubated at 4°C overnight with primary EGFR antibody. Certain tissue sections were incubated with 5% serum in phosphate buffered saline (PBS; Zhongshan Jinqiao Biotechnology Co., Inc.) without antibody as a negative control. EGFR antibody was diluted 1:50 in 5% serum. Sections were then washed with PBS and incubated with biotinylated goat anti-rabbit immunoglobulin G secondary antibody at 37°C for 1 h. Following this initial incubation, cells were incubated a second time with avidin-biotin complex from the kit at 37°C for 45 min. Color was developed using 3,3'-diaminobenzidine tetrahydrochloride (Zhongshan Jinqiao Biotechnology Co., Inc.). Slides were counterstained with hematoxylin.

Immunohistochemical staining for EGFR was evaluated using the following criteria: 0, no discernible staining/background-type staining; 1+, ambiguous discontinuous membrane staining; 2+, moderate intensity membrane staining; and 3+, strong and complete plasma membrane staining (14,20). Immunohistochemical staining scores of 2+ and 3+ were considered to indicate EGFR overexpression.

Fluorescence in situ hybridization (FISH). Commercially available probes for the EGFR gene and centromere 7 (GLP EGFR/CSP 7 Dual Color Probe; Beijing Jinpujia Medical Technology Co., Ltd, Beijing, China) were utilized in the present study. Procedures were performed according to standard protocols (21). Briefly, 3-5 μ m sectioned tissue array slides were deparaffinized, dehydrated, and incubated in 20% sodium bisulphate/2X standard saline citrate (2X SSC; Zhongshan Jinqiao Biotechnology Co., Inc.), at 75°C for 20 min. Following washing in 2X SSC, slides were treated with proteinase K (Amresco LCC, Solon, OH, USA) at 37°C for 20 min, rinsed in 2X SSC at room temperature for 5 min and dehydrated using ethanol (Hongming Chemical Reagent Co.) in a series of increasing concentrations (60, 85, 95 and 100%). EGFR and CEP7 probes were applied to each slide, covered with a glass coverslip and sealed using rubber cement (Citotest Labware Manufacturing Co., Ltd., Nanjing, China). Slides were denatured for 5 min at 83°C in a hybrite chamber (Citotest Labware Manufacturing Co., Ltd.) and hybridized overnight (for \geq 8 h) at 37°C. Following post-hybridization

Table I. Correlation of EGFR expression with clinicopathological parameters.

Clinicopathological parameter	n	EGFR expression			P-value
		-	1+	2+/3+	
Gender					0.315
Male	122	56	48	18	
Female	28	11	15	2	
Age, years					0.113
<65	81	41	33	7	
≥65	69	26	30	13	
Diameter of tumor, cm					0.786
<5	77	35	32	9	
≥5	73	31	31	11	
Tumor location					0.013 ^b
Cardia and fundus	17	2	10	5	
Body	46	19	23	4	
Pylorus and antrum	87	63	20	15	
Differentiation					0.367
Well/moderate	28	9	15	4	
Poor	110	54	41	15	
Mucinous ^a	12	4	7	1	
Invasion depth					0.301
Mucosa/submucosa	12	4	7	1	
Muscular/serosa	25	16	7	2	
Out of the serosa	87	38	35	14	
Other organs	26	9	14	3	
Lymph node metastases					0.086
0	43	14	21	8	
1-6	54	31	20	3	
≥7	53	22	22	9	
Distant metastases					0.150
-	127	61	50	16	
+	23	6	13	4	
Tumor/Node/Metastasis stage					0.525
I	22	9	10	3	
II	31	13	14	4	
III	67	36	23	8	
IV	30	9	16	5	

^aMucinous and signet ring cell carcinoma; ^bstatistically significant. EGFR, epidermal growth factor receptor.

washing, slides were counterstained using 10 μ l DAPI (Beyotime Biotechnology Co., Ltd., Shanghai, China) in antifade solution (Beijing Jinpujia Medical Technology Co., Ltd.), cover-slipped and examined under a fluorescence microscope (BX41; Olympus, Tokyo, Japan).

At least 60 tumor cell nuclei were counted per sample. Numbers of red (EGFR) and green (chromosome 7 centromere) signals were counted manually by Dr Xiaotang Yu and a technique assistant (Miss Li Wang of Beijing Jinpujia Medical Technology Co., Ltd.). For each FISH probe tested, the status of the chromosome was defined by the presence of the centromeric probe; CEP7 signals served as a control. The

ratio of gene probe:centromeric probe was calculated. High levels of polysomy and gene amplification were regarded as a positive FISH result. Gene amplification was considered if tight EGFR gene clusters, a ratio of EGFR gene:chromosome of ≥ 2 or ≥ 15 copies of EGFR/cell in $\geq 10\%$ of analyzed cells was observed. Polysomy was considered when ≥ 4 copies of the EGFR gene were identified in $\geq 40\%$ of cells, and was termed inconclusive if the EGFR:CEP7 signal ratio was observed to be ≥ 2 in $\leq 10\%$ of the analyzed cells (15,22).

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Associations

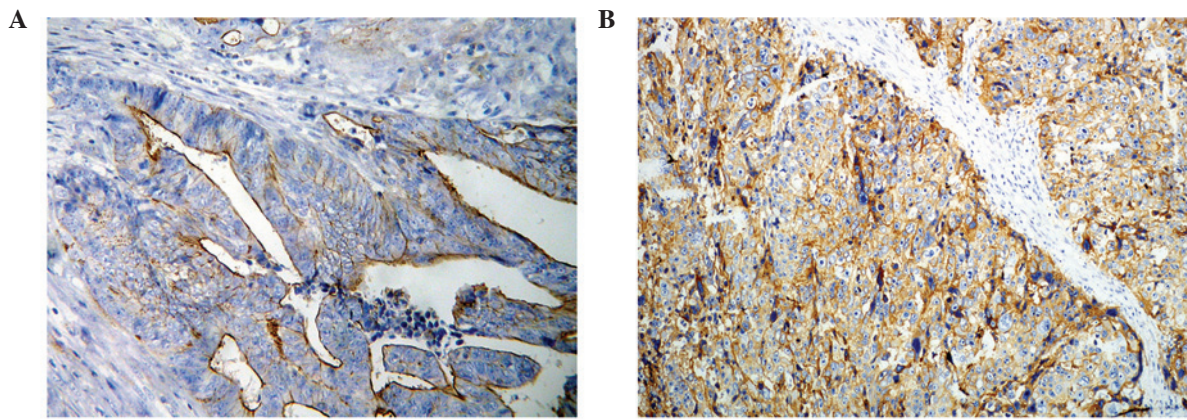


Figure 1. Immunohistochemical analysis of EGFR protein expression in GC. (A) GC exhibiting weak EGFR expression. (B) GC exhibiting EGFR overexpression (magnification, x200). EGFR, epidermal growth factor receptor; GC, gastric carcinoma.

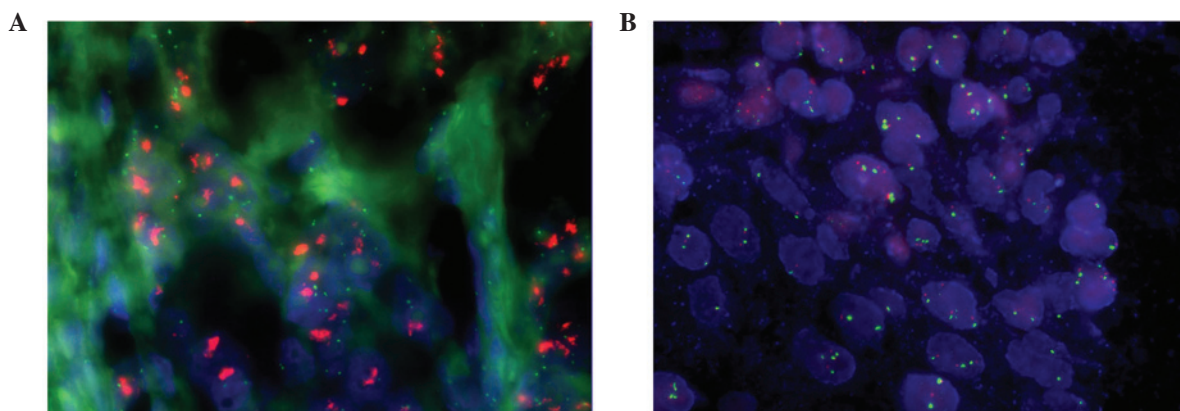


Figure 2. Fluorescence *in situ* hybridization analysis of EGFR gene copy number in GC. EGFR produced a red signal and chromosome 7 centromere produced a green signal; nuclei were stained by DAPI which appeared as a blue signal. (A) GC cells exhibiting gene amplification demonstrated a formation of clusters with numerous signals for EGFR. (B) GC cells exhibiting increased EGFR copy number due to chromosome 7 polysomy (magnification, x100). EGFR, epidermal growth factor receptor; GC, gastric carcinoma.

between EGFR expression and clinicopathological characteristics were assessed by Kruskal-Wallis and Mann-Whitney U test analysis. Groups were compared using the Pearson χ^2 test. Survival curves were constructed using Kaplan-Meier analysis, and the significance of differences between survival curves was determined using the log-rank test. Multivariate analysis was performed using Cox proportional hazards regression. All statistical tests were two-sided. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

High EGFR expression is correlated with the presence of GC at the cardia and fundus. EGFR protein expression was determined using IHC in tissue array slides containing 150 samples of GC (Fig. 1). EGFR staining in GC was detected in the membrane and/or cytoplasm. Out of a total of 150 samples, 67 cases (44.67%) scored 0, 63 (42.00%) scored 1+ and 20 (13.33%) scored 2+ or 3+. A score of 1+ was considered to demonstrate weak EGFR expression, while a score of 2+ or 3+ was considered to demonstrate EGFR overexpression. Associations between EGFR protein expression and clinicopathological parameters were analyzed and

are summarized in Table I. The results of the present study revealed that EGFR is highly expressed in GC located at the cardia and fundus ($P = 0.012$). There were no significant correlations observed between EGFR protein expression and any other clinicopathological features ($P > 0.05$).

Increased EGFR gene amplification is correlated with high levels of EGFR protein expression. EGFR gene amplification was detected using FISH in tissue array slides containing 150 GC samples. In a total of 150 cases of GC, 46 failed to produce a clear signal for evaluation, while 104 cases exhibited a clear signal that was able to be used for enumerating analysis. EGFR protein expression in these 104 cases of GC was as follows: 41 cases scored 0, 45 cases scored 1+ and 18 scored 2+ or 3+. All EGFR signals were compared with signals for centromeric probes for chromosome 7. EGFR amplification was detected in 5.77% (6/104) of the cases, which exhibited red cluster signals for EGFR (Fig. 2A). Four cases demonstrated EGFR protein overexpression (2+/3+) and two cases exhibited weak EGFR expression (1+). An increased gene copy number due to polysomy was detected in 4.81% (5/104) of the cases (Fig. 2B); three of these cases demonstrated EGFR protein overexpression and two

Table II. Univariate analysis of EGFR status, clinicopathological parameters and overall cancer survival in subjects with gastric carcinoma.

Clinicopathological parameter	n	Mean overall survival, months	Overall survival	
			95% CI	P-value
Gender				0.747
Male	122	52.643	37.978-67.308	
Female	28	54.869	47.441-62.297	
Age, years				0.169
<65	81	58.508	49.011-68.001	
≥65	69	50.886	41.558-60.213	
Diameter, cm				0.005 ^b
<5	77	64.602	55.414-73.786	
≥5	73	44.562	35.338-53.785	
Tumor location				0.022 ^b
Cardia and fundus	17	31.882	16.042-47.693	
Body	46	56.043	44.558-67.652	
Pylorus and antrum	87	58.437	49.453-67.420	
Differentiation				0.682
Well/moderate	28	56.357	41.111-71.604	
Poor	110	55.138	47.481-62.795	
Mucinous ^a	12	43.583	18.995-68.172	
Invasion depth				<0.000 ^b
Mucosa/submucosa	12	80.583	65.241-95.925	
Muscular/serosa	25	84.880	71.800-97.960	
Out of the serosa	87	52.243	43.781-60.704	
Other organs	26	19.385	10.732-28.037	
Lymph node metastases				<0.000 ^b
0	43	72.519	60.360-84.679	
1-6	54	57.630	47.716-67.543	
≥7	53	35.679	25.672-45.687	
Distant metastases				<0.000 ^b
-	127	61.643	54.455-68.830	
+	23	18.000	9.222-26.778	
Tumor/Node/Metastasis stage				<0.000 ^b
I	22	94.000	84.029-103.971	
II	31	64.323	50.520-78.117	
III	67	53.836	44.441-63.261	
IV	30	16.567	9.463-23.671	
EGFR				0.011 ^b
-	67	66.635	56.692-76.578	
1+	63	46.507	37.066-55.949	
2+/3+	20	41.650	24.353-58.947	
EGFR gene amplification and polysomy				0.040 ^b
-	93	54.222	45.419-63.024	
+	11	32.182	13.421-50.942	

^aMucinous and signet ring cell carcinoma; ^bstatistically significant. EGFR, epidermal growth factor receptor.

exhibited weak expression. The other 93 cases that produced clear signals for analysis possessed balanced EGFR and CEP7 copy numbers. In the EGFR overexpression and EGFR weak expression groups, the frequencies of gene copy

Table III. Multivariate analysis of predictive indicators for survival of patients exhibiting gastric carcinoma.

Clinicopathological parameter	B	Standard error	Wald ^a	Mean RR	95% CI of RR	P-value
Gender	0.403	0.288	1.961	1.497	0.851-2.632	0.161
Age	0.191	0.224	0.730	1.211	0.781-1.878	0.393
Diameter	-0.156	0.235	0.441	0.855	0.539-1.357	0.507
Site	-0.442	0.168	6.930	0.643	0.462-0.893	0.008 ^b
Differentiation	0.102	0.243	0.178	1.108	0.689-1.782	0.673
Invasive depth	0.341	0.239	2.033	1.407	0.880-2.248	0.154
TNM stage	0.806	0.202	16.005	2.240	1.509-3.325	0.000 ^b
EGFR expression	0.387	0.150	6.640	1.473	1.097-1.976	0.010 ^b

^aWald statistic was calculated using the following equation: $Wald = (B / \text{standard error})^2$; ^bStatistically significant. B, partial correlation coefficient; RR, relative risk; CI, confidence interval; EGFR, epidermal growth factor receptor; TNM, tumor/node/metastasis; EGFR, epidermal growth factor receptor.

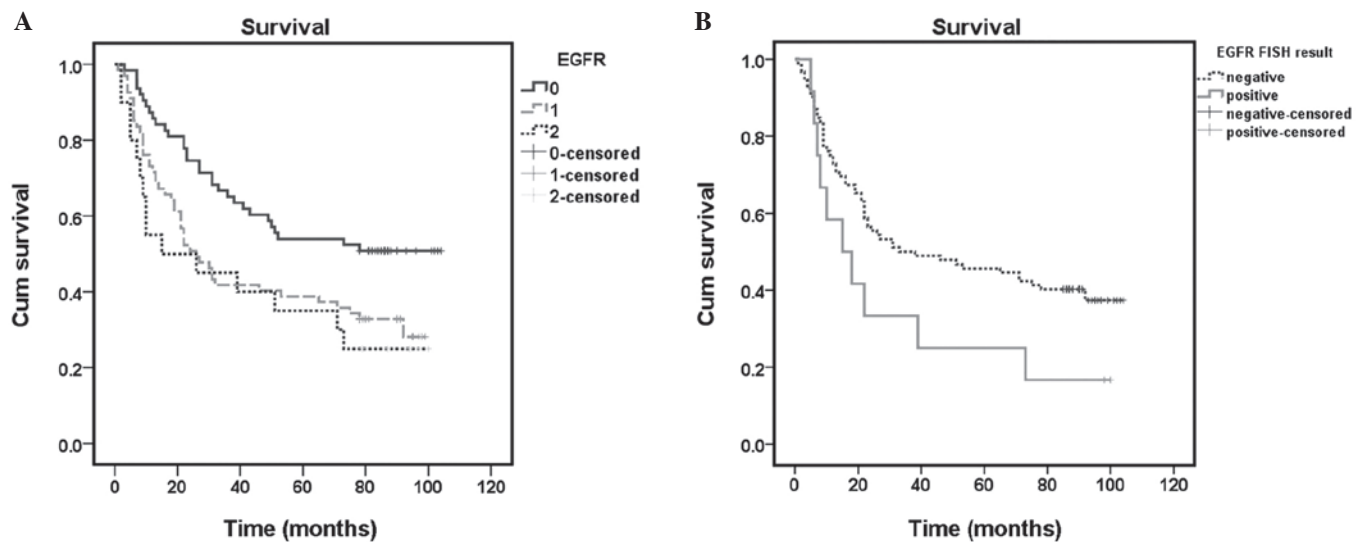


Figure 3. Survival curves constructed using the Kaplan-Meier method and log-rank test. (A) Survival curves revealed that GC patients exhibiting weak EGFR expression (1+) and EGFR overexpression (2+) possessed an unfavorable prognosis compared with EGFR-negative GC patients (0). (B) Survival curves revealed that GC patients demonstrating increased EGFR gene copy numbers, as detected by FISH, possessed an unfavorable prognosis. GC, gastric carcinoma; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; Cum, cumulative.

number abnormalities were 38.89 (7/18) and 8.89% (4/45), respectively. None of the 41 cases demonstrating negative EGFR expression exhibited EGFR gene amplification or high polysomy. Mann-Whitney U test analysis revealed that the correlation between IHC and FISH results was statistically significant ($P < 0.001$). It was concluded that increased copy number of the EGFR gene was associated with GC cases with high EGFR protein expression levels.

Survival analysis. The duration of follow-up was 1-104 months (mean, 48.9 months) subsequent to surgery; 92/150 patients (61.33%) succumbed during this period. Univariate analysis revealed that tumor diameter, site, depth of invasion, presence of lymph node or distant metastases, TNM stage, EGFR expression and EGFR gene amplification were associated with prognosis (Table II). Additional factors, including gender, age and differentiation of GC, were not associated with prognosis ($P > 0.05$). The overall survival rate

of patients exhibiting negative EGFR expression, as determined using the log-rank test, was significantly increased compared with the survival rate of those patients demonstrating EGFR expression ($P = 0.001$; Fig. 3A). However, there was no significant difference in survival rate between patients exhibiting weak EGFR expression and EGFR overexpression. Patients exhibiting EGFR FISH(+) GC possessed a less favorable prognosis compared with those exhibiting EGFR FISH(-) GC ($P = 0.036$; Fig. 3B).

Clinicopathological parameters, including gender, age, tumor diameter, location and differentiation, TNM stage and EGFR expression were included in a multivariate analysis. The results of this analysis revealed that EGFR expression, tumor location and TNM stage were independent prognostic indicators of GC (Table III). However, there were no significant differences between subjects exhibiting EGFR FISH(+) GC and those demonstrating EGFR FISH(-) GC ($P = 0.682$) detected in the multivariate analysis.

Discussion

EGFR inhibitors are utilized in the management of a number of solid malignant tumors, including CRC and metastatic NSCLC (23,24). Due to the development of EGFR-targeted treatments, the EGFR gene has been identified in a variety of studies investigating numerous malignancies (25,26). In the present study, EGFR protein expression and gene amplification were systemically evaluated in GC samples from Chinese patients.

IHC is the typical tool for the determination of EGFR expression levels and for the identification of patients likely to benefit from EGFR-targeted therapies in CRC and NSCLC (27). In GC, EGFR protein expression has been analyzed in several previous studies. Kim *et al* (15) evaluated EGFR status in 511 Korean GC cases; 27.4% of these cases demonstrated EGFR overexpression. Takehana *et al* (28) identified negative EGFR protein expression in 89.6%, low levels in 8.2% and high levels of expression in 2.2% of 413 GC specimens from Japanese patients. Gamboa-Dominguez *et al* (14) investigated EGFR status in 87 cases of GC from Mexican patients; 18.0% demonstrated moderate EGFR expression and 10.1% exhibited strong EGFR expression. The results of the present study revealed that EGFR expression was observed in 83/150 (55.33%) GC cases, and 20 (13.33%) cases demonstrated EGFR overexpression. In the present and previous studies, the frequency of EGFR overexpression, as revealed by IHC, ranged between 2-30%. Potential reasons for this wide variation may include differences in fixation techniques, antibodies and scoring systems used in IHC (29).

EGFR overexpression may occur as a result of the presence of an increased gene copy number. The present study investigated EGFR gene copy number using FISH analysis in tissue array slides. A total of 104 cases of GC exhibited a positive signal; while the remaining 46 cases failed to produce any signal. This low rate of success in the FISH analysis may be a result of the extended storage time of samples, as well as the tissue array slides used in FISH analysis. A number of the wax tissue blocks had been stored for >10 years prior to being utilized for the present study. A longer storage time may lead to fewer positive results, as the following factors may exhibit a considerable impact on the preservation of DNA/mRNA: Oxidation, hydrolysis, sun or light exposure, fixation time and type of fixative (30). This problem may be resolved by punching multiple small cores from different regions to capture the heterogeneity of the tumors more effectively. In addition, the detection of onco-gene amplification by fluorescent *in situ* hybridization on tissue microarray may be a reliable tool for large retrospective studies. Various tissues arranged in one tissue array slide may require alternative experimental conditions to achieve a positive result (31). Furthermore, due to the heterogeneity of various areas of GC tumors, the rate of increased gene copy number that was detected in the tissue array slides may be lower than indicated according to the results of the present study.

In these 104 cases of GC, EGFR gene amplification was detected in six cases and polysomy in five. A number of EGFR overexpression cases (7/18; 38.89%) demonstrated

EGFR gene amplification or high polysomy as revealed by FISH, whereas these features were observed in only 4/45 cases (8.89%) exhibiting weak EGFR expression. The present study confirmed that EGFR IHC scores were significantly correlated with EGFR gene expression levels. This observation also suggested that not all GC cases exhibiting increased EGFR gene copy number demonstrated EGFR protein overexpression; and that a number of cases exhibited weak expression. Therefore, if patients possessing increased EGFR gene copy number are sensitive to EGFR-targeted drugs, certain patients demonstrating weak EGFR expression may also potentially benefit from treatment with these drugs. Univariate analysis revealed that EGFR overexpression and gene copy number were associated with unfavorable prognoses. Multivariate analysis revealed that EGFR expression was an independent prognostic indicator. The potential value of the results of the present study is that they may facilitate the identification of a subset of patients exhibiting tumors that may be sensitive to EGFR-targeted therapy. Patients exhibiting EGFR overexpression possess a poor prognosis, however these patients may benefit from EGFR-targeted therapy.

The EGFR gene status of GC has been analyzed in previous studies. Kim *et al* (15) evaluated the EGFR gene copy number in GC tissues from 511 Korean patients; 13/21 (61.9%) cases demonstrating EGFR overexpression also exhibited EGFR gene amplification or increased polysomy, while only 14/119 (11.8%) cases possessing weak EGFR expression exhibited EGFR gene amplification or high polysomy. Liang *et al* (20) detected the EGFR gene copy number in 100 cases of GC; 16% of these GC specimens demonstrated positive FISH results (20). These results were consistent with those of the present study. The frequency of increased EGFR gene copy number in GC is reduced compared with certain other malignancies, including NSCLC, CRC and high-grade gliomas (13,32,33).

Clinical trials have been undertaken to investigate the effect of EGFR-targeting mAbs in GC. In a multicenter phase II Japanese study, 13/75 metastatic GC patients receiving gefitinib treatment achieved disease control (34). By contrast, a phase II trial of erlotinib conducted in two groups of patients with gastroesophageal junction (GEJ) or cardia and distal gastric adenocarcinomas demonstrated that erlotinib is an effective treatment for patients with GEJ adenocarcinomas, however, it appears ineffective for the treatment of distal GC (35). An additional phase III clinical trial (EXPAND) revealed a small subset of patients that responded to treatment with cetuximab (16). The results of these previous studies emphasize the requirement for the identification of latent responders.

In NSCLC and CRC, patients exhibiting EGFR overexpression and/or an increased gene copy number have been demonstrated to possess a positive response to EGFR-targeted therapies for carcinoma (29,32,36). A number of previous *in vitro* studies have suggested that GC patients exhibiting EGFR overexpression or gene amplification may benefit from EGFR-targeted therapy. Fukuda *et al* (37) identified that the combination of 5-fluorouracil and cetuximab synergistically inhibited cell proliferation and exhibited an enhanced pro-apoptotic effect in GC cells demonstrating

EGFR overexpression. A preclinical trial identified that GC patient-derived xenografts responded to cetuximab, and efficacy was dependent on EGFR overexpression and gene amplification (17). IHC analysis of EGFR expression, including FISH analysis of the EGFR gene, may be a favorable option for the identification of latent patients, who may respond to EGFR-targeted therapies.

In conclusion, the results of the present study provide evidence that EGFR expression may be significantly associated with an unfavorable prognosis in GC. The present study additionally identified that gene amplification and polysomy were low frequency events in GC, although were associated with poor prognosis. An increased copy number of the EGFR gene was significantly correlated with protein overexpression. The results of the present study therefore suggest that there is a potential group of GC patients that may benefit from treatment with EGFR-targeted agents.

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