

# High expression of EGFR predicts poor survival in patients with resected T3 stage gastric adenocarcinoma and promotes cancer cell survival

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**Abstract.** Epidermal growth factor receptor (EGFR) is an essential regulator and biomarker of several types of cancer. However, the association between its expression and prognosis in patients with resected T3 stage gastric adenocarcinoma (RT3-GA) remains to be determined. In total, 683 patients with resectable T3-GA who underwent surgery were retrospectively included in the present study, and their immunohistochemical data for EGFR expression were collected. The associations between the patients' clinicopathologic characteristics and EGFR immunohistochemistry data were analyzed by multiple statistical methods. Annexin V apoptosis and MTT cell viability assays were performed to explore the effect of EGFR on AGS gastric adenocarcinoma cell survival. EGFR expression levels were categorized into two groups: low (406 cases) and high (277 cases). High EGFR was demonstrated to be significantly associated with distant metastasis ( $P=0.043$ ) and severely decreased median overall survival time (MOST) and recurrence-free survival time (MRFST). MOST and MRFST in the low EGFR group were 39 and 37 months, respectively; whereas in the high EGFR group these values were only 18

and 13 months ( $P=3.10 \times 10^{-9}$  and  $P=6.74 \times 10^{-8}$ , respectively). Multivariate analysis confirmed that high EGFR expression levels were associated with poor survival, which was associated with significantly increased recurrence risk and ~2-fold elevation in mortality risk [hazard ratio (HR), 1.73; 95% confidence interval (CI), 1.43-2.10;  $P=2.37 \times 10^{-8}$  and HR, 1.80; 95% CI, 1.50-2.17;  $P=3.80 \times 10^{-10}$ ]. Inhibiting EGFR with AG1478 suppressed its effect on promoting AGS cell survival. These results suggest that high EGFR expression indicates poor survival in patients with RT3-GA, which may be correlated with EGFR promoting GA cell survival.

## Introduction

Human epidermal growth factor receptor (EGFR), which is also known as ErbB-1 or HER-1, is a receptor located on the cell surface belonging to the family of receptor tyrosine protein kinases termed HER (1). Members of this family also include HER-2, HER-3 and HER-4 (1). All have the function of regulating and transducing signals from growth factors, particularly those from EGF (2). Investigations into the association between EGFR and cancer have been widely documented and previous studies have demonstrated that upregulation of the expression or activity of EGFR is associated with numerous types of human cancer (3-5). EGFR is activated by autophosphorylation following the formation of homodimers stimulated by the binding of its ligand, EGF (6). The molecular mechanism involved in EGFR-driven carcinogenesis and cancer development is associated with the upregulation of EGFR, which leads to uncontrolled cell growth and division (7). EGFR typically functions in a driver role, affecting important signaling pathways involved in cell growth, proliferation, differentiation, migration, adhesion and survival, such as mitogen-activated protein kinase-extracellular signal-regulated kinase and phosphoinositide 3-kinase-AKT, which trigger carcinogenesis and cancer progression.

Overexpression of EGFR has been identified in various types of human cancer; therefore, it is regarded as an oncogene

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and a tumor biomarker or target for the treatment of many different types of cancer, among which lung cancer is the most common (8). Aberrant EGFR is also often observed in gastric cancer, and mutations leading to EGFR upregulation are frequently identified in both gastric cancer and lung cancer (8,9). As EGFR has been demonstrated to be closely associated with cancer, and its oncogenic characteristics are clear, various anticancer therapies directed against it have been developed, including gefitinib (10) and icotinib (11) for lung cancer, cetuximab for colon cancer and trastuzumab for HER-2-positive gastric cancer. However, the use of drugs against EGFR in gastric cancer has not yet been fully elucidated or applied clinically.

Gastric cancer is one of the most common cancers worldwide. Although chemotherapy or surgery combined with chemotherapy may prolong survival, the 5-year survival in patients with this cancer, particularly those with advanced stage disease, remains very poor. Due to a lack of specific symptoms in the early stage, the majority of gastric cancers are diagnosed at an advanced stage. EGFR has an important role in gastric cancer; however, whether it may be used as a biomarker, particularly as a prognostic biomarker to indicate survival in T3 stage gastric cancer, or as treatment target in this stage of the disease remains to be determined.

Gastric adenocarcinoma (GA) is the most common type of gastric malignant tumor, accounting for >90% of gastric neoplasms. Therefore, the present study retrospectively investigated EGFR expression levels in 683 tissue samples from patients with resected T3 stage GA (RT3-GA) and evaluated the possibility of using EGFR as a prognostic biomarker for patients with RT3-GA. We also investigated the effect of inhibiting EGFR with the EGFR inhibitor AG1478 on AGS GA cell survival.

## Materials and methods

*Study population and patients' clinicopathologic characteristics.* A total of 683 patients with confirmed, resectable T3 stage GA underwent surgical resection of the GA between July 2003 and December 2009 at Fuzhou General Hospital (Fujian, China) and had their immunohistochemical data and clinicopathologic details logged into the hospital database. These data were collected retrospectively. The present study was approved by the Ethics Committee of Fuzhou General Hospital and written informed consent was obtained from all patients.

Clinicopathologic parameters, including age, gender, histologic grade, tumor size, lymph node metastasis and distant metastasis, were obtained from the patients' medical records. Survival data were calculated from the date of resection to the date that the patient succumbed to cancer (for deceased patients) or the end of follow-up, which was November 2014 (for living patients). Telephone interviews and the Social Security Death Index system were used to confirm patient survival. Recurrence was diagnosed based on the methods and standards described previously (12).

Our previous methods for TNM classification of malignant tumors were conducted to determine the patients' TNM stage (13) and the World Health Organization Classification of Tumors standards (14) were used for histologic classification.

Clinicopathologic parameters were identified by two pathologists.

*Immunohistochemical analysis.* Immunohistochemistry (IHC) was used to determine the expression levels of EGFR in 683 tissue samples from patients with RT3-GA. Previously described methods for the detection of HER-2 expression in gastric cancer tissues by IHC were used to measure EGFR expression (13), the only difference being the use of anti-EGFR antibody (1:100 dilution; catalog no., MAB-0196; Maixin Company, Fujian, China) rather than anti-HER-2 antibody.

Two independent pathologists without any knowledge of the patients' information determined EGFR expression by IHC. Agreement was reached by consensus when differences occurred. EGFR expression was observed in the tumor cell membrane as brownish-yellow granules. Samples without staining or with staining of <10% of GA cells were considered negative (-) for EGFR expression. Samples exhibiting staining of ≥10% of GA cells were considered positive for EGFR, as follows: +, 10-25% positive; ++, 26-50% positive; +++, 50-75% positive; and +++, >75% positive. GA tissues with EGFR staining intensity of -, + or ++ were categorized as having low EGFR expression; GA tissues with staining intensity of +++ or ++++ were categorized as high EGFR expression.

*Cell culture and western blot analysis for EGFR expression.* AGS gastric adenocarcinoma cells and GES-1 normal human gastric epithelial cells were purchased from ATCC (Manassas, VA, USA) and the Beijing Cancer Institute (Beijing, China), respectively. Cells were grown in F12 or Dulbecco's modified Eagle medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) in an incubator at 37°C with 5% CO<sub>2</sub>. Rabbit anti-human EGFR antibody (catalog no., 4267S) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and was diluted 1:1,000 prior to use. Mouse anti-β-actin antibody (catalog no., A2228) from Sigma-Aldrich (Merck Millipore; Darmstadt, Germany) was diluted 1:6,000 and used to detect the expression of β-actin, which served as a loading control. The secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) (dilution 1:4,000; catalog no., M21002) and goat anti-mouse IgG-HRP (dilution, 1:4,000; catalog no., M21001) antibodies used for western blotting were from Abmart Company (Shanghai, China).

Western blot analysis of EGFR expression in both AGS and GES-1 cells was performed as previously described (15). The protein used for western blot analysis was extracted by radioimmunoprecipitation assay (RIPA) reagent (Beyotime Institute of Biotechnology, Haimen, China). Briefly, the cultured supernatant was discarded, and the cells were washed by 1X PBS three times, and then 200 μl of RIPA reagent was added to each well of a 6-well plate. The plate was put on ice for 30 min, during which the plate was rotated every 5 min. Finally, a cell scraper was used to scrape cells off the plate. The cell lysates were transferred into a 1.5 ml tube, which was centrifugated at 12,000 x g at 4°C for 20 min. The supernatant was then transferred into new tubes stored at -80°C until use, and the sediment was discarded. The protein in the lysates was quantified by using a bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology), according

to the manufacturer's instructions. In total, 35  $\mu\text{g}$  of protein from each sample was added to SDS-PAGE gel for the electrophoresis. Subsequently, 5% concentration gel (prepared by pH 6.8 1.0 M Tris-HCl buffer) and 10% separation gel (prepared by pH 8.8 1.5 M Tris-HCl buffer) was used for SDS-PAGE electrophoresis to detect EGFR expression, whereas 5% concentration gel and 12% separation gel was used for SDS-PAGE electrophoresis to detect  $\beta$ -actin expression in the same samples. All gels contained 0.1% SDS, and the running buffer used was 25 mmol/l Tris, 250 mmol/l glycine and 0.1% SDS (pH 8.3) buffer. Samples were prepared using 5X loading buffer that contained 250 mmol/l Tris-HCl (pH 6.8) 500 mM DTT, 10% SDS, 0.02% bromine phenol blue (BPB) and 50% glycerol. The 4 samples were added to 5X loading buffer, and the mixture was then put into a 100°C water bath for 5 min. Subsequently, the mixture was added to the upper layer of the SDS-PAGE gel (5% of concentration gel), which had been put into 1X running buffer. The SDS-PAGE device (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was subjected to electrophoresis with constant voltage at 100V, after the dye of BPB was out of the separation gel. The protein on the gels were then transferred onto polyvinylidene fluoride membranes for western blotting using transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol).

Following the transfer, the membranes were blocked using 20 mM Tris-HCl (pH 7.6) buffer containing 0.8% NaCl and 0.1% Tween-20, as well as 5% non-fat milk powder, for 1 h. The aforementioned primary anti-EGFR and anti- $\beta$ -actin antibodies, diluted in blocking buffer, were added to the membranes for reaction overnight at 4°C. The membranes were then washed with 20 mM Tris-HCl buffer (pH 7.6) containing 0.8% NaCl and 0.1% Tween-20 (washing buffer) 3 times, for 15 min each time. The aforementioned secondary goat anti-rabbit-IgG-HRP and anti-mouse-IgG-HRP antibodies, diluted in blocking buffer, were added to each membrane and allowed to react for 4 h at room temperature. The membranes were then washed with washing buffer another three times, for 15 min each time. Subsequently, the enhanced chemiluminescence substrates (Pierce; Thermo Fisher Scientific, Inc.) were added to each membrane, and the results were checked by X-ray films. The protein in samples was quantified by BCA kits according to the manufacturer's instructions. The absorbance at 562 nm of each sample and standards was measured by Eppendorf Biophotometer (Eppendorf, Hamburg, Germany), the bands in the X-film was scanned and captured by Epson scan, and was quantified by Quantity One software.

**Cell apoptosis assay by Annexin V plus propidium iodide (PI) staining and flow cytometry analysis.** GES-1 or AGS cells were cultured in DMEM or F12 medium supplemented with 10% FBS, respectively. The following day, cells were cultured in low serum medium containing 1% FBS for 12 h. Cells were then divided into two groups. Group 1 was pretreated with DMSO as a control. Group 2 was pretreated with the EGFR inhibitor AG1478 dissolved in DMSO (final concentration, 5  $\mu\text{M}$ ) for 12 h, then the medium in each group was replaced with fresh low serum medium (1% FBS). Subsequently, cells were cultured in an incubator for 24 h and apoptosis was determined by Annexin V plus PI staining and flow cytometry analysis according to the manufacturer's instructions (KenGen

Table I. Patient characteristics.

Characteristics	Cases (n=683)	
	n	%
Age		
<60 years	324	47.4
$\geq$ 60 years	359	52.6
Gender		
Male	505	73.9
Female	178	26.1
Histologic grade		
Well/moderately differentiated	406	59.4
Poorly differentiated	277	40.6
Tumor size		
Small	468	68.5
Large	215	31.5
Lymph node metastasis		
No metastasis	146	21.4
Metastasis	537	78.6
Distant metastasis		
No metastasis	574	84.0
Metastasis	109	16.0
EGFR expression		
Low	406	59.4
High	277	40.6
Survival		
Yes	211	30.9
No	472	69.1
Recurrence		
No	252	36.9
Yes	431	63.1

EGFR, epidermal growth factor receptor.

Biotech, Nanjing, China). At the same time, apoptosis in cells cultured with normal serum (containing 10% FBS) were also assessed, which acted as a control.

**MTT assay for cell viability.** Following GES-1 and AGS cell culture, groups and pretreatment with DMSO or AG1478 were the same as described above. MTT assay for cell viability was conducted based on the method outlined by Nyakern *et al* (16). The MTT assay used in this study was manufactured by Sigma-Aldrich (Merck Millipore). Briefly, an initial 4,000 cells in 100  $\mu\text{l}$  of cell suspension per well were seeded in different wells of a 96-well plate. The following day, cells were cultured in low serum medium containing 1% FBS for 12 h. Cells were then divided into two groups. Group 1 was pretreated with DMSO as a control and group 2 was pretreated with the EGFR inhibitor AG1478 dissolved in DMSO (final concentration, 5  $\mu\text{M}$ ) for 12 h, then the medium in each group was replaced with fresh low serum medium (1% FBS). Cells were then cultured in an incubator for another

Table II. Association between EGFR expression and patient characteristics.

Characteristics	EGFR expression, n (%)		P-value
	Low	High	
Age			0.243
<60 years	185 (45.6)	139 (50.2)	
≥60 years	221 (54.4)	138 (49.8)	
Gender			0.010
Male	315 (77.6)	190 (68.6)	
Female	91 (22.4)	87 (31.4)	
Histologic grade			0.597
Well/moderately differentiated	245 (60.3)	161 (58.1)	
Poorly differentiated	161 (39.7)	116 (41.9)	
Tumor size			0.078
Small	289 (71.2)	179 (64.6)	
Large	117 (28.8)	98 (35.4)	
Lymph node metastasis			0.184
No metastasis	94 (23.2)	52 (18.8)	
Metastasis	312 (76.8)	225 (81.2)	
Distant metastasis			0.043 <sup>a</sup>
No metastasis	351 (86.5)	223 (80.5)	
Metastasis	55 (13.5)	54 (19.5)	
Survival			<0.001 <sup>a</sup>
Alive	150 (36.9)	61 (22.0)	
Dead	256 (63.1)	216 (78.0)	
Recurrence			0.002 <sup>a</sup>
No	169 (41.6)	83 (30.0)	
Yes	237 (58.4)	194 (70.0)	

<sup>a</sup>P<0.05. EGFR, epidermal growth factor receptor.

24 h. Subsequently, 10  $\mu$ l of 5 mg/ml MTT (Sigma-Aldrich; Merck Millipore) dissolved in 1X PBS was added to each well containing cells. The cells were incubated for a further 4 h at 37°C, and 100  $\mu$ l solvent (10% SDS in 10 mmol/l HCl) was then added to each well. The plate was incubated at 37°C overnight. Finally, the absorbance of each well at 570 nm was read by SpectraMax microplate reader (Molecular Devices; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** All statistical analyses were performed using SPSS 17.0 software (SPSS IBM, Armonk, NY). Associations between EGFR expression level and the clinicopathologic parameters of patients with RT3-GA were analyzed by the  $\chi^2$  test. The association of each parameter and EGFR expression with 5-year overall survival and recurrence rates, 5-year median overall survival time (MOST) in months and 5-year median recurrence-free survival time (MRFST) in months were assessed by the  $\chi^2$  test (for rates) and the log-rank test (for months). Kaplan-Meier curves and the log-rank test were used to further evaluate the relationship between EGFR expression in RT3-GA tissues and the patients' 5-year MOST and MRFST. The prognostic correlation of the clinicopathologic parameters and EGFR expression with

death and recurrence risk was determined by univariate and multivariate Cox proportional hazards regression analyses. Variables that exhibited a P-value of <0.1 in the univariate analyses were used to establish a multivariate model to identify whether elevated expression of EGFR was an independent prognostic factor. The relationship between EGFR expression and the risk for both lymph node metastasis and distant metastasis was assessed by univariate and multivariate logistic regression. An independent-sample *t*-test was used to evaluate differences between the groups in cell viability. All statistical tests were two-sided and P<0.05 was considered to indicate a statistically significant difference.

## Results

**Clinicopathologic parameters.** Clinicopathologic parameters of the 683 patients are shown in Table I. Enrolled patients included 505 males (73.9%) and 178 females (26.1%) with a median age of 60.12 years (range, 25-89 years). By the end of the follow-up period in November 2014, 63.1% of the patients (431 cases) had suffered from a recurrence of cancer and 69.1% (472 cases) had succumbed to cancer. For the entire group of patients, MOST was 29 months. No patients enrolled in the



Table III. Association between survival and patient characteristics.

Characteristics	Survival, n (%)		P-value <sup>a</sup>	Median overall survival, months	P-value <sup>b</sup>
	Alive	Dead			
Age			0.214		0.295
<60 years	108 (51.2)	216 (45.8)		30.0	
≥60 years	103 (48.8)	256 (54.2)		28.0	
Gender			0.777		0.698
Male	158 (74.9)	347 (73.5)		30.0	
Female	53 (25.1)	125 (26.5)		27.0	
Histologic grade			0.005 <sup>c</sup>		0.001 <sup>c</sup>
Well/moderately differentiated	142 (67.3)	264 (55.9)		35.0	
Poorly differentiated	69 (32.7)	208 (44.1)		22.0	
Tumor size			<0.001 <sup>c</sup>		<0.001 <sup>c</sup>
Small	166 (78.7)	302 (64.0)		37.0	
Large	45 (21.3)	170 (36.0)		13.0	
Lymph node metastasis			<0.001 <sup>c</sup>		<0.001 <sup>c</sup>
No metastasis	81 (38.4)	65 (13.8)		66.0	
Metastasis	130 (61.6)	407 (86.2)		22.0	
Distant metastasis			<0.001 <sup>c</sup>		<0.001 <sup>c</sup>
No metastasis	204 (96.7)	370 (78.4)		36.0	
Metastasis	7 (3.3)	102 (21.6)		7.0	
EGFR expression			<0.001 <sup>c</sup>		<0.001 <sup>c</sup>
Low	150 (71.1)	256 (54.2)		39.0	
High	61 (28.9)	216 (45.8)		18.0	

<sup>a</sup>P-value obtained from chi-square test; <sup>b</sup>P-value obtained from log-rank test; <sup>c</sup>P<0.05. EGFR, epidermal growth factor receptor.

present study had undergone radiation therapy or chemotherapy before surgery (Table I).

A total of 406 patients (59.4%) had well or moderately differentiated tumors, whereas the remaining patients (277 cases; 40.6%) exhibited poorly differentiated tumors. A total of 468 patients (68.5%) had small tumors (<6 cm) and 215 (31.5%) had large tumors (≥6 cm). A total of 537 patients (78.6%) exhibited lymph node metastasis and 109 (16%) had distant metastasis (Table I). All patients (683 cases) had T3 stage GA.

**Association between EGFR expression and clinicopathologic parameters.** EGFR expression was detected in the GA cell membrane and varied among the RT3-GA tissue samples. Of the 683 samples, 277 (40.6%) exhibited high expression of EGFR (Table I) on IHC (Fig. 1A). Low EGFR expression is illustrated in Fig. 1B.  $\chi^2$  tests demonstrated that high EGFR expression was significantly associated with distant metastasis (P=0.043; Table II). Most importantly, high EGFR expression was strongly associated with poor survival. The survival rate in patients with low EGFR expression was 36.9% (150 cases), whereas this rate in the high EGFR group was 22% (61 cases; P=3.42x10<sup>-5</sup>; Table II). High EGFR expression was also demonstrated to be significantly associated with a high recurrence rate. The recurrence rate in patients with low EGFR expression was 58.4% (237 cases), whereas this rate

in the high EGFR group was 70.0% (194 cases; P=0.002; Table II).

No significant association was detected between EGFR expression and the remaining clinicopathologic parameters, including age, tumor size, lymph node metastasis and histological grade. These results suggest that high expression of EGFR may be correlated with poor prognosis in patients with RT3-GA.

**Factors correlated with overall survival.** Kaplan-Meier and log-rank results showed that MOST in the high EGFR expression group (277 cases) was very poor at only 18 months, whereas MOST in the low EGFR group (406 cases) was significantly higher at 39 months (P<0.001; Fig. 2).  $\chi^2$  test and the log-rank test showed high EGFR expression to be strongly associated with short survival (P=3.42x10<sup>-5</sup> and 3.10x10<sup>-9</sup>, respectively; Table III). These two statistical tests also demonstrated that the patients' clinicopathologic parameters, including histologic grade (P=0.005 and P=0.001), tumor size (P=1.72x10<sup>-4</sup> and P=1.88x10<sup>-10</sup>), lymph node metastasis (P=1.97x10<sup>-12</sup> and P=1.02x10<sup>-13</sup>) and distant metastasis (P=2.36x10<sup>-9</sup> and P=1.33x10<sup>-34</sup>), were associated with overall survival. Univariate analysis showed that tumor size (P=5.37x10<sup>-10</sup>), lymph node metastasis (P=1.03x10<sup>-12</sup>), distant metastasis (P=7.68x10<sup>-30</sup>) and EGFR expression levels (P=6.98x10<sup>-9</sup>) were statistically significantly associated with overall survival (Table IV).

Table IV. Univariate and multivariate Cox regression analysis of overall survival.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age		0.300		0.031
<60 years	1.00		1.00	
≥60 years	1.10 (0.92-1.32)		1.23 (1.01-1.47)	
Gender		0.701		0.600
Male	1.00		1.00	
Female	1.04 (0.85-1.28)		0.95 (0.77-1.66)	
Histologic grade		0.001 <sup>a</sup>		0.001 <sup>a</sup>
Poorly differentiated	1.00		1.00	
Well/moderately differentiated	0.74 (0.62-0.89)		0.74 (0.62-0.89)	
Tumor size		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
Small	1.00		1.00	
Large	1.82 (1.51-2.20)		1.79 (1.48-2.16)	
Lymph node metastasis		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
No metastasis	1.00		1.00	
Metastasis	2.59 (1.99-3.37)		2.22 (1.70-2.90)	
Distant metastasis		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
No metastasis	1.00		1.00	
Metastasis	3.66 (2.93-4.59)		3.28 (2.60-4.13)	
EGFR expression		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
Low	1.00		1.00	
High	1.71 (1.43-2.05)		1.80 (1.50-2.17)	

<sup>a</sup>P<0.05. HR, hazard ratio; CI, confidence interval; EGFR, epidermal growth factor receptor.

After controlling for all of the above adverse factors identified in univariate analyses, multivariate analysis demonstrated that high EGFR expression was an independent risk factor for short patient survival, being associated with a >1.5-fold increased risk of death [hazard ratio (HR) 1.80, 95% confidence interval (CI) 1.50-2.17,  $P=3.80 \times 10^{-10}$ ] (Table IV). These results further confirmed that EGFR expression is an important independent prognostic factor correlated with overall survival and that high EGFR expression indicates poor survival in patients with RT3-GA.

#### Factors associated with recurrence-free survival.

Kaplan-Meier and log-rank analyses showed that patients with RT3-GA with high EGFR expression (277 cases) had a very poor MRFST of only 13 months, compared with 37 months in the low EGFR group (406 cases) ( $P<0.001$ ) (Fig. 3). High EGFR expression was significantly associated with a high recurrence rate on both the  $\chi^2$  test and the log-rank test ( $P=0.002$  and  $P=6.74 \times 10^{-8}$ , respectively) (Table V). Other clinicopathologic parameters-histologic grade ( $P=0.019$  and  $P=0.001$ ), tumor size ( $P=0.394$  and  $P=1.86 \times 10^{-5}$ ), lymph node metastasis ( $P=2.08 \times 10^{-11}$  and  $P=8.42 \times 10^{-14}$ ) and distant metastasis ( $P=2.07 \times 10^{-4}$  and  $P=1.17 \times 10^{-12}$ )-were also found to be associated with elevated recurrence risk by both the  $\chi^2$  test and the log-rank test. Tumor size ( $P=2.81 \times 10^{-5}$ ), lymph node metastasis ( $P=1.55 \times 10^{-12}$ ), distant metastasis ( $P=3.72 \times 10^{-23}$ )

and EGFR expression level ( $P=1.39 \times 10^{-7}$ ) were shown to be statistically significantly related to MRFST by univariate analysis (Table VI). After all of the above adverse factors detected by univariate analysis were controlled for, multivariate analysis showed that high EGFR expression was an independent risk factor for poor recurrence-free survival, being associated with a more than 1.5-fold increased recurrence risk (HR 1.73, 95% CI 1.43-2.10,  $P=2.37 \times 10^{-8}$ ) (Table VI). These results further confirmed that EGFR expression is an important independent prognostic factor correlated with recurrence-free survival and that high EGFR expression indicates a high recurrence risk in patients with RT3-GA.

#### EGFR expression in AGS cells and the effect of EGFR on AGS cell survival.

As shown in Fig. 4A, western blot analysis showed that EGFR was overexpressed in AGS cells compared with GES-1 cells, which are normal human gastric epithelial cells. To determine whether overexpression of EGFR in GA cells affected GA cell viability and survival, viability in AGS cells was examined by MTT. The findings demonstrated that suppressing EGFR with AG1478 significantly decreased AGS cell viability under low serum conditions compared with cells without AG1478 pretreatment ( $P<0.05$ ; Fig. 4B). Apoptosis in AGS cells was induced by low serum culture in the absence or presence of the EGFR inhibitor, AG1478, as determined by

Table V. Association between recurrence and patient characteristics.

Characteristics	Recurrence, n (%)		P-value <sup>a</sup>	Median overall recurrence-free, months	P-value <sup>b</sup>
	No	Yes			
Age			0.942		0.716
<60 years	120 (47.6)	204 (47.3)		25.0	
≥60 years	132 (52.4)	227 (52.7)		25.0	
Gender			0.528		0.538
Male	190 (75.4)	315 (73.1)		27.0	
Female	62 (24.6)	116 (26.9)		23.0	
Histologic grade			0.019 <sup>c</sup>		0.001 <sup>c</sup>
Well/moderately differentiated	166 (65.9)	240 (55.7)		33.0	
Poorly differentiated	86 (34.1)	191 (44.3)		18.0	
Tumor size			0.394		<0.001 <sup>c</sup>
Small	178 (70.6)	290 (67.3)		32.0	
Large	74 (29.4)	141 (32.7)		13.0	
Lymph node metastasis			<0.001 <sup>c</sup>		<0.001 <sup>c</sup>
No metastasis	89 (35.3)	57 (13.2)		60.0	
Metastasis	163 (64.7)	374 (86.8)		17.0	
Distant metastasis			<0.001 <sup>c</sup>		<0.001 <sup>c</sup>
No metastasis	229 (90.9)	345 (80.0)		33.0	
Metastasis	23 (9.1)	86 (20.0)		6.0	
EGFR expression			0.002 <sup>c</sup>		<0.001 <sup>c</sup>
Low	169 (67.1)	237 (55.0)		37.0	
High	83 (32.9)	194 (45.0)		13.0	

<sup>a</sup>P-value obtained from chi-square test; <sup>b</sup>P-value obtained from log-rank test; <sup>c</sup>P<0.05. EGFR, epidermal growth factor receptor.

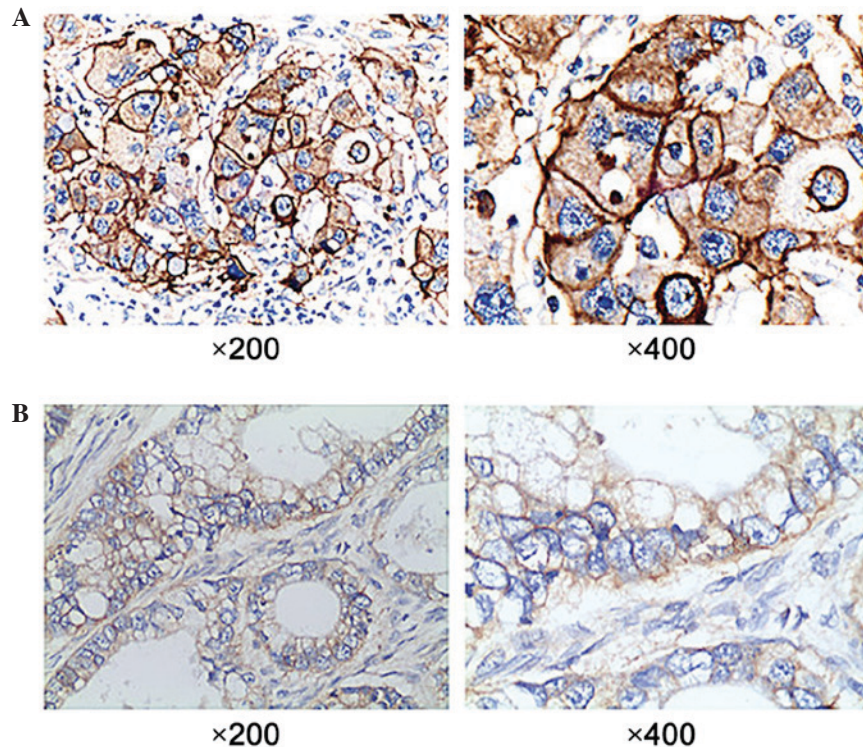


Figure 1. Expression levels of EGFR in resected T3 stage gastric adenocarcinoma tissues, as determined by immunohistochemistry. (A) High and (B) low expression of EGFR. High expression of EGFR was detected in 277 patients (40.6%) and low expression in 406 patients (59.4%). EGFR, epidermal growth factor receptor.

Table VI. Univariate and multivariate cox regression analysis of recurrence.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age		0.720		0.076
<60 years	1.00		1.00	
≥60 years	1.03 (0.86-1.25)		1.19 (0.98-1.44)	
Gender		0.543		0.840
Male	1.00		1.00	
Female	1.07 (0.86-1.32)		0.98 (0.79-1.21)	
Histologic grade		0.001 <sup>a</sup>		0.002 <sup>a</sup>
Poorly differentiated	1.00		1.00	
Well/moderately differentiated	0.73 (0.60-0.88)		0.74 (0.61-0.90)	
Tumor size		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
Small	1.00		1.00	
Large	1.54 (1.26-1.88)		1.47 (1.20-1.81)	
Lymph node metastasis		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
No metastasis	1.00		1.00	
Metastasis	2.74 (2.07-3.63)		2.43 (1.83-3.23)	
Distant metastasis		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
No metastasis	1.00		1.00	
Metastasis	3.42 (2.68-4.36)		2.98 (2.32-3.82)	
EGFR expression		<0.001 <sup>a</sup>		<0.001 <sup>a</sup>
Low	1.00		1.00	
High	1.69 (1.39-2.02)		1.73 (1.43-2.10)	

<sup>a</sup>P<0.05. HR, hazard ratio; CI, confidence interval; EGFR, epidermal growth factor receptor.

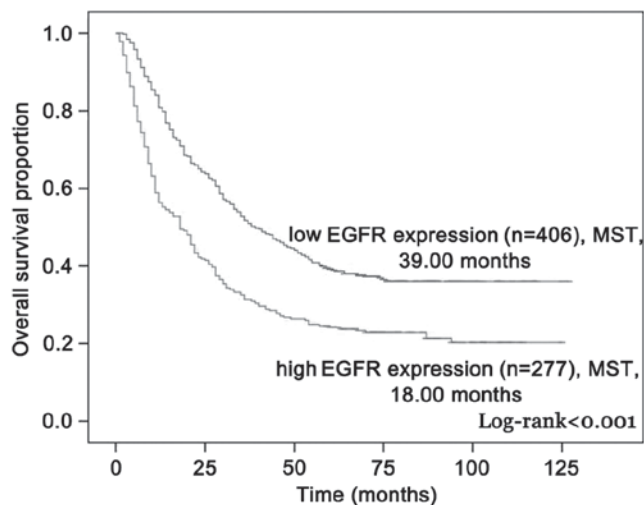


Figure 2. Kaplan-Meier curves obtained from EGFR expression levels for all patients. P-values obtained from log-rank test. EGFR, epidermal growth factor receptor; MOST, median overall survival time.

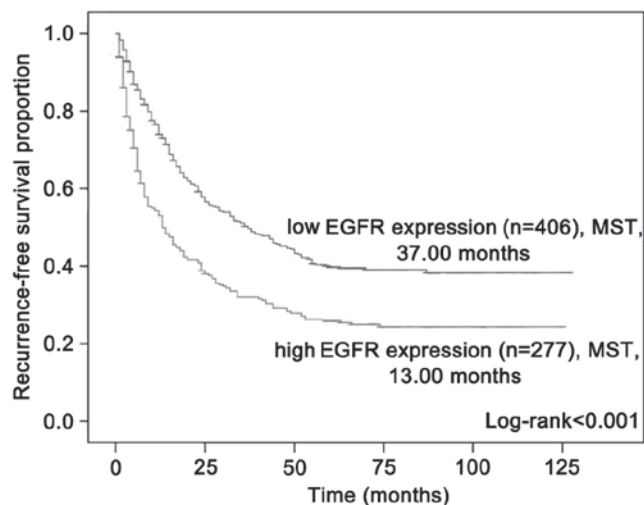


Figure 3. Kaplan-Meier curves obtained from EGFR expression levels for all patients. P-values obtained from log-rank test. EGFR, epidermal growth factor receptor; MRFST, median recurrence-free survival time.

Annexin V plus PI staining and flow cytometry analysis. As shown in Fig. 4C, suppressing EGFR with AG1478 markedly increased AGS cell apoptosis induced by low serum culture. These data indicate that EGFR has an important role in promoting GA cell survival.

## Discussion

Research into potentially suitable anticancer treatments targeted at EGFR in gastric cancer is ongoing. This type of therapy is termed new targeted treatment or personalized



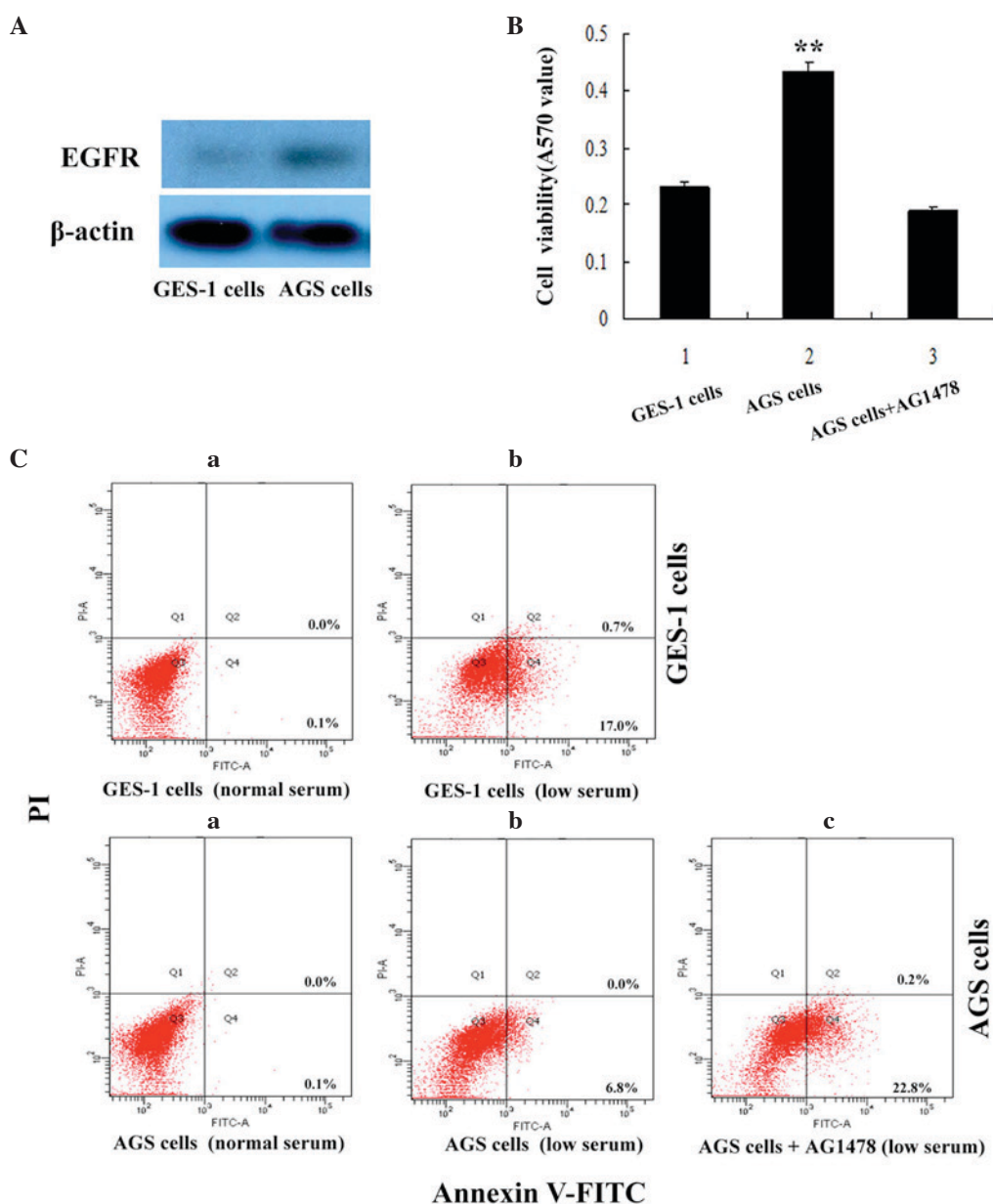


Figure 4. EGFR promotes AGS cell survival. (A) Western blot analysis showed that EGFR was overexpressed in AGS cells. (B) MTT assay for cell viability demonstrated that AGS cells had higher viability than GES-1 cells when both cell types were cultured in low serum conditions, which was blocked by pretreatment of AGS cells with AG1478. \*\* $P < 0.05$  vs. GES-1 cells or AGS cells pre-treated with AG1478. (C) Annexin V plus PI staining and flow cytometry analysis showed that apoptosis under low serum conditions (medium containing 1% fetal bovine serum) was lower in AGS cells than in GES-1 cells, and pretreatment of AGS cells with AG1478 markedly increased apoptosis in AGS cells. (a) Both GES-1 cells and AGS cells cultured in normal serum; (b) both GES-1 cells and AGS cells cultured in low serum; (c) AGS cells pretreated with AG1478 and cultured in low serum. EGFR, epidermal growth factor receptor; GES-1 cells, normal human gastric epithelial cells; AGS cells, gastric adenocarcinoma cells; PI, propidium iodide; FITC, fluorescein isothiocyanate.

treatment and has attracted attention from both experimental and clinical investigators (17-19). Compared with surgical resection combined with traditional radiation therapy or chemotherapy, these methods combined with new targeted treatments may indicate a new chapter in cancer therapy. The latter aim at molecular targets closely associated with the development of cancer, and such molecular therapies are closer to the molecular mechanisms of tumorigenesis and cancer progression. Data from clinical trials have demonstrated that gastric cancer patients with well known tumor biomarkers, such as HER-2, benefit from improved survival rates when treated with trastuzumab (20,21), as can those with EGFR mutation-positive non-small cell lung cancer treated

with gefitinib or erlotinib (22). The most important step in the development of such treatments is to identify biomarkers that are essential for cancer development.

EGFR is valuable as a tumor biomarker because it is often identified in its mutant form during the occurrence and development of cancer (23,24). Mutant EGFR is always highly active or overexpressed and is therefore a sensitive tumor biomarker (23,24); furthermore, EGFR is the driver of several important signaling pathways that are closely associated with cancer (25,26).

Although extensive research has been conducted into the influence of EGFR in cancer (27), to the best of our knowledge the relationship between EGFR expression and prognosis

in patients with RT3-GA remains to be determined. Few biomarkers have been identified to be of particular value in patients at this stage. Identifying biomarkers that are true oncogenes and treatment targets may be important in improving their survival. The findings of the present study demonstrate that EGFR is a prognostic biomarker in patients with RT3-GA. High EGFR expression was shown to be an independent prognostic factor for both poor MRFST and poor MOST in patients with RT3-GA, and that an indicator that patients with high EGFR expression may suffer from short survival durations.

The results of the present study were obtained from 683 patients with RT3-GA, a sample size of sufficient statistical power, and the EGFR expression findings on IHC were stable; thus, our data clearly suggest that the determination of EGFR expression in RT3-GA by IHC may be used as a routine method to evaluate the prognosis of patients with RT3-GA.

In accordance with its overexpression in GA tissues, EGFR was found to be overexpressed in AGS GA cells compared with non-cancer gastric cells (GES-1 cells) on western blot analysis. The effects of overexpression of EGFR in promoting GA cell survival were confirmed by cell apoptosis and viability assays, using cells pretreated with the EGFR inhibitor AG1478. Under low serum conditions, apoptosis was lower in AGS cells than in GES-1 cells; however, apoptosis was significantly increased in AGS cells pretreated with AG1478. Similar results were observed following an MTT assay for cell viability, where the use of AG1478 to pretreat AGS cells significantly decreased the viability of cells exposed to low serum conditions. These findings indicate that EGFR promotes GA cell survival.

AG1478 is an EGFR selective inhibitor that principally blocks EGFR activation at Tyr1173 (28,29). In the present study, AG1478 increased AGS cell apoptosis, thus we speculate that the expression levels of EGFR may be associated with its activity. We also speculate that the significant association between the high expression of EGFR in RT3-GA tissues and poor patient survival may be associated with increased activation of EGFR in cancer tissues.

In conclusion, the present study demonstrated that high EGFR expression levels are significantly associated with poor prognosis in patients with RT3-GA. These findings suggest that EGFR may be used as a prognostic biomarker for evaluating survival in patients with RT3-GA and may be a treatment target in these patients.

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