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Elevated epiregulin expression predicts poor prognosis in gastric cancer

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ABSTRACT

Epiregulin (EREG) is a novel family member of EGF-like ligands and have elevated expression in a variety of human cancers. EREG expression promotes tumor progression and metastasis and reduces patient survival. However, the expression of EREG and its prognostic value are not clear in gastric cancer (GC). We assessed EREG mRNA and protein expression in GC tissues from Chinese patients using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical staining of tissue microarray, and analyzed the correlation between the level of EREG expression and patient clinical characteristics and prognosis. We found that EREG expression was significantly higher in GC tissues than in matched adjacent noncancerous tissues. High EREG protein expression in GC was significantly associated with TNM stage including tumor size, lymph node metastases and distant metastases as well as poor overall survival. These finding demonstrate that EREG is an independent prognostic biomarker for GC.

1. Introduction

Gastric cancer (GC) is the second leading cause of cancer mortality and the fourth most common cancer worldwide [1]. Eastern Asia including China has been considered to have the highest GC prevalence area [2,3]. Unfortunately, the majority of cases of GC are diagnosed at an advanced stage. Despite intensive cytotoxic chemotherapy, the 5-year survival rate is under 20% [4]. However, early detection of GC could lead to a good prognosis with a 5-year survival rate of over 90% [5]. The occurrence of GC is associated with many risk factors including helicobacter pylori infection, salt intake, smoking, alcohol, family history of gastric cancer, atrophic gastritis (AG) and intestinal metaplasia (IM) [6]. Chronic gastritis induced by Helicobacter pylori infection has been demonstrated to be a cause of GC [7]. GC is reported to over express a lot of markers which are associated with survival of patients. However, there has not been an effective prognostic marker which is widely used in clinic. Therefore, it is necessary to identify new biomarkers to predict prognosis in GC.

Epiregulin (EREG) is a novel member of the family of EGF-like ligands isolated from conditioned medium of the mouse fibroblast-derived tumor cell line NIH3T3/clone T7 [8]. It is a 46-amino-acid single chain polypeptide, and its amino acid sequence exhibit 24–50% amino acid sequence identity with sequences of other EGF-related growth factors [8]. The gene coding for EREG is clustered in a region of human chromosome 4q13.3 [9]. EREG is a relatively wide receptor binding ligand, not only directly activates EGFR and ErbB4 homodimers, but also stimulates ErbB2 and ErbB3 heterodimers, result in the phosphorylation of four ErbB receptors tyrosine, and therefore activates

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downstream signaling pathways including the MEK/ERK pathway [10,11]. The intracellular signal transduction is primarily mediated by the EGFR and ErbB4 [12].

The expression of EREG and other EGF family members have been found in human gastric carcinoma cell lines TMK1 and MKN-45 and a large percentage of gastrointestinal stromal tumor (GIST) specimens [13,14]. However, it remains unknown whether EREG was expressed in all or a majority of human GC specimens. In the present study, we explored the expression of EREG in primary GC compared to matched neighboring non-cancerous tissues and analyzed the correlation between EREG expression and patient prognosis. We also demonstrated that the prognostic significance of EREG protein expression in GC and present the potential value of this marker as a prognostic indicator of survival in patients with GC.

2. Materials and methods

2.1. Human tissue specimens and patient clinical information

A total of 830 formalin-fixed paraffin-embedded (FFPE) stomach tissue specimens were collected from 741 patients. These included 600 cancer tissues with 89 matched adjacent noncancerous tissues(the area 3–5 cm beyond the edge of the tumor), 32 chronic gastritis, 30 intestinal metaplasia, 31 low-grade intraepithelial neoplasia and 48 high-grade intraepithelial neoplasia. All paraffin blocks were obtained from Department of Pathology at the Affiliated Hospital of Nantong University between January 2003 and December 2010. Medical records of patients provided information regarding age, sex, Tumor Node Metastasis (TNM) stage, histological type and differentiation grade. All the cancer and normal gastric benign tissues were histologically verified. The patients were not administered radiotherapy chemotherapy or immunotherapy prior to surgery. Overall (OS) was defined as the period from initial diagnosis via biopsy to death. The follow-up process ranged from 2 to 10 years, and data of patients who were alive at the last date of follow-up were censored for data analysis. Disease-free survival (DFS) was defined as the period from follow-up to recurrence. Forty-one frozen gastric cancer tissues and matched neighboring noncancerous tissues were obtained primarily from the First Affiliated Hospital of Nanjing Medical University, Huaian Second People's Hospital and Zhangjiagang Aoyang Hospital. The study protocol was approved by the Human Research Ethics Committee of these hospitals and carried out in accordance with the approved guidelines. All patients provided written informed consent for their stomach tissue samples to be used for research. All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Tissue microarray (TMA) and immunohistochemistry (IHC) analysis

TMA was generated using the Tissue Microarrayer System Quick Ray (UT06, UNITMA, Korea) manual. Core tissue biopsies (2 mm in diameter) were taken from 70 individual in 741 patients randomly. FFPE blocks were made and then arranged in new recipient paraffin blocks. A total 13 gastric TMAs were made. Four-micron sections were cut and placed on super frost-charged glass microscope slides to generate TMA slides. Tissue sections were deparaffinized and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2. Tissues were placed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave for antigen retrieval. EREG was detected by polyclonal goat anti-human EREG antibody (1:200) (R & D Systems, CA, USA). Reactions were detected with an Envision + ™ peroxidase kit (Dako, Carpinteria, CA, USA). Tissues were then incubated in 3,3′-diaminobenzidine plus (Dako, Carpinteria, CA, USA), counterstained with Hematoxylin, dehydrated through graded alcohols, cleared in xylene, and coverslipped with permanent mounting media. Staining was quantified in all tissues without knowledge of clinical characteristics. EREG expression was scored using the semi-quantitative H-score method, which takes into account both the staining intensity and the percentage of cells at that intensity [1]. The following staining intensity scores were used: 0 indicated no staining, 1 + indicated weak staining, 2 + indicated moderate staining and 3 + indicated intense staining. The total number of cells at each intensity level was multiplied by the corresponding intensity score to yield an intensity percentage score. The final staining scores were then calculated by summing the four intensity percentag scores; minimum possible final staining score was 0 (no staining), and a maximum possible score was 300 (100% of cells with 3 + staining intensity) [7].

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR analysis was performed to determine EREG mRNA expression in 41 pairs of human GC tissues and matched neighboring noncancerous tissues. Tissue samples were snap-frozen in liquid nitrogen and stored at -80° C before RNA isolation. Total RNA was isolated from frozen samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using a PrimeScript™ RT reagent kit (Takara, Glen Burnie, MD) according to manufacturer's instructions. Human β-actin served as the internal control for EREG mRNA determination. The following primers were used for PCR reactions: human β-actin forward, 5′- TGGAGAAAATCTGGCACCAC-3′, and reverse, 5′-GATGATGCCTCGTTCTAC-3′, and EREG forward, 5′-ATATTTCGGT GAACGGTGTG-3′ and reverse, 5′ -TGTGAGGATCACAGCAGACA-3′ (Genescript. Nanjing, China). qRT-PCR was performed on an ABI PRISM 7500 H T Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in 96-well plates. The volume for each reaction was 20 μL, which included 2 μL of cDNA template (corresponding to ˜40 ng of retro-transcribed total RNA), the primers (20 nmol/L each) and 2 × SYBR Green PCR Master Mixtures (10 μL, Applied Biosystems). Cycle conditions were as follows: after an initial 2-minute hold at 50 °C to allow AmpErase-UNG activity and 10 min at 95 °C, the samples were cycled 40 times at 95 °C for 15 s and 58 °C for 1 min. All experiments were performed in triplicate. Results were normalized to respective internal controls. The Ct-value for each sample was calculated using the △△Ct method [2], and results were expressed as $2^{-\triangle\triangle Ct}$.

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL). The two groups were compared using unpaired Student's *t*-test. For statistical analysis, the continuous EREG expression data from IHC were fist converted into dichotic data (low vs high) using specific cutoff values, which were selected based on significant differences in OS using the X-tile software program (The Rimm Lab at Yale University; http://www.tissuearray.org/rimmlab) [3]. Student's *t*-test and χ [2] test ($T < 1$ or $n < 40$, Fisher's exact test) were used to determine the statistical significance of differences between the groups. Cumulative patient survival was estimated using the Kaplan-Meier method, and a log-rank test was used to compare the survival curves. A Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios for the variables. Values of P less than 0.05 was considered statistically significant.

3. Results

3.1. Increased EREG mRNA expression in GC tissues

To investigate the expression of EREG mRNA, we performed qRT-PCR in 41 pairs of GC tissues and adjacent noncancerous tissues. EREG mRNA was 1.94 ± 1.45 fold higher in GC tissues than that in matched adjacent noncancerous tissues ($P < 0.001$, Fig. 1).

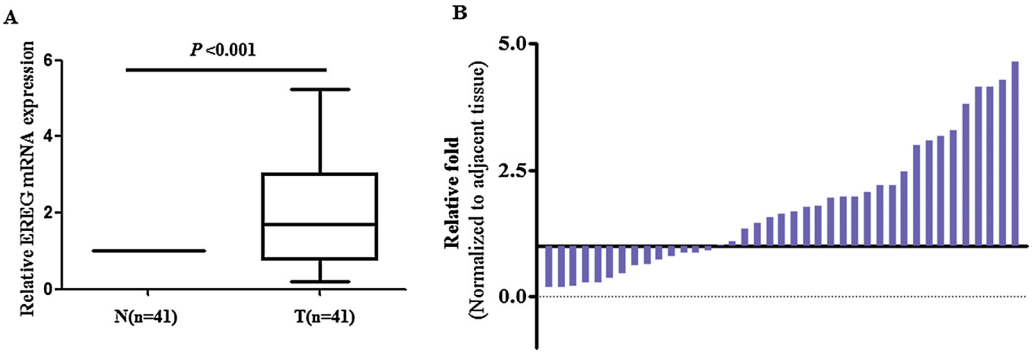

Fig. 1. Expression of EREG mRNA was detected by qRT-PCR and normalized to β-actin expression in 41 pairs of GC tissues. (A) Expression of EREG mRNA tissues was significantly higher in GC tissues (T) than that in matched adjacent noncancerous tissues (N) ($P < 0.001$). (B) EREG mRNA expression in GC tissue compared to paired adjacent noncancerous tissues.

3.2. Clinicopathologic features of patients

Analysis of EREG expression was possible in 750 (87.1%) of the 861 tumors on the TMA slides, 111 (12.9%) sections were lost during antigen retrieval or no tumor cells present in the core. In 750 gastric tissues analyzed, there were 51 chronic gastritis, 22 intestnial metaplasia, 9 low-grade intraepithelial neoplasia, 14 high-grade intraepithelial neoplasia, 104 surgical margin and 550 cancer. The clinicopathologic features of the 550 primary gastric cancer were summarized in Table 2. There were 416 men and 134 women. The average (SD) age was 60.5 years (range, 35 ~ 89 years). The distribution of TNM stage was as follows: 330 patients at stage 0, I and II, 220 at stage III and IV. For the histological type, most GC tissues initially presented as pure tubular (89%, 490/550), 25 were mucinous, 5 were tubular and mucinous carcinoma (mixed), 20 were signet ring cell carcinoma and 10 were "others" including 5 papillary adenocarcinoma, 1 adenosquamous carcinoma and 4 squamous cell carcinoma. For the differentiation status, 167 tumors were well and moderately differentiated, 328 were poorly differentiated, and 55 were other GC.

3.3. EREG protein expression in benign and malignant gastric tissues by IHC

EREG protein expression was localized in the cell membrane and cytoplasm (Fig. 2). Using the X-tile software program for TMA data analysis (http://www.tissuearray.org/rimmlab), we first identified the cutoff point 100 was significant in terms of overall survival in gastric cancer. Score 0–100 was considered no or low expression as negative, while 101–300 was considered high expression as positive [7]. For all subsequent analyses, EREG protein expression were considered either as “Low or no” or “High” using these cutoff values. High EREG protein expression was recorded in 23.1%, 17.6%, 18.2%, 22.2% and 28.6% of the stomach benign tissues in adjacent noncancerous tissues, chronic gastritis, intestinal metaplasia, low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia, respectively (Table 1). In GC tissues, high EREG protein expression was 43.3%, which was significantly higher than benign tissues ($P < 0.001$).

Table 1
EREG expression in gastric benign and malignant tissues.

Characteristics	n	EREG +	Pearsean X^2	P
Total	750		30.513	.000*
Chronic gastritis	51	9(17.6%)		
Intestnial metaplasia	22	4(18.2%)		
Low-grade intraepithelial neoplasia	9	2(22.2%)		
High-grade intraepithelial neoplasia	14	4(28.6%)		
Cancer	550	238(43.3%)		
Surgical margin	104	24(23.1%)		

Note: EREG + represents high EREG expression.
* $P < 0.05$.

3.4. Association of EREG expression and clinicopathologic characteristics in gastric cancer

The correlation between EREG protein expression and clinicopathologic variables in GC patients were statistically analyzed and summarized in Table 2. Increased EREG expression was significantly associated with TNM stage ($P = 0.002$), depth of invasion ($P = 0.001$), lymph node metastasis ($P = 0.048$) and distant metastasis ($P = 0.010$). However, we did not find significant correlation between EREG expression and histological type, tumor differentiation and other clinicopathologic variables including age, gender and preoperative CEA and CA199 ($P > 0.05$). (Table 2)

3.5. Increased EREG expression correlated with poor patients’ survival

In order to investigate the predictive value of EREG expression in GC, Kaplan–Meier survival analysis and log-rank test were performed to

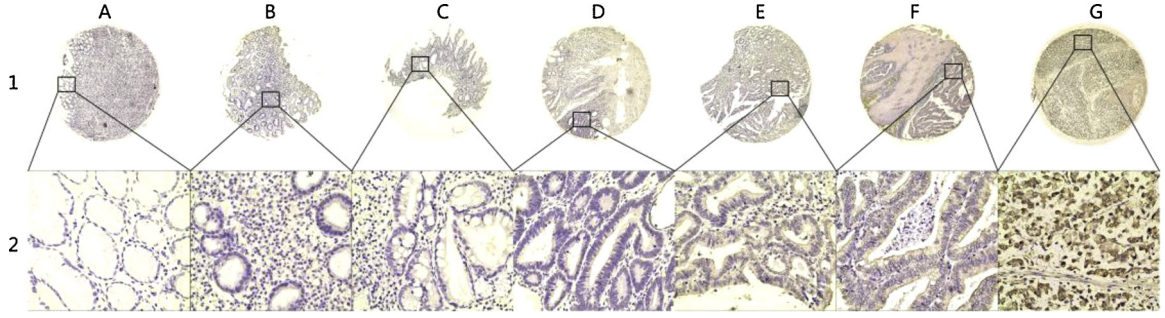

Fig. 2. Representative images of EREG expression on gastric tissue TMA sections. (A) normal surgical margin of gastric cancer with negtive EREG expression (IHC score, 0); (B)chronic gastritis with low EREG expression (IHC score, 20); (C) intestinal metaplasia with low EREG expression (IHC score, 10); (D) low-grade intraepithelial neoplasia with negtive EREG expression (IHC score, 0); (E) high-grade intraepithelial neoplasia with high EREG expression (IHC score, 140); (F) well differentiated gastric cancer with high EREG expression (IHC score, 110); (G) middle differentiated gastric cancer with high EREG expression (IHC score, 240); Row 1 are EREG IHC staining with x 40 magnification, and row 2 are EREG IHC staining with x 400 magnification.

Table 2
Association between EREG expression and clinicopathological Characteristics in GC patients.

Characteristics:	n	EREG +	Pearsean X^2	P
Total	550	238(43.3%)		
Gender			0.158	.691
Male	416	182 (43.8%)		
Female	134	56(41.8%)		
Age			0.027	.870
≤60	224	96(42.9%)		
> 60	326	142(43.6%)		
Histological type			1.823	.768
Tubular	490	212 (43.3%)		
Mucinous	25	10(40.0%)		
Mixed[a]	5	2(40.0%)		
Signet ring cell	20	7(35.0%)		
Others[b]	10	6(60.0%)		
Differentiation			0.573	.751
Well + Middle	167	76(45.5%)		
Poor	328	138(42.1%)		
Others[c]	55	23(41.8%)		
T			11.504	.001*
Tis + T1 + T2	201	68(33.8%)		
T3 + T4	349	170(48.7%)		
N			3.900	.048*
N0 + N1	331	132(39.9%)		
N2 + N3	219	106(48.4%)		
M			6.575	.010*
M0	512	214(41.8%)		
M1	38	24(63.2%)		
TNM stage			9.778	.002*
0 + I + II	330	125(37.9%)		
III + IV	220	113(51.4%)		
Preoperative CEA, ng/ml			1.268	.531
≤5	240	98(40.8%)		
> 5	67	32(47.8%)		
Unknown	243	108(44.4%)		
Preoperative CA199, U/ml			5.928	.052
≤37	251	98 (39.0%)		
> 37	47	27(57.4%)		
Unknown	252	113(44.8%)		

* $P < 0.05$.
[a] Mixed: Tubular and mucinous.
[b] others: Papillary adenocarcinoma, 5 cases; Adenosquamous carcinoma, 1 case; Squamous cell carcinoma, 4 case.
[c] others: besides Tubular and Papillary adenocarcinoma.

study the relationship between EREG expression and OS. Our data revealed that increased EREG overexpression correlated with poor OS ($P < 0.001$, Fig. 3A). The overall cumulative survival rate was 46.0% in low or no EREG expression group compared to 26.9% in high EREG expression group. We also found a poor OS in TNM stage III and IV compared to TNM stage 0, I and II ($P < 0.001$, Fig. 3B).

Both univariate and multivariate analysis were used to investigate the relationship between EREG expression and prognostic factors in GC [7]. Univariate analysis showed high EREG expression was significantly correlated with poor OS (HR, 1.763, 95% CI, 1.253–2.480; P = 0.001) (Table 3). This correlation were also observed in other prognostic markers including tumor differentiation (HR, 0.413, 95% CI, 1.200–2.549; $P = 0.008$), preoperative CEA (HR, 1.450, 95% CI, 1.128–1.863; $P = 0.004$), preoperative CA199 (HR, 1.469, 95% CI, 1.124–1.920; $P < 0.005$) and TNM stage (HR, 4.387, 95% CI, 3.030–6.351; $P < 0.001$) (Table 3). In multivariate analysis, high EREG expression (HR, 1.506, 95% CI, 1.026–2.211; $P = 0.037$) and TNM stage (HR, 3.646, 95% CI, 2.447–5.433; $P < 0.001$) were associated with poor OS, respectively (Table 3). These results suggest that EREG expression is an independent prognostic marker in GC.

4. Discussion

Previous studies demonstrated that GC develops initially from normal mucosa to chronic gastritis then to chronic atrophic gastritis, intestinal metaplasia, and dysplasia including low-grade and high-grade intraepithelial neoplasia, and finally to adenocarcinoma [7]. Although many factors are involved in GC progression, aberrant expression of epidermal growth factor receptor (EGFR) and its cognate ligands including EREG is a cause for malignancy progression and cancer formation [17]. EREG was reported to express predominantly in human peripheral blood macrophages and placenta, and extremely low compared to that of other EGF family members in majority of human organs such as heart, lung, liver, small intestine, colon, ovary and bone marrow [15]. Previous studies revealed that EREG is involved in a range of functions in normal physiological states. EREG contributes to inflammation, cutaneous excisional wound healing, corneal wound healing, airway epithelial differentiation and proliferation, intestinal epithelial proliferation and oocyte maturation by regulating angiogenesis, vascular remodeling and stimulating cell proliferation [8]. On the other hand, EREG also possesses a range of functions in pathological conditions especially malignant carcinomas [8]. EREG regulates

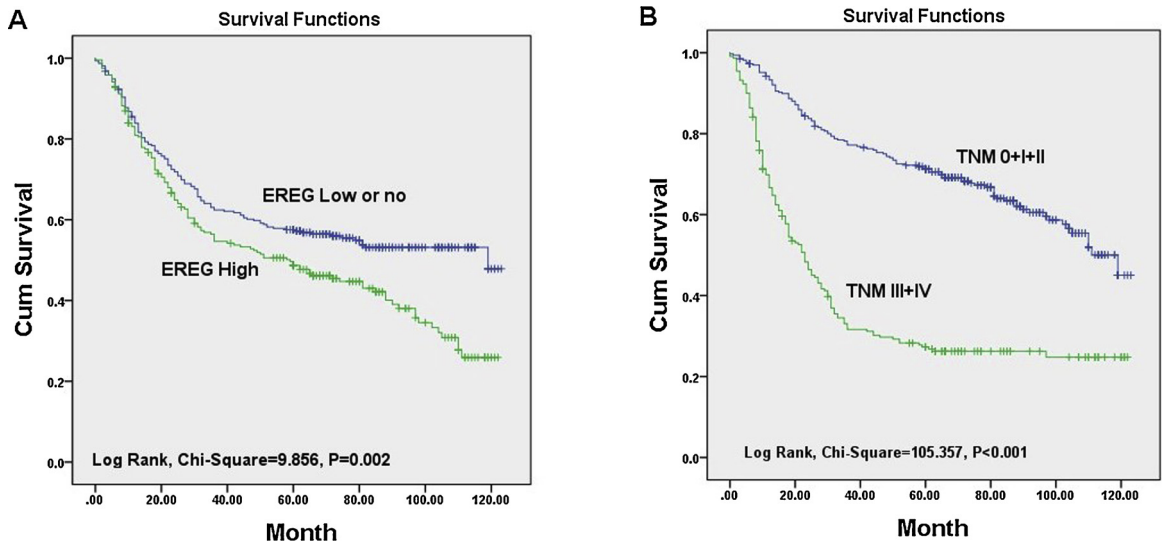

Fig. 3. Survival curves of GC using Kaplan-Meier method and log-rank test. (A) OS curves of EREG + (green line, 1) and EREG - (blue line, 2); (B) OS curves of stage TNM 0 + I + II (blue line, 1) and TNM III + IV (green line, 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Univariate and multivariate analysis of prognostic markers for OS in GC.

	Univariate analysis			Multivariate analysis		
	HR	p > |z|	95% CI	HR	p > |z|	95% CI
EREG expression High vs Low or no	1.763	0.001*	1.235 2.480	1.506	0.037*	1.026 2.211
Age (years) ≤60 vs > 60	1.533	0.100	0.544 1.077			
Gender Male vs Female	1.144	0.607	0.633 1.380			
Histological type Tubular vs Mucinous vs Mixed[a] vs Signet ring cell vs Others[b]	1.219	0.200	0.732 1.086			
Differentiation Well + Middle vs Poor	0.413	0.008*	1.200 2.549	1.459	0.067	0.974 2.186
TNM stage 0+I + II vs III + IV	4.387	< 0.001*	3.030 6.351	3.646	< 0.001*	2.447 5.433
T Tis + T1+T2 vs T3+T4	3.209	< 0.001*	2.230 4.618			
N N0 + N1 vs N2+N3	3.630	< 0.001*	2.524 5.221			
M M0 vs M1	3.869	0.001*	0.174 8.601			
Preoperative CEA, ng/ml ≤5 vs > 5 vs Unknown	1.450	0.004*	1.128 1.863	1.307	0.300	0.788 2.168
Preoperative CA199, U/ml ≤37 vs > 37 vs Unknown	1.469	0.005*	1.124 1.920	1.002	0.995	0.579 1.734

* $p < 0.05$.
[a] Mixed: Tubular and mucinous.
[b] others: Papillary adenocarcinoma, 5 cases; Adenosquamous carcinoma, 1 case; Squamous cell carcinoma, 4 case.

varying cellular processes including cell proliferation, invasion, metastasis, angiogenesis and resistance to apoptosis [16].

EREG plays an important role in tumor carcinogenesis, progression, invasiveness and metastasis. EREG was detected to be overexpressed in various human cancers including colon cancer, oral squamous epithelial cell carcinoma, lung carcinoma, bladder cancer, kidney cancer, androgen-independent prostate cancer, pancreatic cancer, breast cancer, liver cancer, ovarian cancer, thymic cancer, malignant glioma, salivary adenoid cystic carcinoma and malignant fibrous histiocytoma, whereas EREG expression levels were extremely low in the normal tissues corresponding to the tumors [13,14,17–32]. The growth of hepatoma cells was found to be suppressed by siRNA-mediated EREG knockdown in vitro [25], and the size of hepatocellular carcinomas was smaller in EREG knockout mice than that in wild type mice [33]. Moreover, the invading ability of EGFR-mutant NSCLC cells through Matrigel in vitro was inhibited by anti-EREG neutralizing antibody or by transfection with an EREG short hairpin RNA [34]. EREG was identified to be overexpressed in salivary adenoid cystic carcinoma cells with lung metastatic potential, and promoted cellular migration and invasion through the activation of ERK and Akt26. Meanwhile, EREG was identified as a lung metastasis signature (LMS) gene, which confers the potential for lung metastasis in breast cancer [35]. In a mouse model of bladder cancer with lung metastasis, EREG was identified to be one of the upregulated genes by microarray analysis in lung metastatic tumors [24]. Moreover, the EREG overexpression positively correlates with metastatic potential in bladder cancer cases [24]. Furthermore, EREG was identified as a metastasis-associated gene by Gene expression profiling comparing colorectal cancers with and without liver metastasis in 160 specimens [36]. Collectively, these findings indicate that EREG is involved in tumor carcinogenesis, invasiveness and metastasis.

EREG appears to play a role in targeted cancer chemotherapeutic drug sensitivity. The expression of EREG in human colorectal cancer specimens correlates with responsiveness to the anti-EGFR monoclonal antibody cetuximab [37–39]. EREG expression is also correlated with responsiveness to cetuximab in human head and neck tumor cell lines [40]. Meanwhile, EREG might be a therapeutic target for cancer stem cells. A previous study revealed that EREG was expressed in LGR5-positive colon cancer cells which possess cancer stem cell properties by microarray and immunohistochemistry analyses [41]. Moreover, an anti-EREG antibody showed antitumor activity against tumors derived from LGR5-positive colon cancer cells in a metastatic xenograft model [41]. Furthermore, LGR5 was confirmed to be related to big tumor size, advanced stage and worse prognosis in lung adenocarcinoma [42].

Clinicopathological investigations have shown that EREG is related to aggressive tumor phenotypes and poor prognoses in several human cancers. Like in NSCLC, Zhang reported that patients with EREG-positive tumors had worse clinical outcomes than those with EREG-negative tumors [43]. Sunaga reported that EREG is predominantly expressed in aggressive tumor phenotypes of NSCLCs including pleural involvement, vascular invasion and lymphatic permeation [44]. Patients with EREG-high expression lung adenocarcinoma have significantly shorter disease-free survival (DFS) and OS than those with EREG-low tumors [44]. Multivariate analyses suggest that EREG is a significant prognostic marker for DFS and OS in NSCLCs especially KRAS-mutant tumors [44]. In bladder cancer, EREG mRNA was highly expressed in tumors with advanced T stages, and elevated EREG expression is significantly associated with shorter survival [35]. In oral squamous cell carcinoma, elevated EREG expression is also related to shorter survival in patients [45]. In colon cancer, EREG expression is significantly associated with the depth of tumor invasion and distant metastasis [46]. In glioblastoma, EREG expression was correlated with higher tumor grade and worse survival [17]. Taken together, EREG seems to contribute to aggressive tumor phenotypes and works as a prognostic marker.

Regarding the gastric cancer, EREG was reported to be expressed in human gastric carcinoma cell lines TMK1 and MKN-45 and gastrointestinal stromal tumor (GIST) samples. However, we don't know the expression of EREG in human gastric cancer specimens. In this study, we used qRT-PCR and immunohistochemistry to detect the EREG mRNA and protein expression in gastric tissues from Chinese patients. qRT-PCR showed that GC tissues displayed higher EREG mRNA expression compared to that of matched neighboring non-cancerous tissues ($P < 0.001$). This result is in accordance with previous reports in oral squamous epithelial cell carcinoma [4]. In order to analyze the relationship between EREG expression and clinical characteristics of

GC, we performed a tissue microarray analysis that included 830 samples of gastric tissue, 600 of them were GC, with associated clinical and follow-up data. Immunohistochemical staining showed that EREG protein localized in cellular membrane and cytoplasm, and this observation was in line with previous findings in epithelial cells [47]. In this study, we found that EREG protein expression was significantly higher in cancerous tissues (43.3%) than that in matched adjuscent noncancerous normal tissues (23.1%), chronic gastritis (17.6%), intestinal metaplasia (18.2%), low-grade intraepithelial neoplasia (22.2%) and high-grade intraepithelial neoplasia (28.6%) (Table 1). It is still not fully understood the mechanism of deregulation of EGER high expression or low expression, the role of increased EREG expression, whether EREG overexpression influence its downstream and whether EREG expression is influenced by chemotherapy or radiotherapy in GC. Further investigation are needed next step. EREG protein expression were in accordance with that of EREG mRNA expression detected by qRT-PCR in GC and adjuscent normal tissues, indicating that high expressing EREG may play an important role in tumorigenesis of GC. EREG or amphiregulin was reported to stimulate prostaglandin E2 (PGE2) prostaglandin E2 (PGE2) production by stimulating cyclooxygenase 2 expression [48]. The elevated expression of EREG may be the consequence of PGE2 action on the EP4 PGE2 receptor in gastric epithelial cells [49].

There is a correlation between EREG protein expression levels and clinicopathologic variables in GC patients. We found that high EREG protein expression was positively related to tumor TNM stage including tumor size, lymph node metastases and distant metastases in GC (Table 2). Further prospective studies will be necessary to clarify these. However, we couldn't find correlation with age, gender, histological type, GC cell differentiation, preoperative CEA and CA199 (Table 2). Also, we found that increased EREG expression was significantly correlated with poor OS, which indicated that EREG was an independent GC prognostic marker. This finding might lay the basis for further investigation about the biological role of EREG in GC. Therefore, EREG and the elements of the EGF/ErbB signaling network that lie downstream of EREG appear to be good targets for therapeutic intervention of GC. We are interested in the further investigation to inhibit GC cells growth in vitro and in vivo using an EREG inhibitor or by EREG knockdown.

In conclusion, our study appears to support the hypothesis that EREG is a new prognostic biomarker in GC treatment. The increased expression of EREG is correlated with tumor TNM stage including tumor size, lymph node and distant metastasis. EREG is an independent prognostic marker for poor outcome in GC patients.

Statement of author contributions

Hongmei Yong and Huijun Zhu conceived experiments and analysed data. Shu Zhang, Wei Wang, Wei Zhao and Guipeng Dingcarried out experiments. Jin Zhu, Xiaohua Li, Bing Wang and Zhenqing Feng were carried out study design. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Conflict of interest statement

The authors declare no competing financial interests. We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Competing financial interests

The authors declare no competing financial interests.

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