The meta and bioinformatics analysis of GRP78 expression in gastric cancer

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ABSTRACT

GRP78 is a molecular chaperone located in endoplasmic reticulum, and induces folding and assembly of newly-synthesized proteins, proteasome degradation of aberrant proteins, and translocation of secretory proteins, autophagy, and epithelial-mesenchymal transition. We performed a systematic meta- and bioinformatics analysis through multiple online databases up to March 14, 2017. It was found that up-regulated GRP78 expression in gastric cancer, compared with normal mucosa at both protein and mRNA levels ($p < 0.05$). GRP78 expression was positively correlated with depth of invasion, TNM staging and dedifferentiation of gastric cancer ($p < 0.05$), while its mRNA expression was negatively correlated with depth of invasion, histological grading and dedifferentiation ($p < 0.05$). A positive association between GRP78 expression and unfavorable overall survival was found in patients with gastric cancer ($p < 0.005$). A higher *GRP78* mRNA expression was positively correlated with overall and progression-free survival rates of all cancer patients, even stratified by aggressive parameters, or as an independent factor ($p < 0.05$). These findings indicated that GRP78 expression might be employed as a potential marker to indicate gastric carcinogenesis and subsequent progression, even prognosis.

INTRODUCTION

Endoplasmic reticulum (ER) stress is initiated by oxidative stress, glucose deprivation, chemical toxicity, alterations in intracellular Ca^{2+} levels, and blockade of glycosylation and hypoxia, and subsequently activates the expression of glucose-related proteins (GRPs) and CHOP by ER stress response element. It results in cell survival via translational attenuation to limit further accumulation of misfolded proteins, but prolonged or strong ER does apoptosis. GRP78 is a 78 kDa molecular chaperone located in ER lumen, and is also named as Binding immunoglobulin protein (BiP) or heat shock protein 5 (HSPA5). It is responsible for folding and assembly of newly- synthesized proteins, transport retrogradation across the ER membrane, proteasome degradation of aberrant proteins, and translocation of secretory proteins [1, 2].

GRP78 overexpression promotes autophagy, evidenced by increased VPS34 and LC3-II, and decreased p62 and LC3-I [3]. It activates PI3K-mediated autophagy pathway and induces autophagic degradation of IKKβ, which causes inactivation of NF-κB pathway, and subsequently alters the expression of PKM2 and HIF-1α [4]. GRP78 confers drug resistance by aggresome delivery to autophagosomes [5] or c-src/LSF/TS activation [6] respectively. Zhang et al. [7] found that cancer cells promoted cell surface relocalization of GRP78 which interacted with PI3K and enhanced PIP3 production. Cell-surface GRP78 might facilitate the migration and invasion of colorectal cancer cells by regulating cell-matrix adhesion and the degradation of extracellular matrix, which was partly mediated through uPA-uPAR protease system [8]. Blockade of cripto binding to cell surface GRP78 inhibited oncogenic cripto signaling via MAPK/PI3K and Smad2/3 pathways [9]. Cell surface GRP78 directly bound to and phosphorylated c-src for EGFR activation, promoting the invasion and metastasis of hepatocellular carcinoma (HCC) cells [10], and accelerating the proliferation and migration of breast cancer cells by activating STAT3 [11]. Li et al. [12] found that GRP78 was secreted from colon cancer cells via

exosomes. Monoclonal antibody against cell surface GRP78 suppressed tumor growth and metastasis by weakening PI3K/Akt signaling [13]. Histone deacetylase inhibitors blocked GRP78 release by inducing its aggregation in ER [12]. Reportedly, secretory GRP78 facilitated cell proliferation of colon cancer cells via PI3K/Akt signaling [14], and stimulated the differentiation of bone marrow mesenchymal stem cells to cancer-associated fibroblasts by activating TGF-β/Smad signaling pathway [15].

Body weight, organ development and integrity were not impaired, and cancer incidence and inflammation were not affected in GRP78 (+/−) mice [16]. At 3 months, PTEN (f/f)GRP78(f/f) livers showed hepatomegaly, activation of lipogenic genes, exacerbated steatosis and liver injury, whereas HCC was developed at 8–9 months [17]. Dong et al. [18] found that GRP78 heterozygosity impeded cancer growth by suppressing tumor cell proliferation and promoting apoptosis, and exhibited dramatic reduction in the microvessel density of the endogenous mammary tumors. Conditional heterozygous knockout of GRP78 in the host endothelial cells showed severe reduction of tumor angiogenesis and metastatic growth with minimal effect on microvessel density of normal tissue. In gastric cancer cells, GRP78 knockdown inhibited proliferation and enhanced apoptosis, whereas MEK inhibition blocked GRP78 up-regulation and had the same effects [19–20]. Gastric adenocarcinoma patients with the combined GRP78 rs391957 C/T and T/T genotype were at higher risk for

tumor recurrence and death than those with C/C [21]. Our previous study also showed that GRP78 expression was upregulated in gastric cancer, and positively correlated with tumor size, depth of invasion, lymphatic and venous invasion, lymph node metastasis, TNM staging, and poor prognosis of gastric cancer [22]. In the present study, we performed a meta- and bioinformatics analysis to clarify the clinicopathological and prognostic significances of GRP78 expression at both mRNA and protein levels.

RESULTS

Characteristics of eligible studies

Figure 1 is the flow diagram of paper selection in our meta-analysis. As shown in Table 1, a total of 12 articles on the relationship between GRP78 expression and cancer risk, clinicopathological or prognostic parameters of gastric cancer were retrieved for our meta-analysis by immunohistochemistry in PubMed, Web of Science, BIOSIS, SciFinder and CNKI. Only 3 articles contained the samples of normal gastric mucosa [22–24]. There appeared the comparison between GRP78 expression and clinicopathological characteristics of gastric cancer in 12 pieces of paper, including sex, depth of invasion, lymph node metastasis, TNM staging and Lauren's classification [22–33]. Finally, the authors discussed the prognostic significance of GRP78 expression in 6 articles [22, 24, 25, 28, 30, 33].
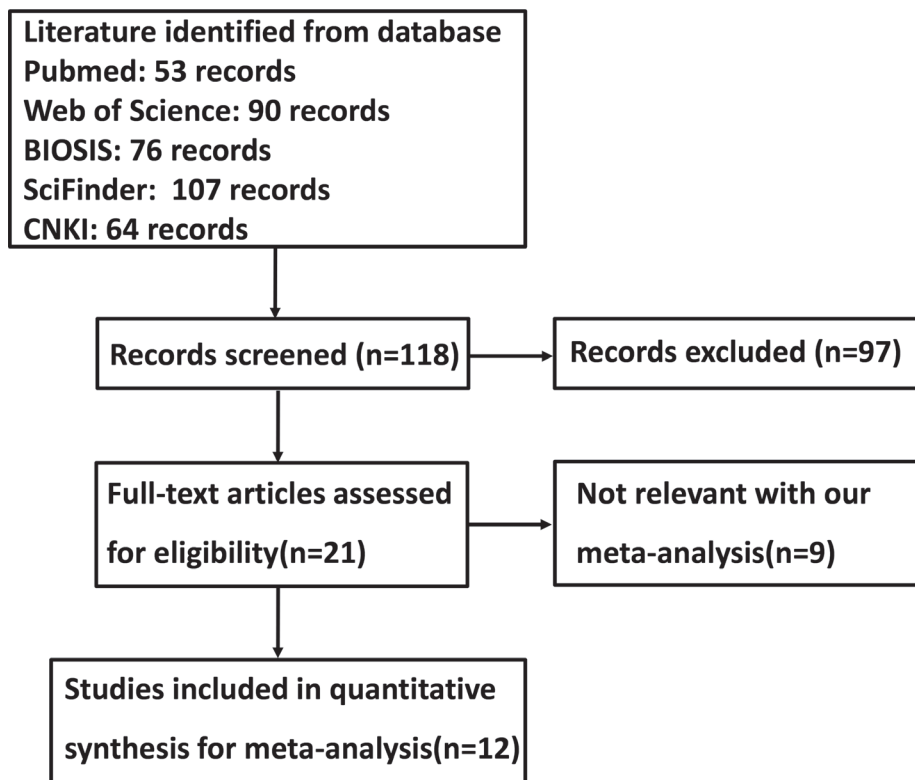

Figure 1: Flow diagram of the selection process in this meta-analysis.

Table 1: Main characteristics of eligible studies

First author	Year	Country	Ethnicity	Antibody source	Cases	Control	Risk to cancer	Outcome	Quality
Zhang J	2006	China	Asian	Santa Cruz	86			Negative	8
Zheng HC	2008	Japan	Asian	Santa Cruz	487	83	Up	Negative	9
Yang L	2014	China	Asian	Zhongshan	237			Negative	8
Zhang XC	2005	China	Asian	Santa Cruz					8
Xu FY	2010	China	Asian	Santa Cruz	65				8
Peng CL	2013	China	Asian	Ruiqi	60	60	Up	Negative	8
Fu ZQ	2014	China	Asian	Abcam	34				8
Wei H	2014	China	Asian	Santa Cruz	80			Negative	8
Li LN	2014	China	Asian	Proteintech	60	20	Up		8
He ZX	2015	China	Asian	Boster	48				8
Hu YW	2016	China	Asian	Santa Cruz	90				8
Ge JJ	2016	China	Asian	Abcam	172				8

Association between GRP78 expression and cancer susceptibility of gastric mucosa

We analyzed the association between GRP78 expression and cancer susceptibility of gastric normal mucosa in 3 studies with 607 cancers and 163 controls. As a result, we found up-regulated GRP78 expression in gastric cancer, compared with normal mucosa (Figure 2A, $p = 0.03$).

Association between GRP78 expression and clinicopathological parameters of gastric cancer

As shown in Figure 2B, the male patients with gastric cancer showed a higher GRP78 expression than female ones ($p < 0.05$). A higher GRP78 expression was detected in T2–4 than T0–1 gastric cancers (Figure 2C, $p = 0.01$), and in T3–4 than T0–2 cancers (Figure 2D, $p < 0.00001$). GRP78 expression was positively related to lymph node metastasis of gastric cancer (Figure 2E, $p < 0.00001$). Gastric cancers with stage II–IV showed GRP78 overexpression, compared with ones with stage 0-I (Figure 2F, $p < 0.00001$). The stage III–IV cancers displayed a higher expression of GRP78 than stage 0–II ones (Figure 2G, $p = 0.00003$). GRP78 protein was less expressed in intestinal-type than diffuse-type carcinoma (Figure 2H, $p = 0.02$).

Association between GRP78 expression and survival rate of gastric cancer

As indicated in Figure 2I, the pooled result from 6 datasets demonstrated a negative association between GRP78 expression and unfavorable overall survival in patients with gastric cancer (HR = 0.78, 95% CI: 0.63–0.97, $p = 0.02$).

Publication bias

The heterogeneity test was performed as shown in Figure 3. Sensitivity analysis was used to evaluate individual study's influence on the pooled results by deleting one single study each time from pooled analysis. As a result, the carcinogenesis risk of GRP78-positive mucosa from Li's study had a significant effect on the pooled OR. When this study was excluded, the heterogeneity test was significantly reduced (data not shown).

## The clinicopathological and prognostic significances of *GRP78* mRNA expression in gastric cancers

Then, we used DErrico's and Cui's datasets to perform bioinformatics analysis and found that *GRP78* mRNA expression was higher in gastric cancer than normal tissues, even in intestinal-type carcinoma (Figure 4A, $p < 0.05$). In TCGA data, *GRP78* mRNA expression was higher in female with male gastric cancers (Figure 4B, $p < 0.001$). It was negatively correlated with depth of invasion and histological grading of gastric cancer as well (Figure 4B, $p < 0.05$). A higher *GRP78* mRNA expression was detectable in intestinal-type carcinoma than diffuse-type counterpart (Figure 4B, $p < 0.001$).

According to Kaplan-Meier plotter, we found that a higher *GRP78* mRNA expression was positively correlated with overall and progression-free survival rates of all cancer patients (Figure 4C, $p < 0.05$). As summarized in Table 2, the cancer patients with high *GRP78* mRNA expression showed a long overall survival time than those with its low expression, even stratified by gender, treatment, Lauren's classification and Her2 immunoreactivity ($p < 0.05$). The overall or progression-free survival rate of the patient with

T2–T4, lymph-node-positive, and M0 cancers was higher in the group of high *GRP78* mRNA expression than that of its low expression ($p < 0.05$).

According to TCGA's database, univariate analysis showed a positive link between *GRP78* mRNA expression and the overall better prognosis of the patients with gastric cancer (Figure 4C, $p < 0.05$). Multivariate analysis using Cox's hazard proportional model indicated that *GRP78* mRNA expression was independent prognostic factor for gastric cancer (Table 3, $p < 0.05$).
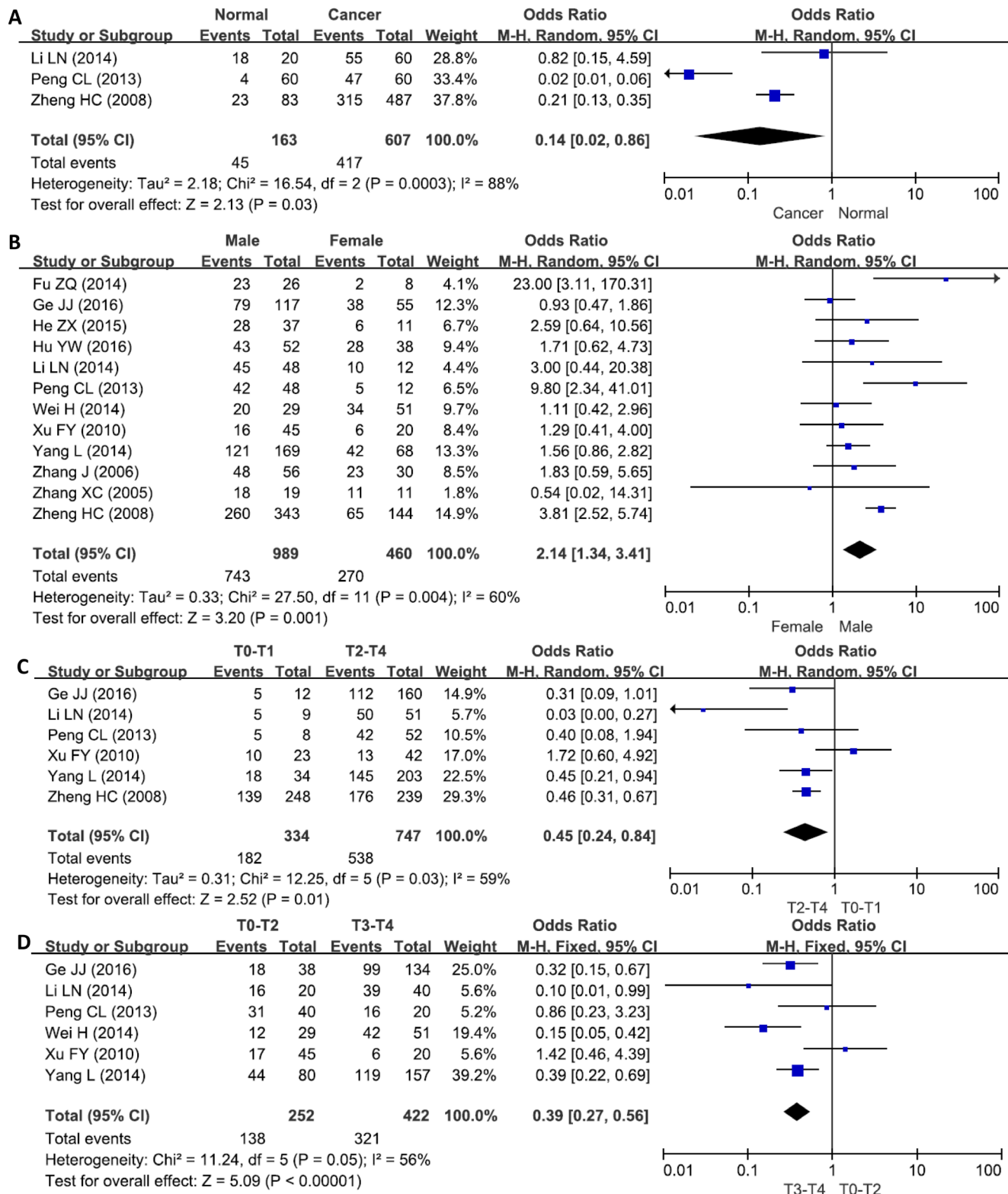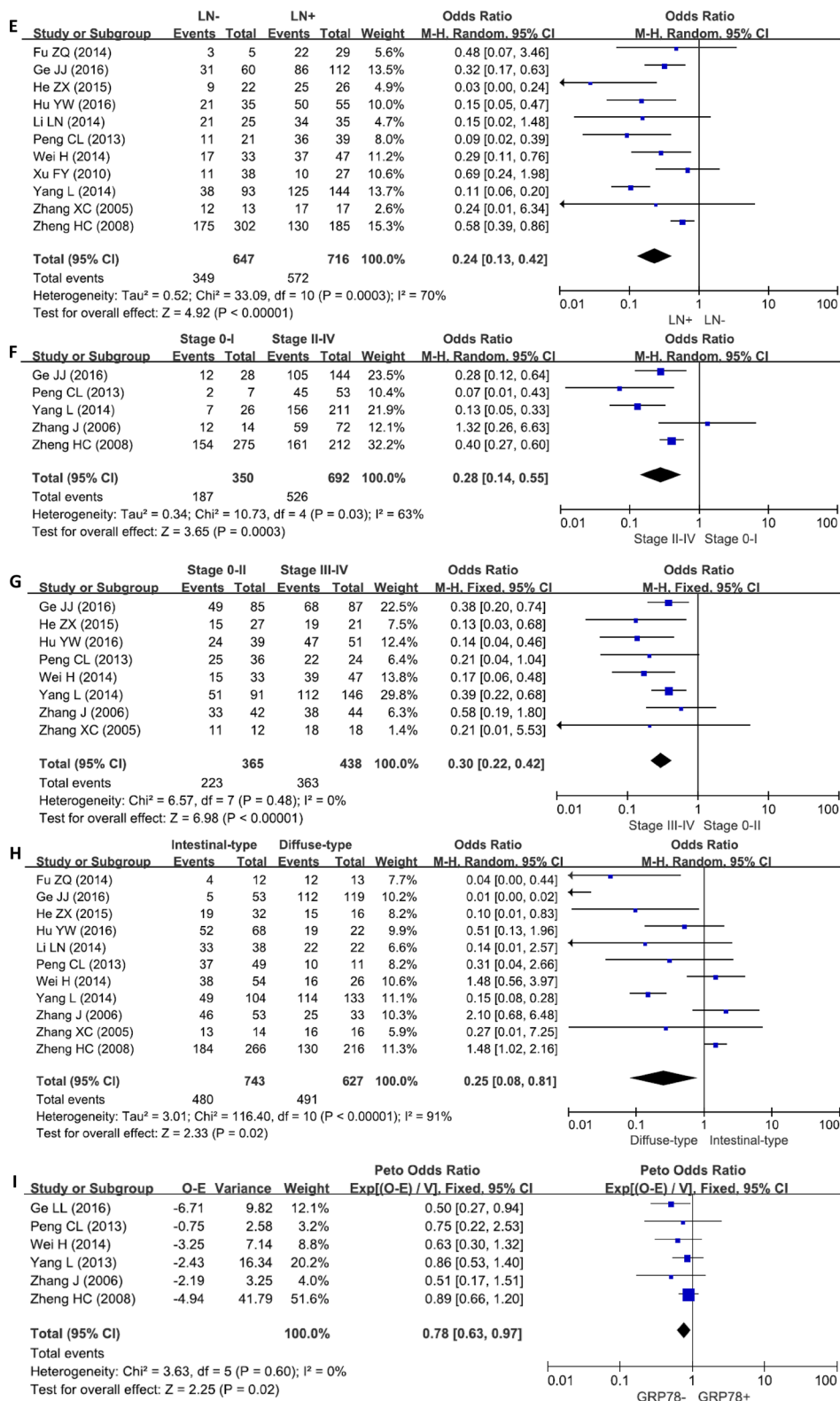
Figure 2: Forest plot for the relationship between GRP78 expression and clinicopathological parameters of gastric cancer. (**A**) gastric carcinogenesis (cancer *vs* normal mucosa); (**B**) correlation between sex and GRP78 expression (male *vs* female); (**C**) correlation between depth of invasion and GRP78 expression (T0–1 *vs* T2–4); (**D**) correlation between depth of invasion and GRP78 expression (T0–2 *vs* T3–4); (**E**) correlation between lymph node metastasis (LN) and GRP78 expression (LN− *vs* LN+); (**F**) correlation between TNM staging and GRP78 expression (stage 0–I *vs* stage II–IV); (**G**) correlation between TNM staging and GRP78 expression (stage 0–II *vs* stage III–IV); (**H**) correlation between differentiation and GRP78 expression (intestinal-type *vs* diffuse-type); (**I**) correlation between survival and GRP78 expression (GRP78+ *vs* GRP78−).

DISCUSSION

GRP78 silencing was reported to increase essential polyunsaturated fat accumulation of breast cancer cells, due to an inhibition of mitochondrial fatty acid transport and a reduction of fatty acid oxidation [34]. In pancreatic ductal cancer cells, GRP78 enhanced the proliferation, migration and invasion by up-regulating the expression of CyclinD1, CDK 4, CDK6, p-STAT3, JAK2, RhoA, ROCK1, and Smad4 [35]. GRP78 promoted cancer metastasis via up-regulation of MMP-2 and MMP-9, the induction of epithelial-mesenchymal transition or activation of NRF-2/HO-1 pathway [36–38]. Xie et al. [39] reported that GRP78 inhibited apoptosis and attenuated gemcitabine chemosensitivity in breast cancer cell via Akt/ mitochondrial apoptotic pathway. Li et al. [40] found that GRP78 mediated radiotherapeutic resistance of a stem cell-like subpopulation within breast cancer cells. To investigate the clinicopathological and prognostic significances of GRP78 expression, we analyzed 12 studies, which met specific inclusion criteria and had moderate to high quality according to their NOS scores.

Ciortea et al. [41] found that plasma GRP78 level was significantly higher in patients with endometrial cancer than the control group. Android fat distribution had a positive correlation with plasma GRP78 level, but versa for gynoid fat. Raiter et al. [42] demonstrated that anti-GRP78 titer in patients with colorectal polyp or cancer was higher than that in healthy subjects. Consistent with the data about esophageal cancer, medullary thyroid carcinoma, pancreatic ductal adenocarcinoma, colorectal cancer, renal clear cell carcinoma, astrocytoma, prostate cancer, lung cancer and hepatocellular carcinoma [35, 43–50], we found up-regulated GRP78 expression in gastric cancer, compared with gastric mucosa at both mRNA and protein levels, suggesting that GRP78 hyperexpression was positively linked to gastric carcinogenesis. Although anti-GRP78 antibodies come from 6 companies, and different statistical methods are employed, GRP78 expression and its correlation with clinicopathological parameters are comparatively consistent, indicating that
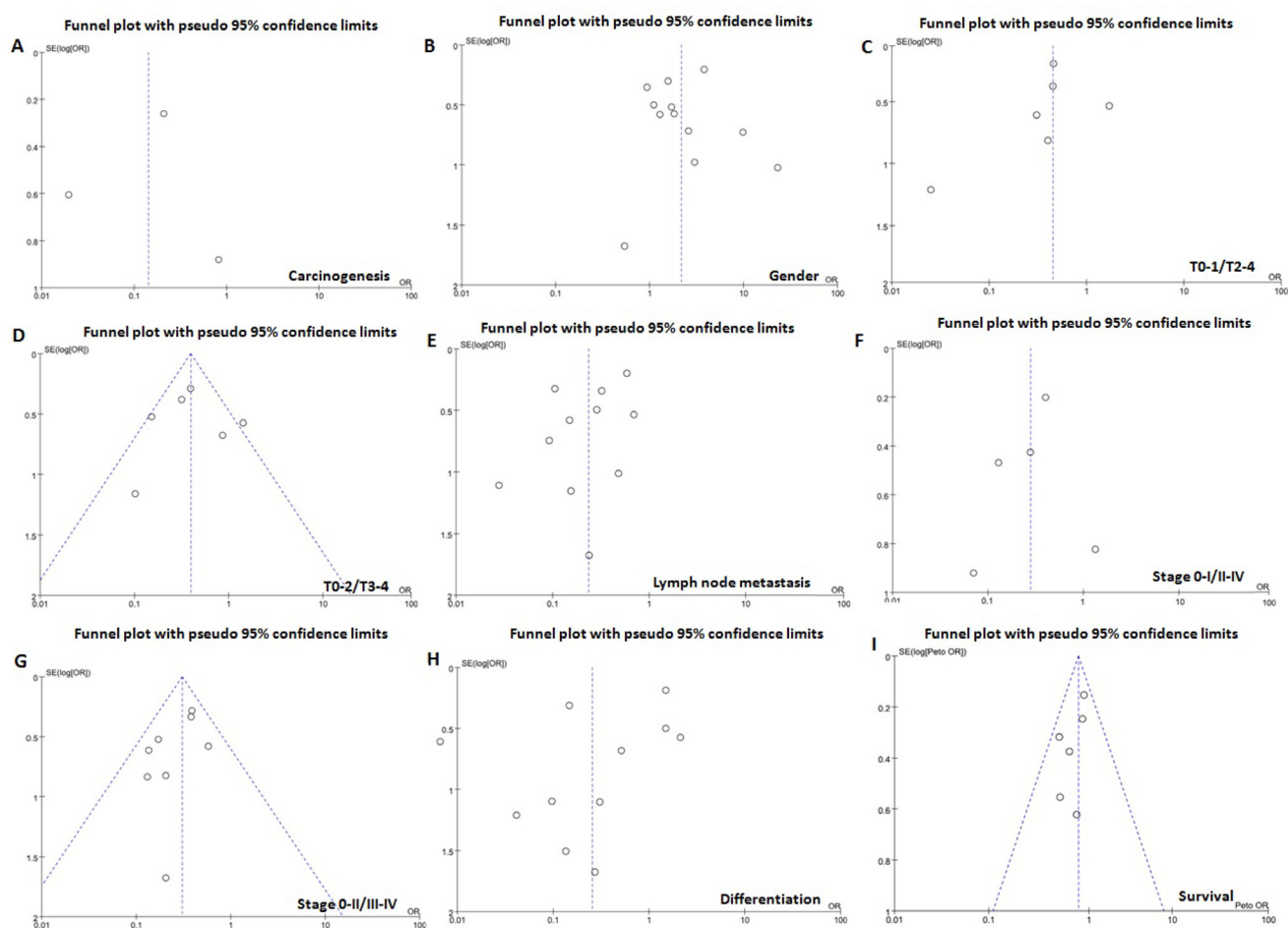

Figure 3: Funnel plot for publication bias test between GRP78 expression and gastric carcinogenesis or progression. The bias was analyzed about risk degrees of GRP78 expression in gastric mucosa (**A**) afor gastric carcinogenesis. Additionally, it was tested between GRP78 expression and clinicopathological features of gastric cancer, including age (**B**), depth of invasion (**C** and **D**), lymph node metastasis (**E**), TNM staging (**F** and **G**), and differentiation (**H**) and prognosis (**I**).

these antibodies mainly recognizes GRP78 protein and its expression trend is not determined by statistics. Here, there appeared a positive correlation between GRP78 protein expression and aggressiveness, such as depth of invasion, lymph node metastasis and TNM staging, but the converse was true for *GRP78* mRNA expression, indicating that aberrant GRP78 expression might be employed to indicate the pathological behaviors of gastric cancer. This is not surprising since mRNA levels from an expressed gene do not usually predict the corresponding protein levels because it takes a long distance from mRNA to protein by translation and posttranslational modification.

Reportedly, GRP78 expression was positively related to the poor prognosis of the patients with pancreatic and rectal cancers [35, 51]. It might be also demonstrated to indicate the worse prognosis of esophageal cancer, melanoma, advanced laryngeal squamous cell carcinoma, tongue cancer, and glioblastoma as an independent factor [43, 47, 52–54]. Ma et al. [55] reported that the overall survival of patients with high serum GRP78 level was significantly poorer than those with low GRP78 level. Our meta-analysis showed that GRP78 expression was positively linked to the worse prognosis of the patients with gastric cancer. However, our bioinformatics data indicated that *GRP78* mRNA expression was positively associated with overall and progression-free survival rates of the patient with gastric cancer, even stratified by clinicopathological features or as an independent factor. The paradoxical findings might be attributable to their correlation with aggressive behaviors of gastric cancer.
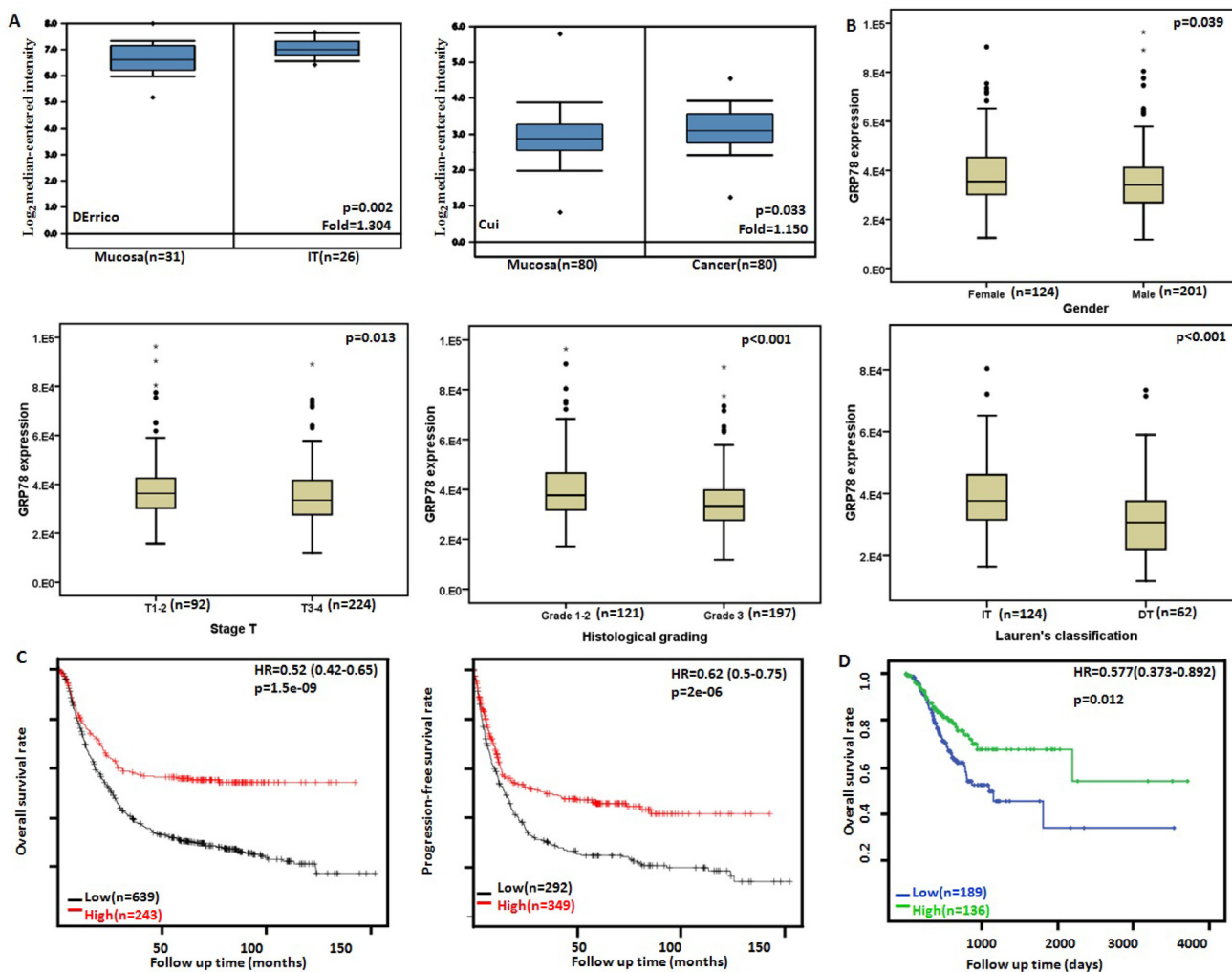

Figure 4: *GRP78* mRNA expression in gastric carcinogenesis and subsequent progression. Cui's and DErrico's datasets were employed for bioinformatics analysis to analyze *GRP78* mRNA expression during gastric carcinogenesis. A higher *GRP78* mRNA expression was detectable in gastric cancer than that in normal gastric mucosa (**A**) $p < 0.05$, even in intestinal-type (IT) carcinoma. TCGA database shows that *GRP78* mRNA was more expressed in female than male gastric cancers (**B**) $p < 0.05$. *GRP78* mRNA expression was negatively correlated with T staging, histological grading and differentiation of gastric cancers (B) $p < 0.05$. According to the data from KM plotter, *GRP78* mRNA expression was positively related to both overall and progression-free survival rates of the patients with gastric cancer (**C**). According to TGCA database, *GRP78* mRNA expression was positively linked to overall survival rate of the patients with gastric cancer (**D**). HR, hazard ratio.

Table 2: The prognostic significance of *GRP78* mRNA in gastric cancer

Clinicopathological features	Overall survival		Progression-free survival	
	Hazard ratio	*p*	Hazard ratio	*p*
Sex				
Female	0.48 (0.32−0.7)	9.1e−05	0.53 (0.36−0.79)	0.0013
Male	0.55 (0.42−0.72)	7.4e−06	0.58 (0.44−0.76)	5.7e−05
T				
2	0.42 (0.27−0.65)	7.1e−05	0.49 (0.32−0.75)	0.00077
3	0.58 (0.38−0.89)	0.011	0.69 (0.46−1.02)	0.063
4	0.36 (0.14−0.91)	0.025	0.51 (0.22−1.17)	0.11
N				
0	0.54 (0.22−1.35)	0.18	0.57 (0.21−1.58)	0.28
1−3	0.45 (0.34−0.6)	3e−08	0.52 (0.4−0.69)	2.2e−06
1	0.49 (0.32−0.74)	0.00055	0.53 (0.36−0.78)	0.0012
2	0.32 (0.18−0.59)	0.00011	0.39 (0.23−0.67)	0.00042
3	0.28 (0.14−0.56)	1e−04	0.38 (0.2−0.73)	0.0024
M				
0	0.45 (0.33−0.61)	1.2e−07	0.53 (0.4−0.7)	9.5e−06
1	0.59 (0.3−1.17)	0.12	1.89 (0.95−3.76)	0.066
TNM staging				
I	0.46 (0.14− 1.46)	0.18	0.65 (0.19−2.18)	0.48
II	0.24 (0.09−0.61)	0.0012	0.31 (0.13−0.73)	0.0045
III	0.57 (0.41−0.79)	0.00068	0.55 (0.35−0.87)	0.0099
IV	0.49 (0.31−0.76)	0.0013	0.63 (0.41−0.96)	0.032
Differentiation				
Well-differentiated	-	-	-	-
Moderately-differentiated	0.68 (0.35−1.32)	0.25	0.69 (0.36−1.3)	0.24
Poorly-differentiated	1.24 (0.83−1.87)	0.29	1.22 (0.77−1.92)	0.4
Lauren's classification				
Intestinal-type	0.44 (0.3−0.62)	3e−06	0.57 (0.39−0.82)	0.002
Diffuse-type	0.5 (0.34−0.72)	0.00017	0.52 (0.36−0.76)	5e−04
Her2 positivity				
−	0.49 (0.37−0.65)	3.3e−07	0.56 (0.43−0.75)	4.4e−05
+	0.7 (0.52−0.93)	0.014	0.68 (0.49−0.93)	0.016
Perforation				
−	0.79 (0.5−1.23)	0.29	0.8 (0.52−1.25)	0.33
Treatment				
Surgery alone	0.55 (0.4−0.76)	2e−04	0.67 (0.5−0.9)	0.0073
Other adjuvant	0.07 (0.01−0.5)	0.00047	0.28 (0.12−0.63)	0.0011

In conclusion, GRP78 expression underwent an up-regulation from gastric carcinogenesis at both protein and mRNA levels. GPR78 protein expression was positively correlated with depth of invasion, lymph node metastasis, TNM staging and dedifferentiation of gastric cancer, but versa for *GRP78* mRNA. GRP78 protein expression might be employed as a good potential marker for worse prognosis of gastric cancer patients. *GRP78* mRNA expression has the opposite results, even stratified by aggressive features or as an independent factor. Several limitations should be noted in our meta-analysis. Firstly, the potential publication bias stems from published results being predominantly positive. Secondly, patient populations in our study were limited, as patients came only from Asia. Thirdly, all of the survival data were extracted from survival curves, which may introduce

Table 3: Multivariate analysis of hazard factors of the prognosis of the patients with gastric cancer

Clinicopathological features	Hazard ratio (95% CI)	*p*
Gender (female/ male)	0.786 (0.515–1.199)	0.264
Stage T (T1–2/T3–4)	1.006 (0.577–1.756)	0.983
Lymph node status (−/+)	0.999 (0.557–1.792)	0.997
Distant metastasis (−/+)	1.605 (0.557–4.619)	0.381
TNM staging (I–II/III–IV)	0.741 (0.399–1.375)	0.342
Histological staging(G1–2/3)	1.121 (0.879–1.429)	0.357
GRP78 mRNA expression (low/ high)	0.572 (0.357–0.916)	0.020

subjective bias. Fourthly, this small sample size limits the power to detect the associations in some articles.

MATERIALS AND METHODS

Identification of eligible studies and data extraction

We performed a publication search using PubMed, Web of Science, BIOSIS and SciFinder updated on March 14, 2017. The following search terms were used: (GRP78 OR Bip OR HSPA5) AND (gastric OR stomach) AND (cancer OR carcinoma OR adenocarcinoma). Searching was done without restriction on language or publication years. Inclusion criteria for studies: (1) articles to observe the alteration in GRP78 expression in gastric cancer by immunohistochemistry; (2) papers to compare GRP78 expression with pathobiological behaviors and prognosis of gastric cancer by immunohistochemistry. Exclusion criteria included: (1) abstract, comment, review and meeting; (2) duplication of the previous publications; (3) Western blot, RT-PCR, cDNA microarray, or transcriptomic sequencing for GRP78 expression; (4) lack of sufficient information.

Data extraction

Based on the inclusion criteria, two reviewers (HC Zheng and BC Gong) independently extracted information from all eligible publications. The following information was included in each study: name of first author, year of publication, country, ethnicity, antibody company, numbers of cases and controls, expression alteration, and follow-up outcome. Regarding survival analysis, we used Engauge Digitizer software to extract data from Kaplan-Meier curves and calculated the Hazard ratios (HR) and their corresponding 95% confidence intervals (CI). Any disagreement was resolved through discussion until the two reviewers reached a consensus.

Quality score assessment

Two reviewers (HC Zheng and BC Gong) independently assessed the quality of the included studies according to Newcastle Ottawa Scale (NOS) (http://www. ohri.ca/programs /clinical_ epidemiology/oxford.htm). The scale consists of three components related to sample selection, comparability and ascertainment of outcome.

Bioinformatics analysis

The individual gene expression level of *GRP78* was analyzed using Oncomine (www. oncomine.org), a cancer microarray database and web-based data mining platform for a new discovery from genome-wide expression analyses. We compared the differences in *GRP78* mRNA level between gastric normal tissue and cancer. All data were log-transformed, median centered per array, and standard deviation normalized to one per array. The expression (RNA-seqV2) and clinicopathological data of 392 gastric cancer patients were downloaded from the Cancer Genome Atlas (TCGA) database by TCGA-assembler in R software. We integrated the raw data, analyzed *GRP78* mRNA expression in gastric cancer, and compared it with clinicopathological and prognostic data of the patients with gastric cancer. Additionally, the prognostic significance of *GRP78* mRNA was also analyzed using Kaplan-Meier plotter (http://kmplot.com).

Statistic analysis

HWE was evaluated using Chi-square test in control groups of each study. Strength of association between GRP78 expression and cancer risk was assessed by odds ratios with 95% confidence intervals. Statistical significance of the pooled OR was determined by *Z* test. If there was no significant heterogeneity, the fixed effect model (Mantel-Haenszel method) would be employed. Otherwise, the random effect model (DerSimonian and Laird method) would be used excluding prognostic analysis. Heterogeneity effect was then quantified by I^2 test, which was subdivided into low, moderate and high

degrees of heterogeneity according to the cut-off values of 25%, 50% and 75% respectively. Publication bias was evaluated by funnel plot and quantified by Begg's test and Egger's test to assess funnel plot asymmetry. Meta-analyses were performed with Revman software 5.3 and data from TCGA database was dealt with SPSS 10.0 software using student t test. *Kaplan-Meier* survival plots were generated and comparisons between survival curves were made with the log-rank statistic. Cox's proportional hazards model was employed for multivariate analysis. Two-sided $p < 0.05$ was considered as statistically significant.

CONFLICTS OF INTERESTS

The authors have declared that no competing interests exist.

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