ORIGINAL RESEARCH

ZNF418 overexpression protects against gastric carcinoma and prompts a good prognosis

Hong-xia Hui[1,*]
Zhong-wu Hu[2,*]
Chao Jiang[1]
Jian Wu[3]
Yong Gao[1]
Xiao-wei Wang[1]

[1]Department of Medical Oncology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, People's Republic of China; [2]Department of Thoracic Surgery, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, People's Republic of China; [3]Department of Pathology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, People's Republic of China

*These authors contributed equally to this work

Correspondence: Xiao-wei Wang; Yong Gao
Department of Medical Oncology, Huai'an First People's Hospital, Nanjing Medical University, 6 Beijing Road West, Huai'an, Jiangsu 223300, People's Republic of China
Tel +86 139 1207 3286
Email wxw7352@sina.com; hayy_gy@163.com

Background: This study aimed to investigate the prognostic power of zinc-finger protein 418 (*ZNF418*) in gastric cancer (GC) and its potential role in GC development and progression.

Patients and methods: A total of 10 GC patients' individual plasmas were collected and screened for dysregulated mRNA using human microarray. Among these dysregulated mRNAs, *ZNF418* was found to be significantly downregulated in IIIA–IV stage GC patients compared to IA–IIA stage GC patients. Subsequently, the *ZNF418* levels were detected by quantitative reverse transcription-polymerase chain reaction in both GC plasmas and tissues in a larger sample, and the association between *ZNF418* expression level and clinicopathological features as well as overall survival (OS) of GC patients was further analyzed. Finally, a network of *ZNF418* interactions with other molecules was predicated in STRING and GEPIA databases.

Results: Human mRNA microarray was performed to screen for abnormally expressed mRNAs between five IIIA–IV stage GC patients' plasma and five IA–IIA stage GC patients' plasma. A total of 662 mRNAs were differentially expressed in the IIIA–IV stage GC plasma vs IA–IIA stage GC plasma among all the candidate mRNAs according to the Student's *t*-test. Results showed that a decrease in the *ZNF418* expression level was associated with the presence of GC and also with higher tumor–node–metastasis stage and lower OS rates compared with that in adjacent noncancerous tissues. Cox regression analysis results demonstrated that the OS was independently correlated with *ZNF418* expression. Finally, the prediction results showed that a total of eight mRNAs might have an interaction with *ZNF418* in both STRING and GEPIA databases.

Conclusion: *ZNF418* was first identified to be significantly downregulated in GC. Our study indicated that *ZNF418* might serve as a novel biomarker for GC and was involved in GC development.

Keywords: plasma, diagnosis, prognosis, biomarker

Introduction

Gastric cancer (GC) is the most common digestive system tumor and is still the primary cause of human death in developing countries, with its incidence continuously increasing in recent years.[1] GC can be divided into early GC and advanced GC based on the malignant degree and invasion depth. However, due to the lack of specific biomarkers for early diagnosis, the confirmed diagnosis patient was mainly in the advanced stage, which has a poor survival rate.[2] Thus, it is necessary to identify appropriate molecular biomarkers for stage diagnosis and potential targets for GC therapy.[3]

The Cys2His2 (C2H2)-type zinc finger proteins (ZFPs) are a class of well-defined eukaryotic DNA-binding proteins.[4] There are ~700 genes in the human genome

encoding C2H2 motifs, composed of 21 amino acids with a consensus sequence of CX2CX3FX5LX2HX3H.[5] In C2H2-type ZFPs, there is a highly conserved consensus sequence TGE (K/R)P(Y/F)X, referred to as the H/C link between adjacent zinc finger motifs.[6] Each zinc finger can bind three to five base pair subsites specifically in the major groove of the DNA and constitutes the single largest class of transcription factors, which have been proved to be involved in tumor suppression, neoplastic transformation, cell differentiation, proliferation and apoptosis.[7,8] A previous study has confirmed the expression of zinc-finger protein 418 (*ZNF418*) in various adult tissues, including the placenta, pancreas, kidney, liver, brain, muscle, lung and heart.[6] However, up to date, no study has provided information regarding the expression patterns of *ZNF418* and its function in GC.

The present study utilized human mRNA microarray analysis to screen mRNA expression profiles in GC plasmas and discovered a significantly downregulated mRNA of *ZNF418*. Based on the microarray findings, a series of validation experiments were performed to explore the role of *ZNF418* in both gastric tissues and plasma. Then, the association between the expression level of *ZNF418* and the clinicopathological features as well as overall survival (OS) of patients with GC was analyzed. Finally, a network of *ZNF418* interactions with other molecules was predicated in STRING and GEPIA databases.

Patients and methods
mRNA microarray analysis
CapitalBio Technology Human mRNA Array v4 was designed with four identical arrays per slide (4×180 K format), with each array containing probes interrogating ~34,000 human mRNAs. Total RNA containing small RNA was extracted from plasmas by using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. Complementary DNA (cDNA) was produced by the Eberwine's linear RNA amplification method and subsequent enzymatic reaction. After completion of double-stranded cDNA (dsDNA) synthesis using DNA polymerase and RNase H, the dsDNA products were purified using a polymerase chain reaction (PCR) NucleoSpin Extract II Kit (MN) and eluted with 30 µL elution buffer.

Ethics statement
The study was approved by the ethics committee of Huai'an First People's Hospital, Nanjing Medical University, China. Permission for using the information in the medical records of the patients for research purposes was obtained from the Huai'an First People's Hospital.

Patients and samples
A total of 84 pairs of GC tissues and corresponding samples were obtained from patients with GC who underwent surgery during 2008–2016 at the Huai'an First People's Hospital, Nanjing Medical University. Before the operation, the patients have not received radiotherapy or chemotherapy. These tissue specimens were immediately stored in RNA-fixer reagent after removal from patients' stomachs and were kept at −80°C in a refrigerator until use. Following the principle, the paired adjacent non-tumor tissues were confirmed to have no tumor cells through pathological analysis and were located 5 cm away from the edge of the GC. According to the tumor–node–metastasis (TNM) (V.8.2016) staging system of the International Union against Cancer, all tumors were staged accurately.

Peripheral blood (3 mL) of 50 GC patients was obtained before the operation, and then, the plasma was isolated. Following the age- and gender-matched criteria, fresh normal plasma samples were collected from 10 healthy people at the Huai'an First People's Hospital, Nanjing Medical University, in February 2016. All peripheral blood samples used ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. Written informed consent was obtained from each patient before recruitment.

Follow-up
For the OS rate study, each patient saw his or her doctor during the follow-up period for assessment every 3 months in the first year, every 6 months in the second year and annually thereafter until December 2017. Dates of death were obtained from inpatient and outpatient records or from the patients' families through telephone follow-up. The OS was defined as the length of time between diagnosis and death or the last follow-up examination.

RNA isolation, reverse transcription and quantitative real-time PCR
Total RNA from paired tissues were extracted by using TRIzol reagent. All cDNAs were obtained with the Prime-Script™ RT-PCR kit following the manufacturer's instructions. The sequences of *ZNF418* divergent primers were as follows: forward: 5′-TGGACAGAGGAAAGTCGTCAT-3′ and reverse: 5′-AAAGTTCACAGCCACATCTTCA-3′. The primers' sequences of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control, were 5′-TCGACAG TCAGCCGCATCTTCTTT-3′ and 5′-ACCAAATCCG TTGACTCCGACCTT-3′. These primers were synthesized by PrimerBank. We used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on the ABI7500

System (Thermo Fisher Scientific) to measure the expression of mRNA. The PCR conditions were 95°C for 30 s and then 40 cycles of 95°C for 5 s and 60°C for 30 s. Finally, annealing and extension at 95°C for 15 s, 60°C for 60 s and 95°C for 15 s were carried out. Determining the reproducibility of the qRT-PCR method for detecting *ZNF418* levels in GC tissue, we performed qRT-PCR three times to measure differences between experimental batches.

Statistical analysis

Comparison of continuous data was analyzed using an independent Student's *t*-test between the two groups. A receiver operating characteristic (ROC) curve was established to evaluate their diagnostic values. The correction between *ZNF418* expression and OS time was evaluated by Kaplan–Meier plots and log-rank tests. All statistical analyses were performed using SPSS for Windows v.17.0 (SPSS Inc., Chicago, IL, USA). For all results, $P<0.05$ was considered to be statistically significant.

Results

mRNA expression profiles in GC

High-throughput human mRNA microarray was conducted to assess the differences in mRNA expression profiles between five advanced GC patients' plasma and five early GC patients' plasma. A total of 662 mRNAs were differentially expressed in IIIA–IV stage GC plasma vs IA–IIA stage GC plasma among all the candidate mRNAs according to the Student's *t*-test. Of 662 mRNAs, 69 were significantly upregulated in IIIA–IV stage GC plasma, whereas 593 were significantly downregulated. We selected a total of 10 upregulated and downregulated mRNAs from high rank to low rank based on the multiple fold difference between the expression of IIIA–IV stage GC plasma and IA–IIA stage GC plasma. Subsequently, we used qRT-PCR to detect the expression levels of these dysregulated mRNAs in GC plasma and found that compared to other mRNAs, the expression of *ZNF418* was significantly increased in IIIA–IV stage GC plasma vs IA–IIA stage GC plasma, which was selected as a candidate target for GC for further study.

ZNF418 was significantly downregulated in GC plasma

The expression of *ZNF418* in 40 GC plasmas was measured, and fresh normal plasma samples from 10 healthy individuals were used as control. The results indicated that *ZNF418* expression was downregulated in GC plasma compared with normal cases (Figure 1A). To evaluate the potential diagnostic value of *ZNF418*, the ROC curve has been used, and the

larger the area under the ROC curve (AUC), the higher the diagnostic value. As shown in Figure 1B, we found that in plasma, the AUC was 0.7025 (Figure 1B). Down-expression of the *ZNF418* level was negatively associated with the TNM stage. Figure 1C describes the relationship between *ZNF418* level and TNM stage, and as the TNM stage increased, the expression of *ZNF418* gradually decreased (Figure 1C).

ZNF418 was significantly downregulated in GC tissues

To further test the expression level of *ZNF418*, qRT-PCR was used to measure its expression in 84 paired primary cancerous and adjacent noncancerous tissues from GC patients. The results indicated that *ZNF418* expression was significantly downregulated than those in corresponding non-tumorous tissues (Figure 2A). As shown in Figure 2B, the ratio of *ZNF418* low expression was 61/84 (72.6%), while the ratio of *ZNF418* high expression was 23/84 (27.4%; Figure 2B). We further searched the *ZNF418* expression in the GEPIA database and found that the expression of *ZNF418* in 408 GC tissues was much lower than that in 211 noncancerous tissues, consistent with our findings (Figure 2C). Figure 2D reveals that the AUC was 0.7060.

As shown in Table 1, abnormal expression of *ZNF418* level was not aberrantly associated with age, gender, diameter, differentiation or lymph node metastasis in patients with GC (Table 1). However, down-expression of *ZNF418* level was negatively associated with T stage and TNM stage, and Figure 2E describes the relationship between *ZNF418* level and TNM stage in more detail.

ZNF418 was correlated with OS of patients with GC

Kaplan–Meier survival curves indicated that patients with low *ZNF418* expression exhibited a greater probability of attaining shorter OS (Figure 3) than those with high *ZNF418* expression. The mean OS time in patients with low expression for *ZNF418* was 38 months, which was significantly lower than that in patients with high expression for *ZNF418* (57 months). Cox regression analysis showed that survival was independently correlated with *ZNF418* expression ($P=0.043$; Table 2).

Prediction and annotation of *ZNF418* with other molecules' network

To identify the other molecules that might have an association with *ZNF418*, we performed an interaction network prediction on the STRING database (https://string-db.org) and further forecast in the GEPIA database (http://gepia.cancer-pku.cn/index.html)
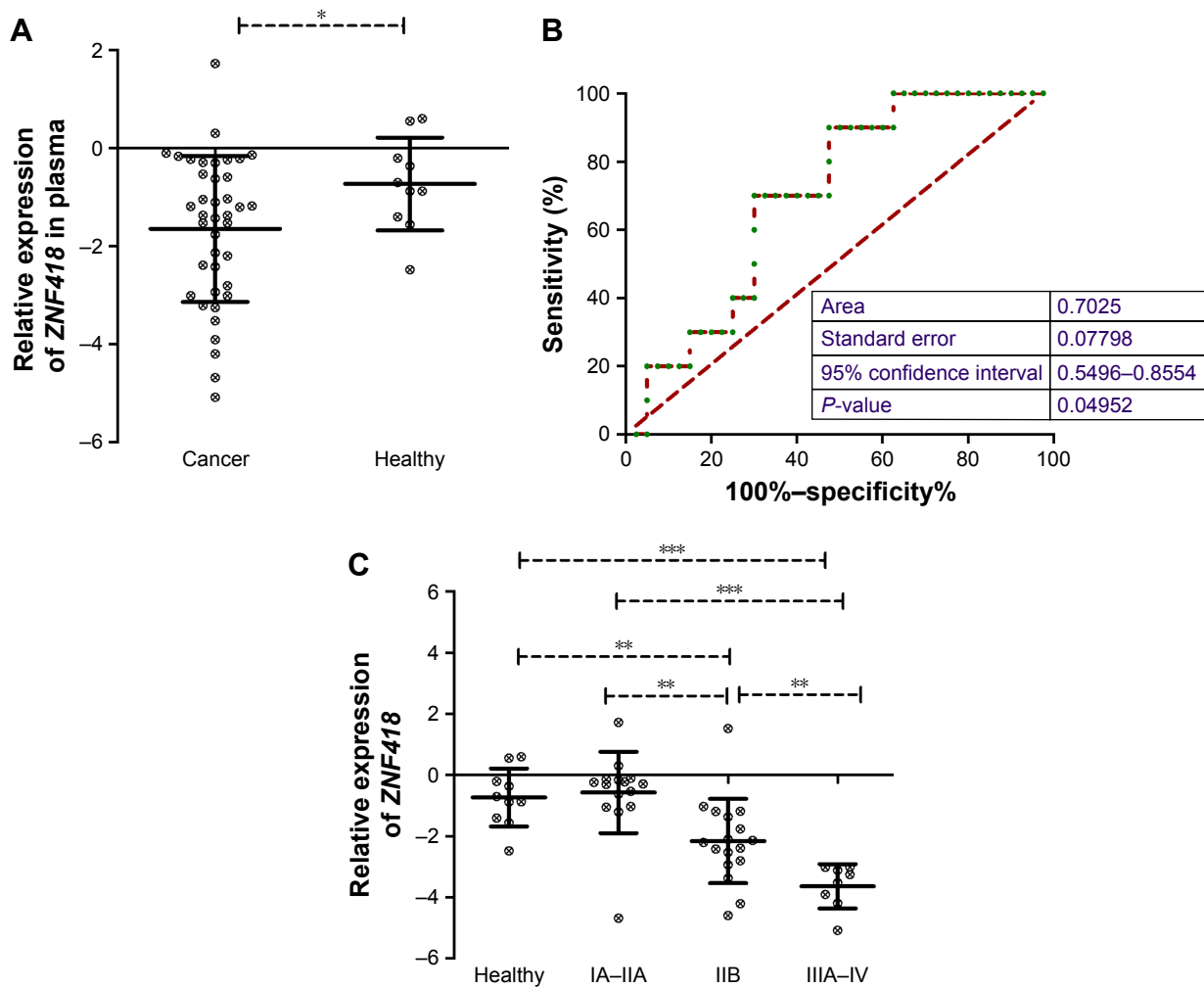
Figure 1 *ZNF418* was significantly downregulated in GC plasma compared to healthy cases.
Notes: (**A**) *ZNF418* expression was downregulated in GC plasma compared with normal cases. (**B**) The AUC was 0.7025. (**C**) Down-expression of *ZNF418* level was negatively associated with TNM. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.
Abbreviations: *ZNF418*, zinc-finger protein 418; GC, gastric cancer; AUC, area under the ROC curve; TNM, tumor–node–metastasis; ROC, receiver operating characteristic.

to increase the credibility of the conclusion. A total of 10 candidate mRNAs (KIAA1984 with another name of CCDC183, PPP1R37, POTEE, POTEI, POTEJ, POTEF, TRIM28, AGAP4, RIPK4, PHLPP2) might have an interaction with

ZNF418 in this study (Figure 4A). Subsequently, we look up the relationship between *ZNF418* and 10 molecules in the GEPIA database. We used the non-log scale for calculation and the log-scale axis for visualization. The results revealed
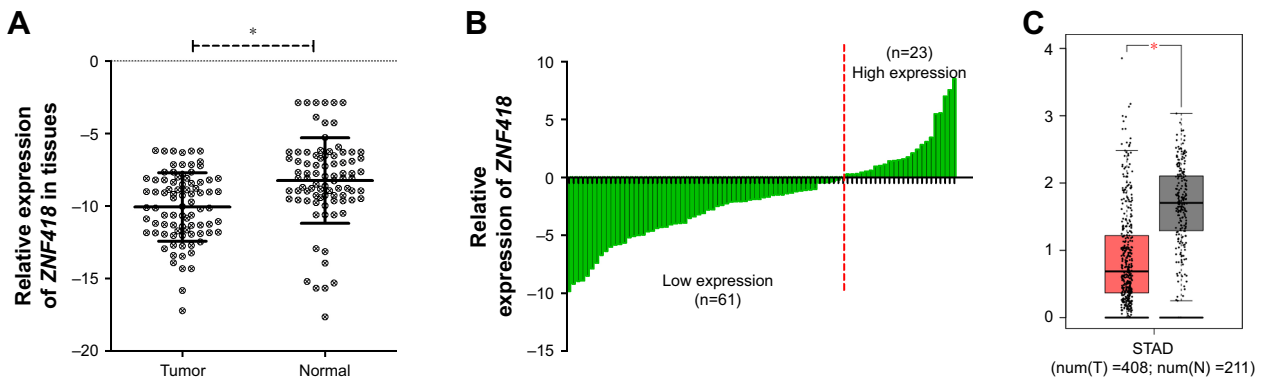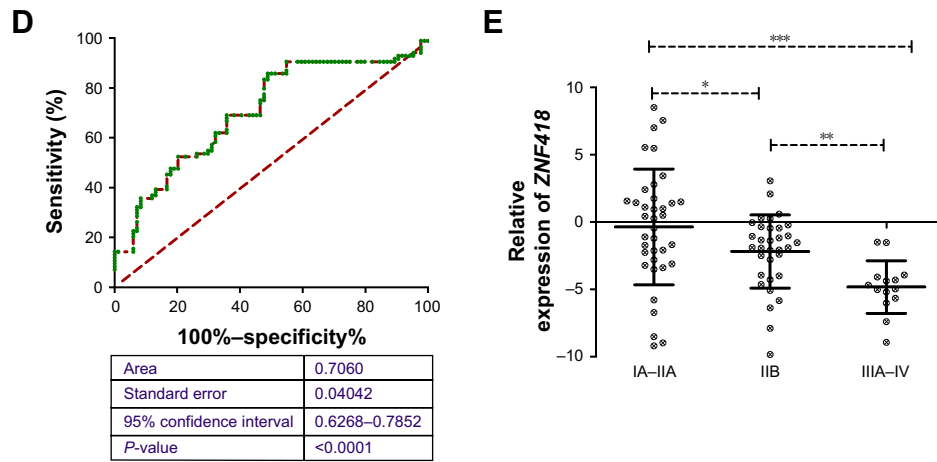

Figure 2 (*Continued*)

D

Area	0.7060
Standard error	0.04042
95% confidence interval	0.6268–0.7852
P-value	<0.0001

E

Figure 2 *ZNF418* was significantly downregulated in GC tissues compared to adjacent noncancerous tissues.
Notes: (**A**) *ZNF418* expression was downregulated in GC tissues. (**B**) The ratio of *ZNF418* low expression was 61/84 (72.6%), while the ratio of *ZNF418* high expression was 23/84 (27.4%). (**C**) The GEPIA database revealed that *ZNF418* expression was significantly downregulated in GC tissues. (**D**) The AUC was 0.7060. (**E**) Down-expression of *ZNF418* level was negatively associated with TNM. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.
Abbreviations: *ZNF418*, zinc-finger protein 418; GC, gastric cancer; TNM, tumor–node–metastasis; AUC, area under the ROC curve; ROC, receiver operating characteristic; STAD, stomach adenocarcinoma.

that the forecasts for both database sites were consistent except for PPP1R37 and POTEJ (Figure 4B).

Discussion

Early detection and disease progression monitoring remain the effective treatment against GC. Novel GC detection and

Table 1 The relationship between *ZNF418* expression levels ($-\Delta\Delta C_t$) in GC tissues and clinicopathological factors of patients

Variables	No of patients	Mean±SD	*P*-value
Age (years)			0.133
≥60	70	−2.09±3.64	
<60	14	−0.44±4.09	
Gender			0.261
Female	38	−2.33±3.73	
Male	46	−1.40±3.74	
Diameter (cm)			0.237
≥5	57	−2.15±3.53	
<5	27	−1.11±4.13	
Differentiation			0.293
Low–middle	69	−2.02±3.56	
High	15	−0.89±4.52	
T stage			0.000*
T1–T2	37	−0.35±4.30	
T3	33	−2.18±2.72	
T4a–T4b	14	−4.82±1.95	
Lymphatic metastasis			0.285
No	14	−2.80±4.42	
Yes	70	−1.62±3.60	
TNM stage			0.000*
IA–IIA	37	−0.35±4.30	
IIB	33	−2.18±2.72	
IIIA–IV	14	−4.82±1.95	

Note: *$P<0.05$.
Abbreviations: *ZNF418*, zinc-finger protein 418; GC, gastric cancer; TNM, tumor–node–metastasis.

monitoring methods are urgently needed because of current biomarkers, such as CA199 or CEA, lacking enough specificity. Zinc finger-containing transcription factors have been implicated as critical regulators of multiple cardiac-expressed genes and are thought to be important for various human diseases including GC and development. A previous study reported that ZNF143 was overexpressed in specimens of GC compared with adjacent normal tissues and ectopic overexpression of ZNF143 enhanced GC migration, whereas ZNF143 knockdown suppressed this effect in vitro. Moreover, the authors of that study found that ZNF143 enhanced GC cell migration by promoting the process of epithelial–mesenchymal transition (EMT) through the PI3K/AKT signaling pathway.[9] In addition to this, Chen et al[10] found
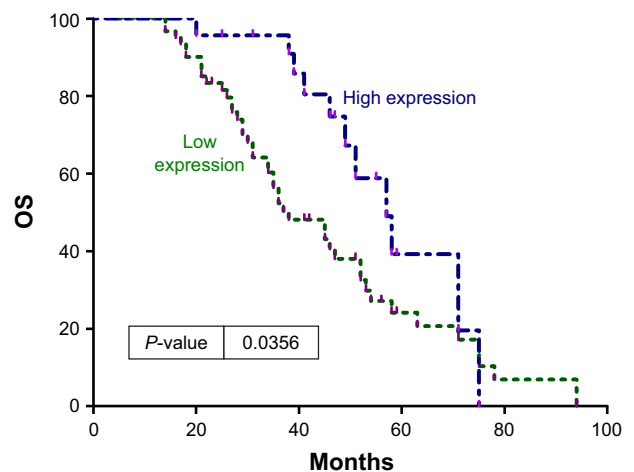

Figure 3 *ZNF418* was correlated with OS of patients with GC.
Abbreviations: *ZNF418*, zinc-finger protein 418; OS, overall survival; GC, gastric cancer.

Table 2 Cox regression analysis of factors associated with OS in GC

Factor	Hazard ratio	P-value	95% confidence interval	
			Lower limitation	Upper limitation
Gender	0.729	0.259	0.422	1.261
Age	1.511	0.228	0.773	2.954
Diameter	1.205	0.522	0.681	2.132
Differentiation	1.094	0.809	0.527	2.270
T stage	1.270	0.184	0.893	1.805
Lymphatic metastasis	1.837	0.054	0.321	1.224
TNM stage	2.213	0.000*	1.487	3.293
ZNF418 expression	0.500	0.043*	0.255	0.978

Note: *$P<0.05$.
Abbreviations: OS, overall survival; GC, gastric cancer; TNM, tumor–node–metastasis; ZNF418, zinc-finger protein 418.

that knockdown of zinc-finger E-box-binding homeobox 1 (ZEB1) expression by RNA interference can decrease lncRNA HOTAIR expression and restrain cell proliferation, invasion and migration in GC cells.

With regard to *ZNF418*, no relevant research on GC was found. However, Li et al[8] reported that overexpression of *ZNF418* in COS-7 cells inhibited the transcriptional activity of SRE and AP-1, which may be silenced by siRNA and may act as a negative regulator in MAPK signaling pathway. Pan et al[11] found that *ZNF418* was markedly downregulated in hearts of cardiac hypertrophy and hypertrophic primary cardiomyocytes and downregulated *ZNF418* exacerbated the myocyte size and fibrosis and moreover increased the mRNA levels of ANP, BNP, β-MHC, MCIP1.4, collagen 1a, collagen III, MMP-2 and fibronectin in hearts of AB-treated *ZNF418* knockout mice or Ang II-treated cardiomyocytes with *ZNF418*.

In our study, microarray revealed that *ZNF418* was involved in the progression and development of GC. The results showed that *ZNF418* expression was downregulated in GC plasma compared with normal cases with an AUC of 0.7025, which was consistent with the study of Pan et al.[11] Down-expression of *ZNF418* level was negatively associated with the TNM stage, which exactly matched the result of the microarray. Besides, qRT-PCR was used to measure its expression in 84 paired primary cancerous and adjacent noncancerous tissues from GC patients. The results showed that *ZNF418* expression was significantly downregulated than that in corresponding non-tumorous tissues. To further
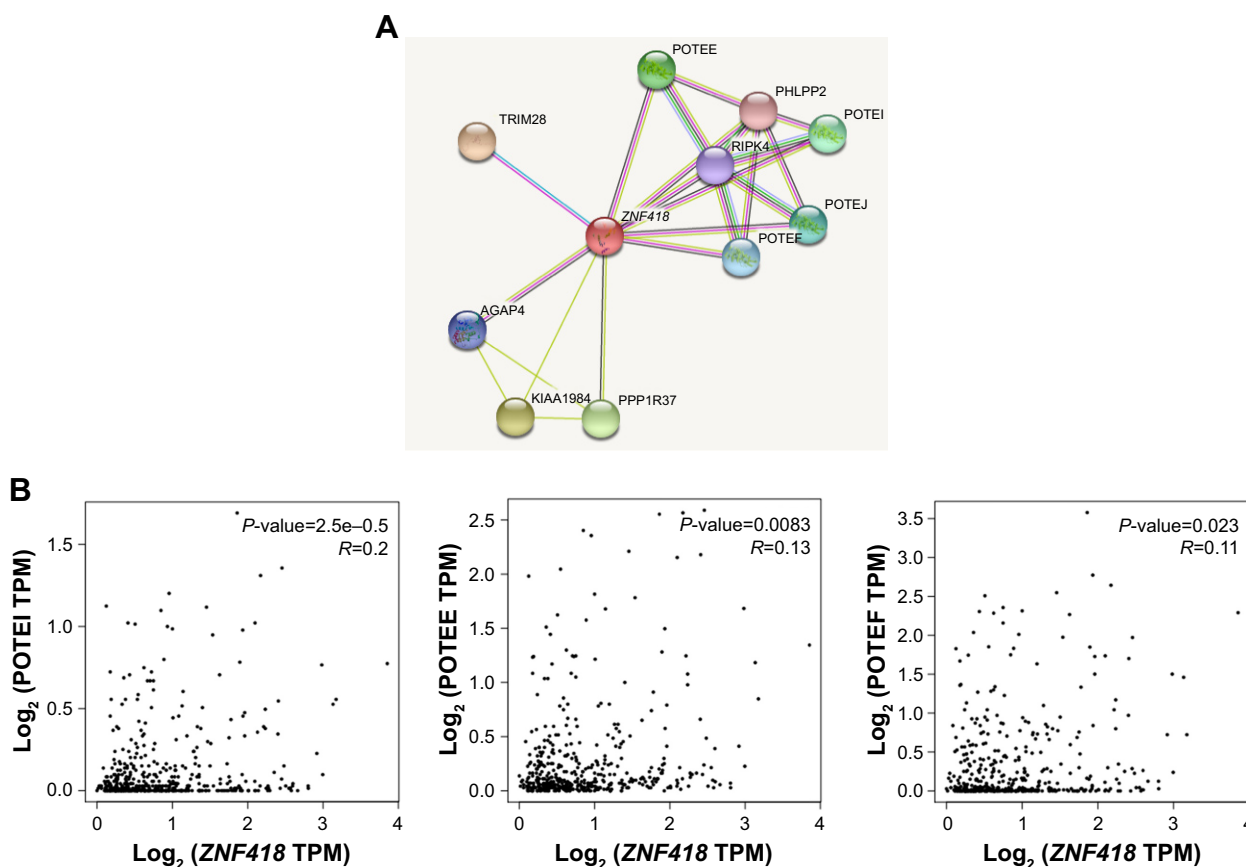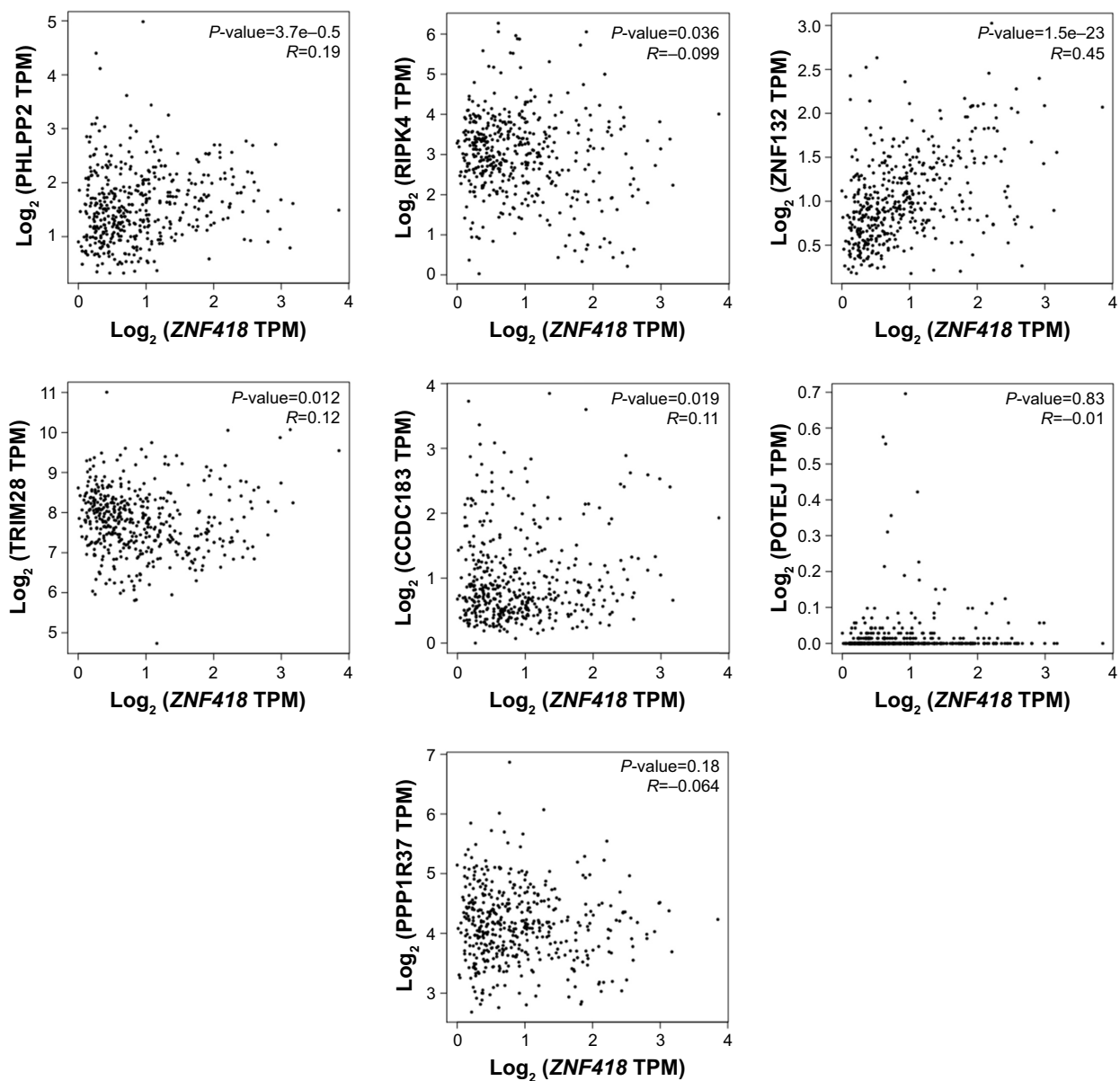

Figure 4 (*Continued*)

Figure 4 Network prediction and annotation of *ZNF418* with other molecules.
Notes: (**A**) Ten candidate mRNAs might have an interaction with *ZNF418*. (**B**) The GEPIA database revealed that PPP1R37 or POTEJ might not be associated with *ZNF418*.
Abbreviations: *ZNF418*, zinc-finger protein 418; TPM, transcripts per million.

confirm our results, we searched the *ZNF418* expression in the GEPIA database and found that the expression of *ZNF418* in 408 GC tissues was much lower than that in 211 noncancerous tissues, which was consistent with our findings. Moreover, clinical analysis indicated that *ZNF418* expression was significantly downregulated than that in corresponding non-tumorous tissues and down-expression of *ZNF418* level was negatively associated with the TNM stage as well. Kaplan–Meier survival curves indicated that patients with low *ZNF418* expression exhibited a greater probability of attaining shorter OS than those with high *ZNF418* expression. This result suggested the possible

association of *ZNF418* down-expression with poor clinical prognosis. In addition to *ZNF418* expression, tumor stage was also a significant predictor of OS. These results were supported further in Cox regression analysis, suggesting that the *ZNF418* may be involved in the invasion and progression of human GC. The current studies have provided evidences that *ZNF418* might serve as a novel biomarker for GC and was involved in GC development.

To identify the other molecules that might have an association with *ZNF418*, we performed network prediction and annotation of *ZNF418* with other molecules in the STRING database and further forecast in the GEPIA database. A total

of 10 candidate mRNAs showed a close interaction with *ZNF418* in the STRING database. However, when we subsequently looked them up in the GEPIA database one by one, we found that only eight mRNAs might be associated with *ZNF418* except for PPP1R37 and POTEJ. Overall, the predictive value in the database plays an important role in our understanding of the linkages between *ZNF418* and other molecules. Although the present study shows inconsistencies in the predictions of the two databases, most of the results are still consistent and valid. Multi-database predictions and joint analyses enhance the credibility of the conclusions and provide clues and directions for our follow-up studies.

Several limitations should not be ignored when interpreting the results. First, all blood samples did not correspond to tissue samples and if the follow-up study can be the two together, the research will be of greater significance. Second, all cases were from one hospital and our results were limited to one location. Therefore, more studies containing more locations of possible differences are needed for further study. Third, this study did not do more in-depth functional studies but drew conclusions only from data analysis. As a result, further biologic and functional evidence is needed to confirm the function of *ZNF418* in GC.

Conclusion

We first identified that *ZNF418* was significantly downregulated in GC. Our study indicated that *ZNF418* might serve as a novel biomarker for GC and was involved in gastric carcinoma development. However, the detailed molecular mechanism by which *ZNF418* contributed to GC proliferation, invasion and metastasis requires further research.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Torre LA, Bray F, Siegel RL, et al. Global cancer statistics, 2016. *CA Cancer J Clin*. 2016;66(1):7–30.
2. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136(5):E359–E386.
3. Pan L, He M, Ma J, et al. Phase and size controllable synthesis of NaYbF4 nanocrystals in oleic acid/ionic liquid two-phase system for targeted fluorescent imaging of gastric cancer. *Theranostics*. 2013;3(3):210–222.
4. Klug A, Schwabe JWR. Protein motifs 5. Zinc fingers. *FASEB J*. 1995;9(8):597–604.
5. Lander ES, Linton LM, Birren B, et al; International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860–921.
6. Dang DT, Pevsner J, Yang VW. The biology of the mammalian Kruppel-like family of transcription factors. *Int J Biochem Cell Biol*. 2000;32(11–12):1103–1121.
7. Nolte RT, Conlin RM, Harrison SC, Brown RS. Differing roles for zinc finger in DNA recognition: structure of a six-finger transcription factor IIIA complex. *Proc Natl Acad Sci U S A*. 1998;95(6):2938–2943.
8. Li Y, Yang D, Bai Y, et al. ZNF418, a novel human KRAB/C2H2 zinc finger protein, suppresses MAPK signaling pathway. *Mol Cell Biochem*. 2008;310(1–2):141–151.
9. Wei S, Wang L, Zhang L, et al. ZNF143 enhances metastasis of gastric cancer by promoting the process of EMT through PI3K/AKT signaling pathway. *Tumour Biol*. 2016;37(9):12813–12821.
10. Chen D, Chu Y, Zheng Q. Knock-down of ZEB1 inhibits the proliferation, invasion and migration of gastric cancer cells. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi [Chin J Cell Mol Immunol]*. 2017;33(8):1073–1078.
11. Pan L, Sheng M, Huang Z, et al. Zinc-finger protein 418 overexpression protects against cardiac hypertrophy and fibrosis. *PLoS One*. 2017;12(10):e0186635.