# Identification and verification of the prognostic value of the glutathione S-transferase Mu genes in gastric cancer

YEYANG CHEN[1*], BOPEI LI[1*], JUNFU WANG[1], JINLU LIU[1], ZHEN WANG[1], YUANTIAN MAO[1], SIYU LIU[1], XIWEN LIAO[2] and JUNQIANG CHEN[1]

Departments of [1]Gastrointestinal Surgery and [2]Hepatobiliary Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

Received October 9, 2019; Accepted June 23, 2020

DOI: 10.3892/ol.2020.11961

**Abstract.** Gastric cancer (GC) is one of the most frequently diagnosed gastrointestinal cancer types in the world. Novel prognostic biomarkers are required to predict the progression of GC. Glutathione S-transferase Mu (GSTM) belongs to a family of phase II enzymes that have been implicated in a number of cancer types. However, the prognostic value of the GSTM genes has not been previously investigated in GC. The Cancer Genome Atlas (TCGA) was used to evaluate mRNA expression levels of GSTMs in GC tissue samples. Overall survival (OS) rates, hazard ratios (HRs) and 95% CIs were calculated using the Cox logistic regression model and Kaplan-Meier (KM) analysis was performed. In addition, the KM plotter online database was used to validate mRNA expression and the prognostic value of GSMT family members in patients with GC. To predict the function of GSTM genes in these patients, several bioinformatics tools, including the Database for Annotation, Visualization and Integrated Discovery, gene multiple association network integration algorithm, Search Tool for the Retrieval of Interacting Genes/Proteins, Gene Set Enrichment Analysis (GSEA), nomogram and genome-wide co-expression analysis were used. In the present study, high expression of GSTM5 was indicated to be strongly associated with lower OS in patients with GC, according to the TCGA and KM plotter online databases (HR=1.47, 95% CI: 1.06-2.04, P=0.021; and HR=1.69, 95% CI: 1.42-2.01, P=1.6x10$^{-9}$, respectively). The results from the GSEA and genome-wide co-expression analysis indicated that GSTM5 expression associated with several biological process terms, including 'adhesion', 'angiogenesis', 'apoptotic process', 'cell growth', 'proliferation', 'migration', 'Hedgehog signaling', 'MAPK signaling' and the 'TGF-β signaling pathway'. In conclusion, the present results indicated that GSTM5 may serve as a biomarker for GC prognosis and may be a potential therapeutic target for GC.

*Correspondence to:* Dr Junqiang Chen, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning, Guangxi 530021, P.R. China
E-mail: gxhans@163.com

*Contributed equally

*Key words:* glutathione S-transferase Mu, The Cancer Genome Atlas, gastric cancer, prognosis, biomarker

## Introduction

According to Global Cancer Statistics for 2018, gastric cancer (GC) is the fifth most prevalent cancer type and the third leading cause of cancer-associated mortality worldwide (1). Of note, its prevalence is markedly elevated in Eastern Asia. The number of newly diagnosed GC cases in 2018 was 1,033,701 worldwide and the estimated number of deaths was 782,685, translating to 1 in every 12 deaths globally (1). Risk factors commonly associated with GC include chronic infection with *Helicobacter pylori* (*H. pylori*), environmental factors, low fruit and vegetable intake, consumption of preserved foods, smoking and alcohol use (2,3). At present, surgery and chemotherapy are the major therapeutic strategies used to treat GC (4). However, only a limited number of patients with GC are diagnosed at early stage, whereas the majority of patients are diagnosed at advanced stage (5). The 5-year overall survival (OS) rate for patients with GC was only 27.4% in China in 2010, and 29% in the United States in 2009 (6,7). Therefore, it is important to explore the molecular mechanisms involved in the tumorigenesis and progression of GC, which may identify novel prognostic biomarkers and treatment targets.

The glutathione S-transferase Mu (GSTM) gene family consists of five genes identified in humans that are numbered M1-5. These genes occur as a cluster on chromosome 1p13, which are arranged in tandem, spanning a 97-kb region, and encode one of eight distinct classes of glutathione transferases (8-10). The GSTM gene family is arranged in a 5'-GSTM4-M2-M1-M5-M3-3' sequence (9). These genes share a sequence homology of 60-80% (11). GSTM genes are generally recognized as detoxifying enzymes involved in the deactivating conversion of carcinogenic reactive metabolites, suggesting that these enzymes may have a role in carcinogenesis (12). To date, there is a lack of studies focusing on the value of the GSTM family of genes as prognostic biomarkers in GC.

To investigate the prognostic value and potential functions of GSTM genes in patients with GC, gene expression data and survival information from The Cancer Genome Atlas (TCGA) were analyzed. Subsequently, the Kaplan-Meier (KM) plotter online database was used to validate mRNA expression levels and the prognostic value of individual GSTM genes in patients with GC. Several bioinformatics tools were also used to explore the potential functions of GSTM genes.

## Materials and methods

*Functional and co-expression analyses*. To analyze the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of GSTM genes, the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8; (https://david.ncifcrf.gov/home.jsp; accessed March 1, 2018) was used (13,14). The functional examination based on GO includes the categories molecular function (MF), biological process (BP) and cellular component (CC). To evaluate gene-gene networks, the Gene Multiple Association Network Integration Algorithm (GeneMANIA) version 3.6.0 (http://www.genemania.org; accessed May 20, 2019), which predicted gene functions, was used (15,16). The Search Tool for the Retrieval of Interacting Genes (STRING) version 11.0 (https://string-db.org; accessed May 20, 2019) database was used to search and analyze protein-protein interaction (PPI) networks (17).

*Co-expression matrix*. A co-expression matrix of GSTM genes was constructed using mRNA expression data from TCGA cohort of GC tumor tissues. Pearson's correlation coefficient analysis was used to analyze mRNA co-expression correlations. The co-expression matrix was constructed using the corrplot package in the R 3.4.4 platform (18). $P<0.05$ was considered to indicate a statistically significant difference.

*TCGA*. RNA sequencing and clinical information (including tumor stage, age of patient and sex) linked with GC were downloaded from TCGA (https://portal.gdc.cancer.gov; accessed August 22, 2018). The TCGA data portal contained 407 patients diagnosed with GC, which included 375 tumor tissues and 32 adjacent normal tissues. After removing cases with missing follow-up profiles, a total of 351 patients with GC from TCGA were analyzed. Clinical data including clinical tumor-node-metastasis (TNM) stage (19), Lauren classification (20), differentiation grade, human epidermal growth factor receptor 2 (HER2) status and clinical treatment were also collected.

*Survival analysis*. KM survival analyses and log-rank tests were used to calculate the OS rate and significance. Patients with GC were separated into high- and low-expression groups of GSTM based on the median values of expression. To perform univariate and multivariate survival analyses, the Cox proportional hazards regression model was used to calculate the hazard ratio (HR), 95% confidence interval (CI) and log-rank P-values. $P<0.05$ was considered to indicate a statistically significant difference.

*KM plotter online database*. The associations between the mRNA levels of individual GSTM genes and OS rates were calculated using the KM plotter online database (http://kmplot.com/analysis/index.php?p=service&cancer=gastric) (21) based on gene expression data and survival information of 875 patients with GC downloaded from the Gene Expression Omnibus (datasets GSE14210, GSE15459, GSE22377, GSE29272, GSE51105 and GSE62254) (22). This tool was used for the identification and validation of survival biomarkers. In brief, GSTM1-5 were entered into the KM plotter online database and analyzed. Based on the median of mRNA expression for each GSTM according to the Gene Expression Omnibus, all GC patients were separated into two groups (high vs. low). Statistical parameters such as survival plot, HRs, 95% CIs and log-rank P-values were obtained from KM plotter. $P<0.05$ was considered to indicate a statistically significant difference.

*Nomogram and stratified analyses*. Based on the survival analysis of TCGA and KM plotter, only GSTM5 was significantly associated with prognosis. A nomogram was developed and used to evaluate the contribution of GSTM5 expression and prognostic clinical parameters, including sex, age and tumor stage, in GC OS. Based on prognostic clinical indicators and the survival analysis of the Cox regression model, sex, age, stage and GSTM5 levels were entered into the risk model. The points against each factor were counted, and 1-, 3- and 5-year survival rates were also calculated (23). The nomogram was constructed using the rms package (https://CRAN.R-project.org/package=rms) (24).

To assess the prognostic value of GSTM5 in different GC strata, a stratified analysis method was performed. The association between GSTM5 and OS in TCGA and KM plotter online database were stratified in the GC cohort for sex, age, clinical stage, Lauren classification, differentiation grade, clinical treatment and HER2 status.

*Gene Set Enrichment Analysis (GSEA)*. To investigate how the prognostic GSTM5 gene participates in GC, GSEA v.3.0 software (http://software.broadinstitute.org/gsea/msigdb/index.jsp) was used to identify the potential biological functions and signaling pathways associated with low vs. high expression levels of GSTM5. The Molecular Signatures Database of GSEA used the c2 (c2.cp.kegg.v6.2.symbols.gmt) and c5 (c5.all.v6.2.symbols.gmt) reference gene sets (25). A value of 1,000 was set as the number of permutations. $P<0.05$, normalized enrichment score >1 and false discovery rate <0.25 were considered to indicate a statistically significant difference.

*Genome-wide co-expression analysis of the prognostic GSTM5 gene and functional enrichment*. To assess gene-gene co-expression interaction of prognostic genes at the mRNA level, Pearson's correlation coefficient was calculated using the cor function on the R 3.4.4 platform. Significant differences were defined as $|r|>0.6$ and $P<0.05$. In addition, DAVID version 6.8 was used to determine the GO functional term and KEGG pathway enrichment of GSTM and its co-expressed genes.

*Statistical analyses*. All data were analyzed using SPSS version 25.0 software (IBM, Corp.). Vertical scatterplots and survival curves were generated using GraphPad Prism 7.0 software (GraphPad Software, Inc.). Vertical scatterplots were

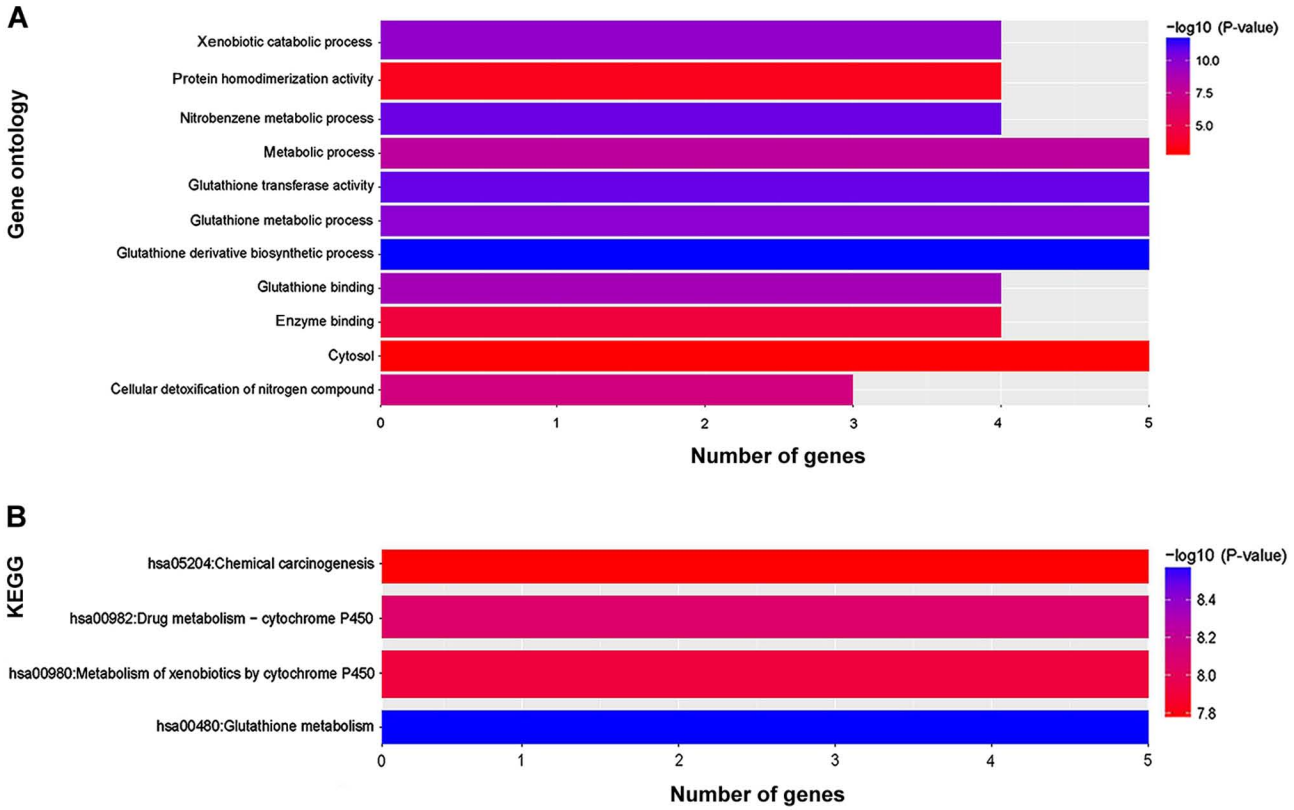


Figure 1. GO and KEGG analysis of GSTM genes. (A) GO enrichment analysis of GSTM genes. (B) KEGG enrichment analysis of GSTM genes. GSTM, glutathione S-transferase Mu; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization and Integrated Discovery; hsa, *Homo sapiens*.

analyzed using independent t-tests. In addition, nomograms and correlation plots were generated using R software.

## Results

*GSTM family functional enrichment and co-expression analysis*. To evaluate the biological functions of GSTM genes, GO functional terms were determined in the categories BP, MF and CC and a KEGG pathway analysis was performed using DAVID (Fig. 1). GO analysis indicated that genes of the GSTM family were enriched in 'protein homodimerization activity', 'enzyme binding' and in the 'cytosol' (Fig. 1A). The results from the KEGG analysis suggested that the functions of the GSTM gene family were enriched in 'chemical carcinogenesis', 'metabolism of xenobiotics by cytochrome P450' and 'drug metabolism-cytochrome P450' (Fig. 1B). Gene-gene interaction networks of GSTM genes are presented in Fig. 2A, which revealed that GSTM1-5 were co-expressed. In addition, the GSTM genes were associated with other genes with the relationship of predicted interactions, physical interactions, co-expression, and shared pathway. Based on the information in the STRING database, PPI interaction networks revealed that GSTM family members were directly and indirectly connected to one another (Fig. 2B). The PPI network revealed that GSTM1 was only associated with GSTM2, and GSTM2 was the only gene that associated with all other family members. In addition, co-expression of the GSTM genes was observed in GC tissues, although the correlation coefficients appear to be weak (<0.4) (Fig. 3).

*Survival analysis*. Significant differences were obtained in the vertical scatterplots between high and low expression of GSTM genes obtained from TCGA (all P<0.001; Fig. 4). A survival analysis comparing patients with GC with different expression levels of GSTM is presented in Fig. 5. High GSTM5 expression was significantly associated with a worse prognosis for patients with GC (HR=1.47, 95% CI: 1.06-2.04, P=0.021). A significant association between high GSTM4 expression and favorable OS was observed (HR=0.63, 95% CI: 0.45-0.87, P=0.006).

Multivariate survival analysis was also performed to investigate the prognostic value of GSTM in GC. Age and tumor stage were two factors identified to be significantly associated with prognosis, as an age of ≥65 years and advanced stages were significantly associated with a worse OS (HR=1.56, 95% CI: 1.12-2.17, P=0.011; HR=1.92, 95% CI: 1.37-2.70, P<0.001, respectively; Table I). To examine survival, tumor stage and age were analyzed using the multivariate Cox proportional hazards regression model. These analyses revealed that high GSTM5 expression was significantly associated with worse OS (HR=1.48, 95% CI: 1.05-2.08, adjusted P=0.027; Table II). These results were consistent with those of the univariate survival analysis (Table II). However, no significant differences were identified for GSTM4 in the multivariate survival analysis (Table II), which was not consistent with the results obtained in the univariate survival analysis (HR=0.63, 95% CI: 0.45-0.87, P=0.006; Table II and Fig. 5D).

*Validation of the GSTM cohort using the KM plotter online database*. The GSTM cohort was validated using the KM

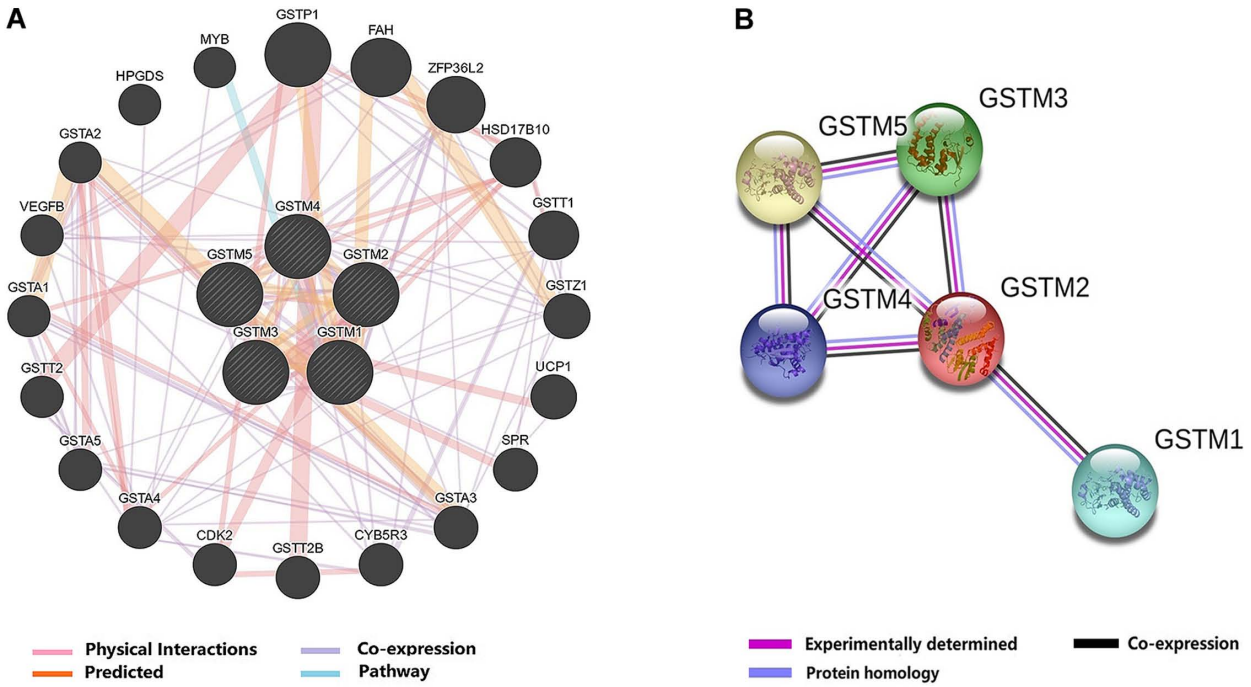


Figure 2. Gene and protein interaction networks of GSTM genes. (A) GSTM genes multiple association network integration algorithm. The size of the circle represents the strength of the co-expression. (B) Protein-protein interaction networks. GSTM, glutathione S-transferase Mu.

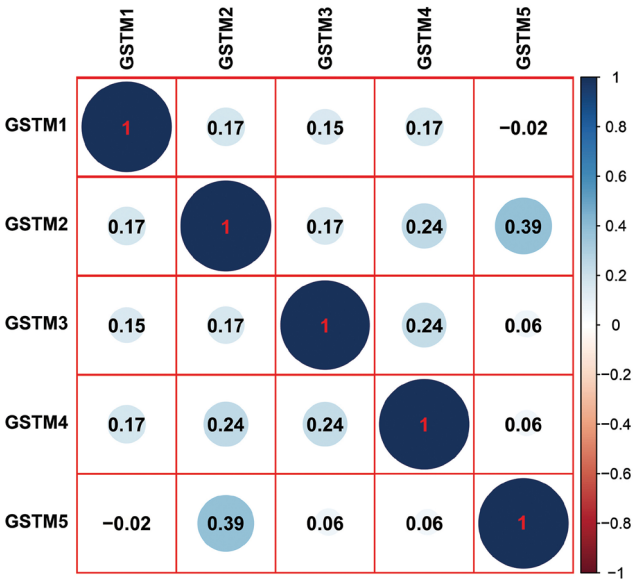


Figure 3. Co-expression heatmap of GSTM genes. The numbers presented are the r-values (Pearson correlation coefficients). The size of the circle represents the strength of the correlation. GSTM, glutathione S-transferase Mu.

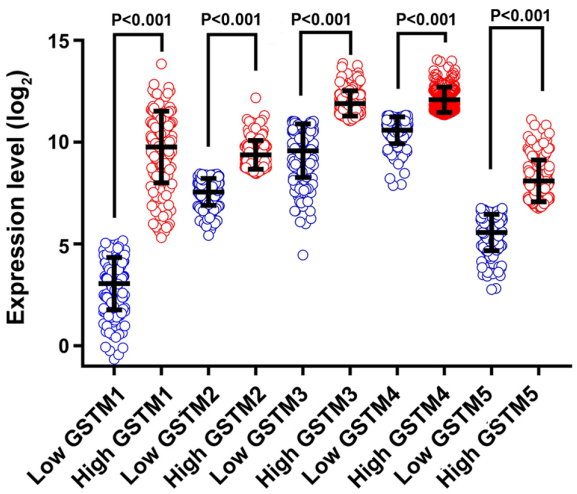


Figure 4. Scatterplots for GSTM gene family expression levels in The Cancer Genome Atlas. Red, high expression; blue, low expression. GSTM, glutathione S-transferase Mu.

plotter online database. KM curves of GSTM genes are presented in Fig. 6. These analyses revealed that high GSTM1, GSTM2, GSTM4 and GSTM5 mRNA levels were associated with a significantly worse OS for patients with GC (HR=1.44, 95% CI: 1.21-1.71, P=2.6x10$^{-5}$; HR=1.57, 95% CI: 1.32-1.86, P=2x10$^{-7}$; HR=1.23, 95% CI: 1.04-1.46, P=0.015; and HR=1.69, 95% CI: 1.42-2.01, P=1.6x10$^{-9}$, respectively). The only result consistent with the TCGA analyses was that GSTM5 was significantly associated with a worse prognosis (HR=1.47, 95% CI: 1.06-2.04, P=0.021; Table II and Fig. 5E).

*Nomogram and stratified analysis*. A nomogram for GC was developed based on GSTM5 expression levels, sex, age, tumor stage and 1-, 3- and 5-year survival rate (Fig. 7). The 1-, 3- and 5-year survival rates were higher for patients with a lower number of total points compared with those with a higher number of total points. In accordance with the nomogram, it was observed that the contribution of GSTM5 expression in prognosis prediction was lower compared with that of age and tumor stage, but higher compared with that of sex. The nomogram analyses revealed that GSTM5 contributes to the prognosis prediction for patients with GC. In addition, the prognostic value of GSTM5 in GC was analyzed using stratification analysis and the association between GSTM5

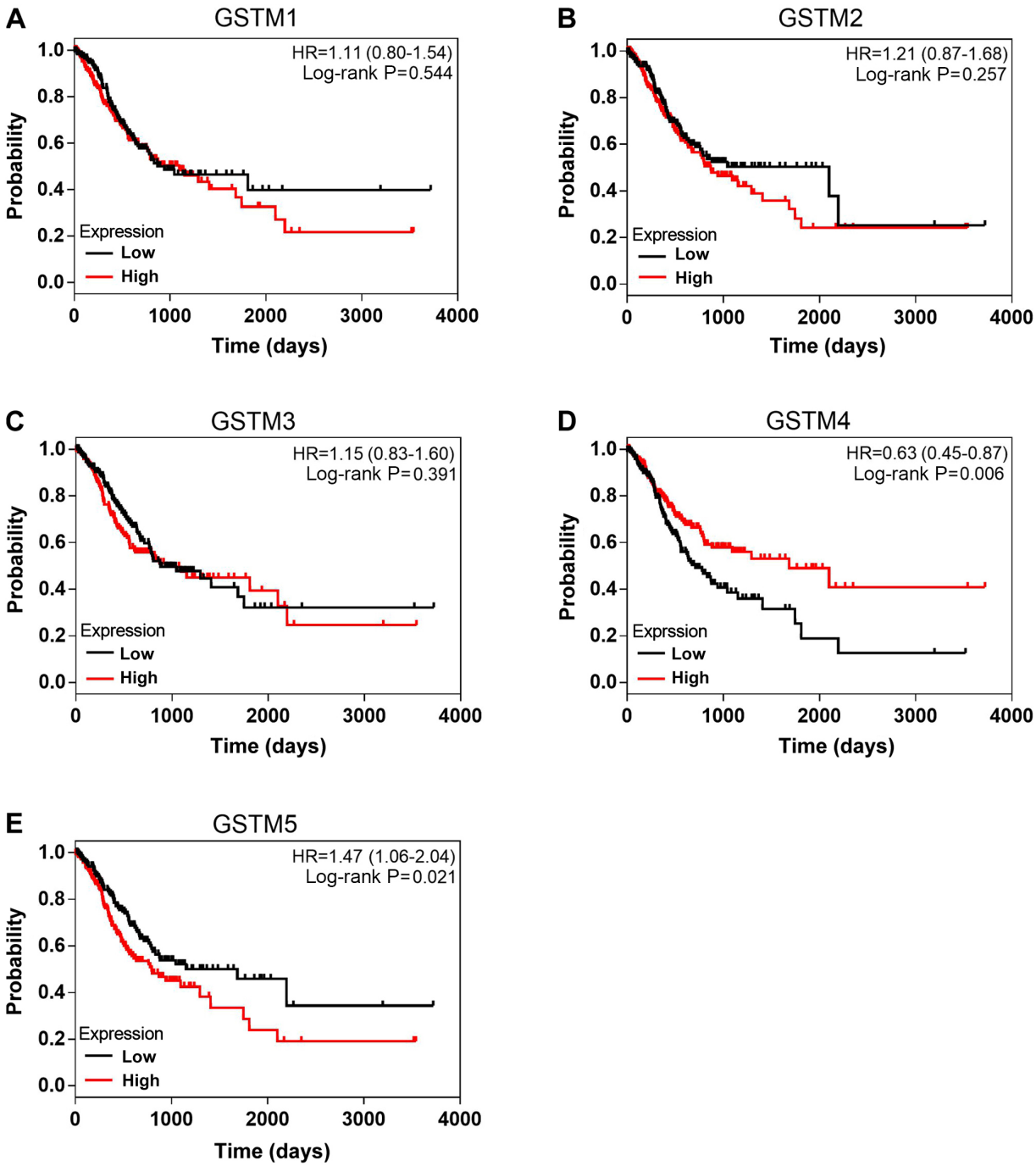


Figure 5. Prognostic graphs illustrating the impact of GSTM expression on overall survival in The Cancer Genome Atlas. Kaplan-Meier survival curves for patients with gastric cancer according to median expression of GSTM1-5 (n=351). (A) Survival curves of GSTM1. (B) Survival curves of GSTM2. (C) Survival curves of GSTM3. (D) Survival curves of GSTM4. (E) Survival curves of GSTM5.GSTM, glutathione S-transferase Mu; HR, hazard ratio (95% CI).

and OS was analyzed using the TCGA (Table III) and KM plotter online database (Table IV) GC cohorts, which included analyses for sex, age, clinical stage, Lauren classification, differentiation grade, clinical treatment and HER2 status. As presented in Table III, high GSTM5 mRNA levels were significantly associated with poor prognosis in patients with GC who were males and <65 years old based on the TCGA GC cohort (HR=1.59, 95% CI: 1.07-2.36, P=0.020; HR=1.86, 95% CI: 1.07-3.25, P=0.030, respectively). As presented in Table IV, high GSTM5 mRNA levels were significantly associated with worse survival for both female and male patients with GC, clinical stages I, II and III, all Lauren classifications, treatment by surgery and other adjuvant therapies, as well as a negative HER2 status, according to the KM plotter analysis.

*GSEA analysis of GSTM5 in GC cases*. To explore the mechanisms underlying GSTM5 function, GSEA was used to assess differences in relative GSTM5 expression levels among TCGA specimens (Fig. 8, Tables SI and SII). In the GSEA, high levels of GSTM5 were significantly enriched in the following processes: 'regulation of cell matrix adhesion' (P<0.001), 'angiogenesis' (P<0.001), 'regulation of cell growth'

Table I. Demographic and clinical data in The Cancer Genome Atlas gastric cancer cohort (n=351).

| Variable | N | Events, n (%) | MST, days | HR (95% CI) | Log-rank P-value |
|---|---|---|---|---|---|
| Sex | | | | | 0.152 |
| Male | 226 | 99 (43.8) | 869 | Ref. | |
| Female | 125 | 44 (35.2) | 1,043 | 0.77 (0.55-1.09) | |
| Age (years) | | | | | 0.011 |
| <65 | 148 | 50 (33.8) | 1,811 | Ref. | |
| ≥65 | 197 | 92 (46.7) | 779 | 1.56 (1.12-2.17) | |
| NA | 6 | | | | |
| Tumor stage | | | | | <0.001 |
| Early (I+II) | 156 | 44 (28.2) | 1,811 | Ref. | |
| Advanced (III+IV) | 180 | 89 (49.4) | 675 | 1.92 (1.37-2.70) | |
| NA | 15 | | | | |
| Tumor stage | | | | | <0.001 |
| I | 47 | 11 (23.4) | 2,197 | Ref. | |
| II | 109 | 33 (30.3) | 1,686 | 1.55 (0.78-3.08) | |
| III | 145 | 67 (46.2) | 782 | 2.38 (1.26-4.51) | |
| IV | 35 | 22 (62.9) | 476 | 3.81 (1.85-7.86) | |
| NA | 15 | | | | |

MST, median survival time; HR, hazard ratio; Ref., reference; NA, not available.

Table II. Analysis of the association between GSTM genes and the risk of death in The Cancer Genome Atlas gastric cancer cohort (n=351).

| Gene expression status | Median expression | N | Events, n (%) | MST, days | Crude HR (95% CI) | Crude P-value | Adjusted HR[a] (95% CI) | Adjusted P-value[a] |
|---|---|---|---|---|---|---|---|---|
| GSTM1 | 36.0 | | | | | 0.544 | | 0.877 |
| Low | | 176 | 68 (38.6) | 869 | Ref. | | Ref. | |
| High | | 175 | 75 (42.9) | 1,095 | 1.11 (0.80-1.54) | | 0.97 (0.69-1.38) | |
| GSTM2 | 349.7 | | | | | 0.257 | | 0.263 |
| Low | | 176 | 66 (37.5) | 2,100 | Ref. | | Ref. | |
| High | | 175 | 77 (44.0) | 869 | 1.2 (0.87-1.68) | | 1.22 (0.86-1.71) | |
| GSTM3 | 2105.3 | | | | | 0.391 | | 0.247 |
| Low | | 176 | 70 (39.8) | 881 | Ref. | | Ref. | |
| High | | 175 | 73 (41.7) | 940 | 1.15 (0.83-1.60) | | 1.23 (0.87-1.74) | |
| GSTM4 | 2570.1 | | | | | 0.006 | | 0.067 |
| Low | | 176 | 84 (47.7) | 712 | Ref. | | Ref. | |
| High | | 175 | 59 (33.7) | 1,686 | 0.63 (0.45-0.87) | | 0.72 (0.50-1.02) | |
| GSTM5 | 107.5 | | | | | 0.021 | | 0.027 |
| Low | | 176 | 64 (36.4) | 1,153 | Ref. | | Ref. | |
| High | | 175 | 79 (45.9) | 794 | 1.47 (1.06-2.04) | | 1.48 (1.05-2.08) | |

[a]Adjusted for age and tumor stage. GSTM, glutathione S-transferase Mu; MST, median survival time; HR, hazard ratio; Ref., reference.

(P<0.001), 'regulation of endothelial cell apoptotic process' (P=0.004), 'extracellular matrix' (P<0.001), 'smoothened signaling pathway' (P<0.001), 'Hedgehog signaling pathway' (P<0.001), 'mitogen-activated protein kinases (MAPK) signaling pathway' (P<0.001), 'transforming growth factor (TGF)-β signaling pathway' (P=0.025), 'calcium signaling pathway' (P<0.001) and other KEGG pathways associated with cancer.

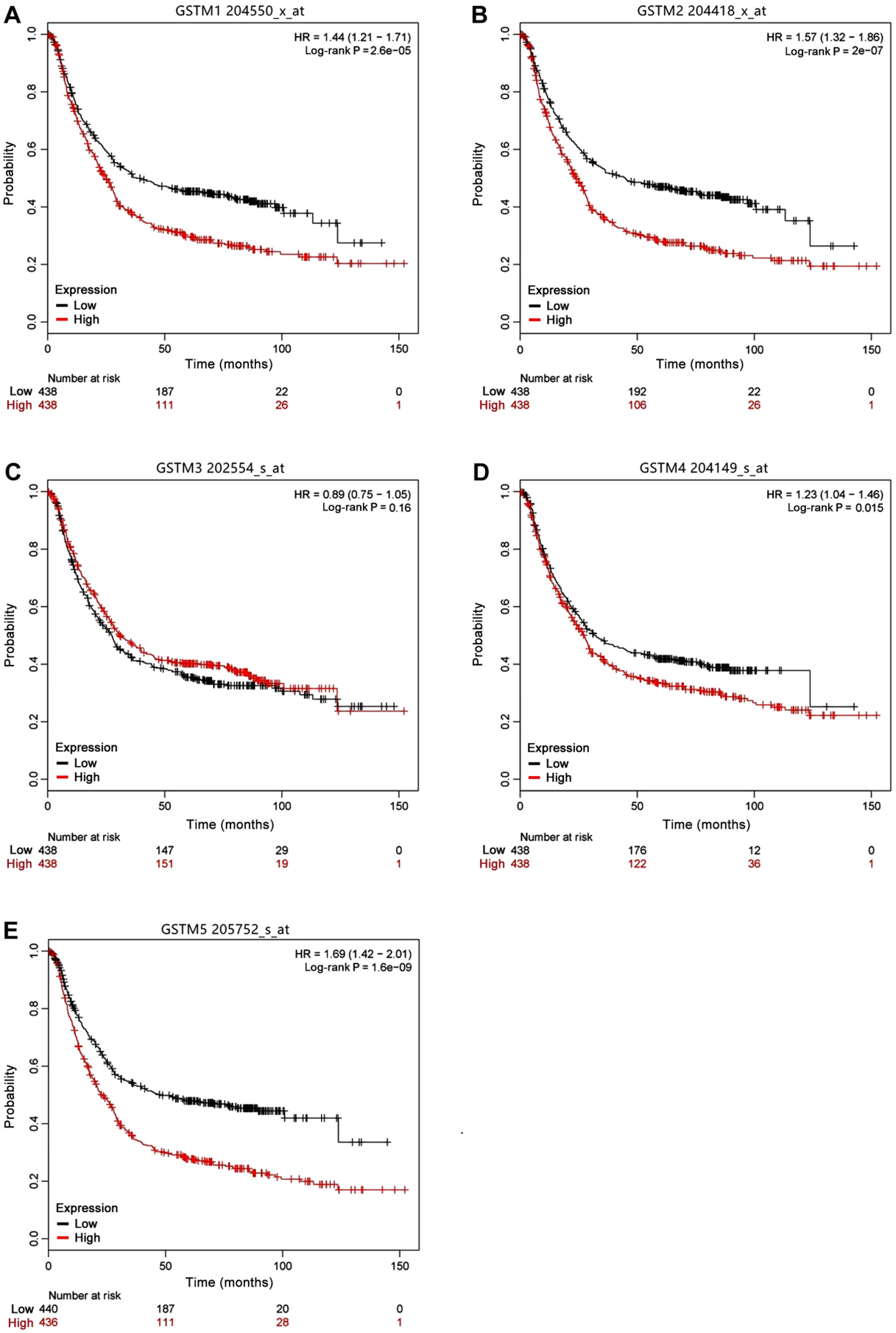


Figure 6. Prognostic graphs of GSTM median expression for overall survival generated using the Kaplan-Meier plotter online database. (A) Survival curves of GSTM1. (B) Survival curves of GSTM2. (C) Survival curves of GSTM3. (D) Survival curves of GSTM4. (E) Survival curves of GSTM5. GSTM, glutathione S-transferase Mu; HR, hazard ratio (95% CI).

Table III. Stratified analysis of the association between GSTM5 expression levels and overall survival in The Cancer Genome Atlas gastric cancer cohort (n=351).

| Variable | Total, n | Low GSTM5, n | High GSTM5, n | HR (95% CI) | Log-rank P-value |
|---|---|---|---|---|---|
| Sex | | | | | |
| Male | 226 | 113 | 113 | 1.59 (1.07-2.36) | 0.020 |
| Female | 125 | 63 | 62 | 1.06 (0.58-1.91) | 0.860 |
| Age, years | | | | | |
| <65 | 148 | 74 | 74 | 1.86 (1.07-3.25) | 0.030 |
| ≥65 | 197 | 99 | 98 | 1.11 (0.74-1.688) | 0.603 |
| NA | 6 | | | | |
| Stage | | | | | |
| Early | 156 | 78 | 78 | 1.48 (0.82-2.68) | 0.189 |
| Advanced | 180 | 90 | 90 | 1.34 (0.88-2.04) | 0.164 |
| NA | 15 | | | | |
| Stage | | | | | |
| I | 47 | 24 | 23 | 1.31 (0.40-4.29) | 0.651 |
| II | 109 | 55 | 54 | 1.52 (0.77-3.02) | 0.217 |
| III | 145 | 73 | 72 | 1.49 (0.92-2.42) | 0.098 |
| IV | 35 | 18 | 17 | 1.15 (0.49-2.68) | 0.744 |
| NA | 15 | | | | |

GSTM5, glutathione S-transferase Mu 5; HR, hazard ratio; NA, not available.

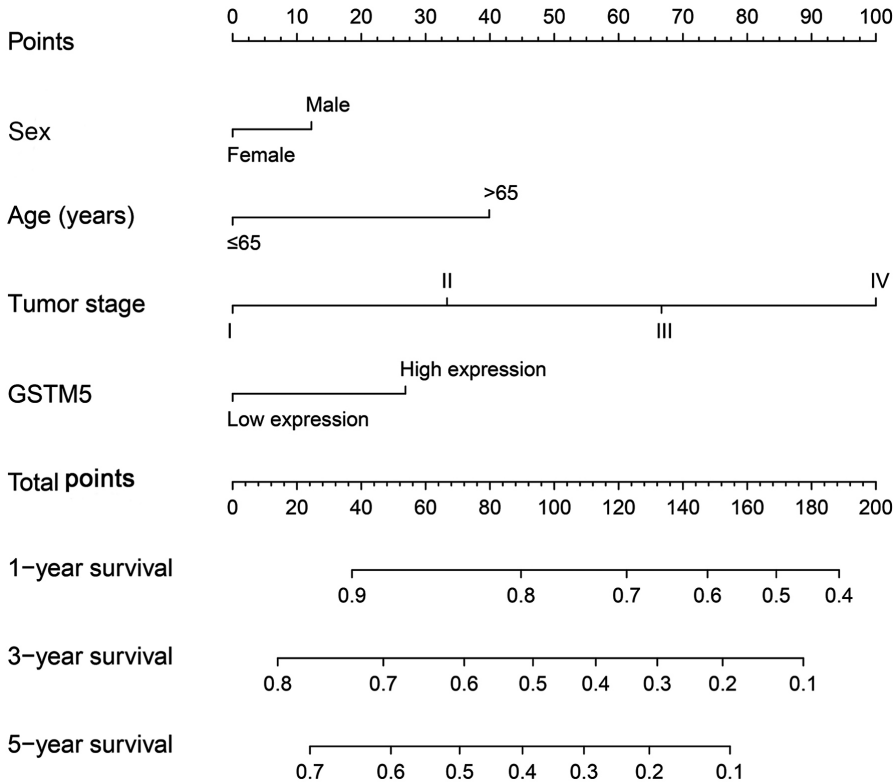


Figure 7. Nomogram for the association between clinicopathological data and risk score. GSTM, glutathione S-transferase Mu.

*Genome-wide co-expression analysis of GSTM5 in GC and potential functional enrichment*. To determine the potential mechanism underlying GSTM5 function in GC, genome-wide co-expression analysis was performed. Regulatory networks of GSTM5 and its co-expressed correlated genes identified in GC tumor tissues from the TCGA cohort are presented

Table IV. Stratified analysis of the association between GSTM5 expression levels and overall survival in the Kaplan-Meier plotter gastric cancer cohort (n=875).

| Variable | Total, n | Low GSTM5, n | High GSTM5, n | HR (95% CI) | Log-rank P-value |
|---|---|---|---|---|---|
| Sex | | | | | |
| Female | 236 | 120 | 116 | 1.87 (1.31-2.68) | <0.001 |
| Male | 544 | 275 | 269 | 1.84 (1.48-2.29) | $2.4x10^{-8}$ |
| NA | 95 | | | | |
| Stage | | | | | |
| I | 67 | 34 | 33 | 3.02 (0.96-9.55) | 0.048 |
| II | 140 | 71 | 69 | 2.20 (1.17-4.15) | 0.012 |
| III | 305 | 152 | 153 | 1.47 (1.11-1.96) | 0.008 |
| IV | 148 | 74 | 74 | 1.41 (0.96-2.08) | 0.075 |
| NA | 215 | | | | |
| Lauren classification | | | | | |
| Intestinal | 320 | 161 | 159 | 2.42 (1.74-3.36) | $7.1x10^{-8}$ |
| Diffuse | 241 | 121 | 120 | 1.95 (1.37-2.76) | <0.001 |
| Mixed | 32 | 16 | 16 | 4.26 (1.34-13.55) | 0.007 |
| NA | 282 | | | | |
| Differentiation | | | | | |
| Poor | 165 | 82 | 83 | 0.71 (0.47-1.05) | 0.085 |
| Moderate | 67 | 34 | 33 | 1.01 (0.52-1.94) | 0.978 |
| Well | 32 | 16 | 16 | 2.04 (0.84-4.94) | 0.106 |
| NA | 611 | | | | |
| Treatment | | | | | |
| Surgery alone | 380 | 190 | 190 | 1.81 (1.35-2.43) | $5.9x10^{-5}$ |
| 5-Fu based adjuvant | 152 | 76 | 76 | 0.86 (0.61-1.22) | 0.389 |
| Other adjuvant | 76 | 38 | 38 | 7.37 (2.15-25.21) | <0.001 |
| NA | 267 | | | | |
| HER2 status | | | | | |
| Negative | 532 | 266 | 266 | 1.93 (1.53-2.44) | $1.3x10^{-8}$ |
| Positive | 343 | 172 | 171 | 1.22 (0.94-1.58) | 0.139 |

GSTM5, glutathione S-transferase Mu 5; HR, hazard ratio; NA, not available; HER2, human epidermal growth factor receptor 2; 5-Fu, fluorouracil.

in Fig. 9 and Table SIII. GO analysis suggested that GSTM5 and its co-expressed genes were significantly enriched in processes including 'cell adhesion' ($P=1.97x10^{-5}$), 'single organismal cell-cell adhesion' ($P=3.83x10^{-5}$), 'focal adhesion' ($P=3.24x10^{-4}$), 'positive regulation of cell-substrate adhesion' (P=0.003), 'angiogenesis' (P=0.004), 'apoptotic process involved in luteolysis' (P=0.048), 'negative regulation of cell growth' ($P=2.75x10^{-5}$), 'negative regulation of cell proliferation' ($P=8.51x10^{-5}$), 'negative regulation of cell migration' ($P=8.89x10^{-4}$) and 'negative regulation of Wnt signaling pathway' (P=0.010). Enrichment of GSTM5 and its co-expressed genes in the 'plasma membrane' (P=0.002) and 'extracellular matrix' ($P=1.88x10^{-10}$) was observed by GO cell component analysis (Tables V and SIV). Furthermore, GSTM5 and its co-expressed genes were enriched in the following KEGG pathways associated with prognosis: 'Cyclic guanosine monophosphate-protein kinase G (cGMP-PKG) signaling pathway' ($P=3.04x10^{-9}$), cyclic adenosine monophosphate (cAMP) signaling pathway' (P=0.004), 'calcium signaling pathway' (P=0.008), 'focal adhesion' (P=0.017) and 'cell adhesion molecules (CAMs)' (P=0.026) (Table VI).

## Discussion

In the present study, the expression levels of GSTM genes and their prognostic value in GC were assessed. A positive correlation was observed between GSTM5 expression and poor OS in GC. In addition, GSEA analysis and genome-wide co-expression analysis were used to predict potential mechanistic roles of GSTM5 in GC.

There are five members of the GSTM class of genes that encode phase II metabolic enzymes found primarily in the cytosol that co-operate with phase I enzymes in carcinogen metabolism (26). These genes are involved in the detoxification of electrophilic compounds, including carcinogens, therapeutic

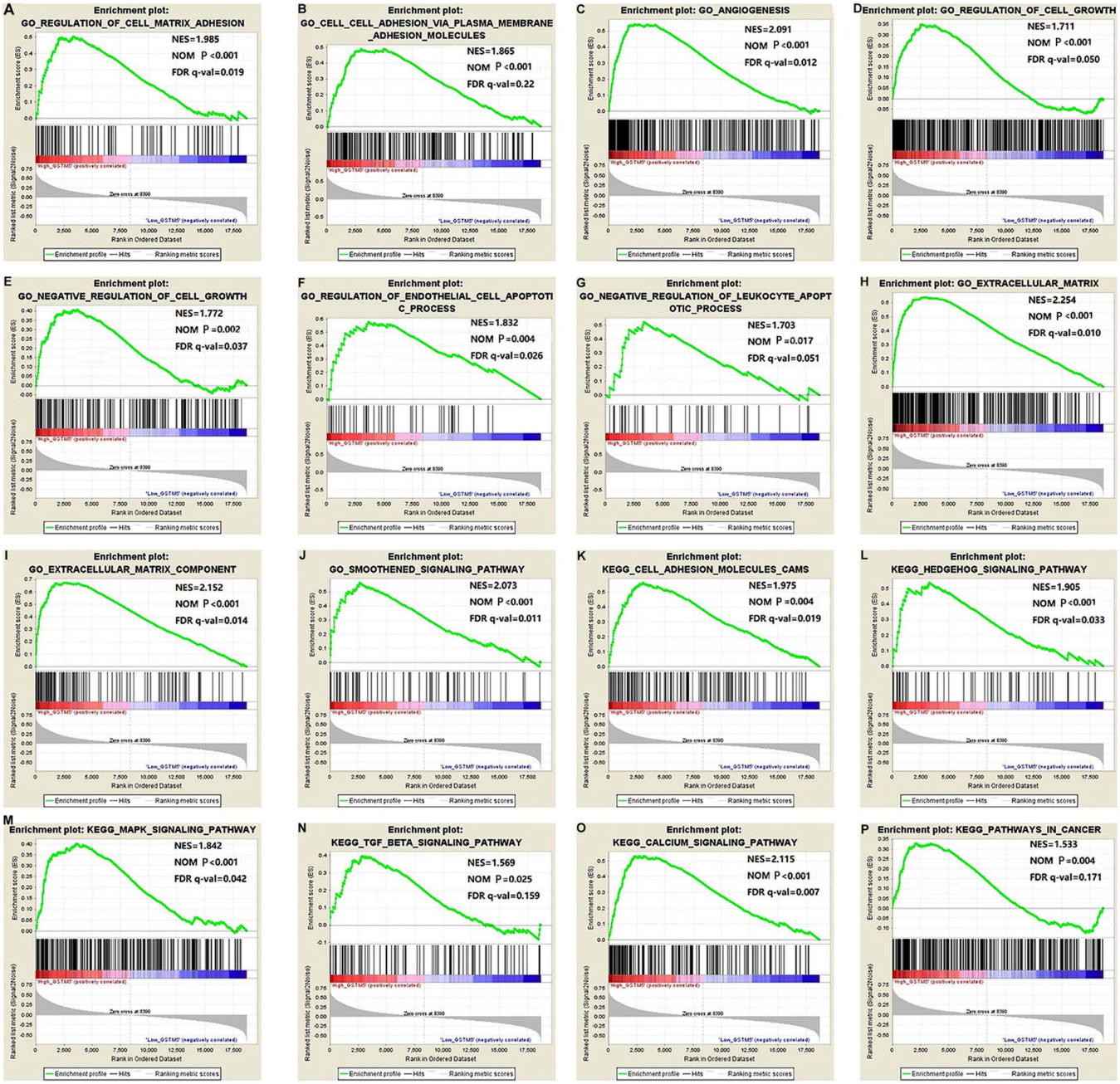


Figure 8. Gene set enrichment analysis results of c2 and c5 reference gene sets for higher expression of GSTM5 in gastric cancer tissues. (A) Regulation of cell matrix adhesion. (B) Cell-cell adhesion via plasma membrane adhesion molecules. (C) Angiogenesis. (D) Regulation of cell growth. (E) Negative regulation of cell growth. (F) Regulation of endothelial cell apoptotic process. (G) Negative regulation of leukocyte apoptotic process. (H) Extracellular matrix. (I) Extracellular matrix component. (J) Smoothened signaling pathway. (K) Cell adhesion molecule. (L) Hedgehog signaling pathway. (M) MAPK signaling pathway. (N) TGF-β signaling pathway. (O) Calcium signaling pathway. (P) Pathways in cancer. GSTM, glutathione S-transferase Mu; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate.

drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione (27).

Previous studies revealed that the GSTM family of genes have critical roles in several cancer types. GSTM1 is highly polymorphic in humans and is associated with multiple cancer types, such as bladder and breast cancer, metabolic disorders and autoimmune diseases, as well as anticancer drugs response and resistance (28) GSTM1 deletion in humans was indicated to have a key role in bladder (29) and breast cancer (30,31), multiple sclerosis (32), severe early-onset mental disorders including schizophrenia-spectrum disorder, bipolar disorder with psychotic symptoms or first-episode psychosis (33) and acute myeloid leukemia (34). Csejtei *et al* (35) identified that GSTM1 may be a prognostic biomarker in clinical diagnostics as well as a potential therapeutic candidate in colorectal cancer. A recent study detected null GSTM1 genotypes in the tumor area of GC, in contrast to the presence of both genes in the proximal and distal margins of the tumor (36). Therefore, GSTM1 polymorphisms may be a potential prognostic marker for certain types of cancer. A

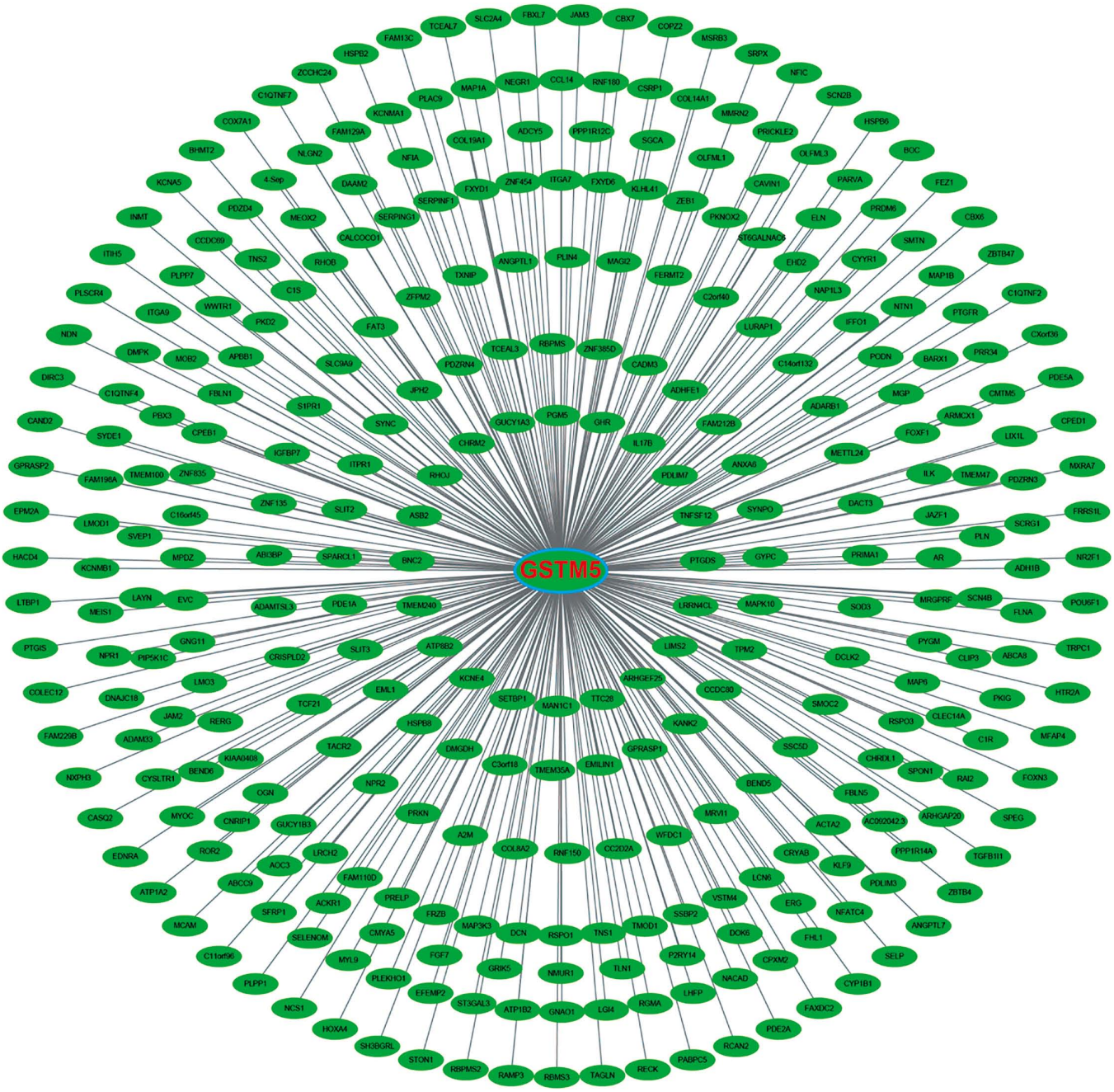


Figure 9. Regulation network of GSTM5 and its co-expressed genes in gastric cancer tumor tissue in The Cancer Genome Atlas cohort. GSTM5, glutathione S-transferase Mu 5.

protective effect of GSTM2 on oxidative stress was identified in studies performed in hepatic carcinoma, colon cells and spontaneously hypertensive rat (37-39). Previous studies have revealed an association between GSTM3 and certain cancer types, including laryngeal (40), oral (41,42), esophageal (43), breast (44), bladder (45), multiple cutaneous basal cell (46) cancer and childhood acute lymphoblastic leukemia (12). GSTM4 is a less studied member of the GSTM family and has been demonstrated to recognize the same standard glutathione S-transferases substrate 1-chloro-2,4-dinitrobenzen as other GSTMs, only with lower specific activity (47). The possible role of GSTM5 in the development of cancer warrants further exploration. Peng *et al* (48) reported that GSTM5 is involved in the detoxification of reactive electrophiles and is associated with Barrett's adenocarcinoma. Pankratz *et al* (49) observed that high-risk tag single-nucleotide polymorphisms in GSTM5 and ATP binding cassette subfamily C member 4 genes may be a good combined predictor of mortality in low-stage non-small cell lung cancer. Gene-based analysis also revealed that the expression of GSTM5 was involved in the OS of patients diagnosed with low-stage non-small cell lung cancer (49). Similar results were observed in a study by Kap *et al* (50), reporting that GSTM5 was differentially expressed in colon cancer tissues compared with normal colon tissues. In addition, high expression of GSTM5 is associated with poor OS in patients with colorectal cancer treated with oxaliplatin (HR=1.50, 95% CI: 1.03-2.19) (50). These conclusions suggested that GSTM5 may be an important prognostic

Table V. GO term enrichments of co-expressed genes of glutathione S-transferase Mu 5 in gastric cancer.

| Term | Count | P-value | Genes |
|---|---|---|---|
| GO:0007155~cell adhesion | 22 | $1.97 \times 10^{-5}$ | SELP, SVEP1, CYP1B1, ATP1B2, MPDZ, IGFBP7, MCAM, EMILIN1, ITGA9, PGM5, SRPX, S1PR1, COL19A1, ITGA7, RHOB, TGFB1I1, MFAP4, BOC, PARVA, FEZ1, AOC3, SPON1 |
| GO:0016337~single organismal cell-cell adhesion | 10 | $3.83 \times 10^{-5}$ | COL14A1, LIMS2, COL19A1, FOXF1, PIP5K1C, NLGN2, JAM2, COL8A2, NTN1, NEGR1 |
| GO:0005925~focal adhesion | 18 | $3.24 \times 10^{-4}$ | TLN1, LIMS2, PDLIM7, FHL1, FERMT2, PIP5K1C, CSRP1, MCAM, FLNA, ANXA6, TNS2, TNS1, PGM5, LAYN, ILK, RHOB, TGFB1I1, PARVA |
| GO:0010811~positive regulation of cell-substrate adhesion | 5 | 0.003 | SMOC2, FOXF1, CCDC80, ABI3BP, EMILIN1 |
| GO:0001525~angiogenesis | 11 | 0.004 | CYP1B1, S1PR1, MEOX2, RHOB, TMEM100, TNFSF12, MCAM, COL8A2, MEIS1, JAM3, MMRN2 |
| GO:0061364~apoptotic process involved in luteolysis | 2 | 0.048 | SLIT2, SLIT3 |
| GO:0031012~extracellular matrix | 25 | $1.88 \times 10^{-10}$ | LTBP1, IGFBP7, DCN, ABI3BP, MMRN2, OGN, ILK, COL8A2, MYOC, SPON1, MGP, CPXM2, SOD3, FLNA, EMILIN1, PRELP, FBLN1, COL14A1, SERPINF1, SFRP1, FBLN5, TGFB1I1, MFAP4, SSC5D, CLEC14A |
| GO:0030198~extracellular matrix organization | 18 | $2.11 \times 10^{-8}$ | RECK, ELN, CCDC80, DCN, ABI3BP, EMILIN1, ITGA9, SMOC2, FBLN1, COL14A1, COL19A1, CRISPLD2, FOXF1, FBLN5, ITGA7, JAM2, JAM3, COL8A2 |
| GO:0005578~proteinaceous extracellular matrix | 21 | $2.62 \times 10^{-8}$ | LTBP1, PODN, SPARCL1, ADAMTSL3, ELN, MGP, SLIT2, PRELP, SLIT3, EMILIN1, OGN, SMOC2, FBLN1, COL14A1, COL19A1, SFRP1, CRISPLD2, FBLN5, COL8A2, MYOC, SPON1 |
| GO:0005576~extracellular region | 52 | $6.02 \times 10^{-6}$ | NXPH3, TLN1, A2M, LTBP1, FGF7, CXORF36, IGFBP7, TNFSF12, OLFML1, OGN, ST3GAL3, RSPO1, RSPO3, ITIH5, LGI4, GHR, SERPING1, SLIT2, FLNA, SLIT3, PRELP, CHRDL1, PTGDS, SERPINF1, MFAP4, ELN, C1R, DCN, C1S, C1QTNF7, METTL24, ANGPTL7, IL17B, CRISPLD2, COL8A2, VSTM4, SVEP1, EFEMP2, PTGFR, FRZB, NTN1, SOD3, PLAC9, EMILIN1, FBLN1, COL14A1, COL19A1, CCL14, SFRP1, FAM198A, LCN6, FBLN5 |
| GO:0005201~extracellular matrix structural constituent | 8 | $1.02 \times 10^{-4}$ | FBLN1, COL14A1, COL19A1, EFEMP2, ELN, MGP, COL8A2, PRELP |
| GO:0050840~extracellular matrix binding | 4 | 0.008 | SPARCL1, ELN, DCN, SSC5D |
| GO:0005886~plasma membrane | 92 | 0.002 | RHOJ, SLC9A9, GYPC, CADM3, TLN1, JPH2, ATP1B2, TACR2, ADCY5, GRIK5, NCS1, TNFSF12, FRRS1L, DMPK, EDNRA, ST6GALNAC6, S1PR1, SLC2A4, NMUR1, ILK, GUCY1A3, RHOB, ATP8B2, TMEM100, FAM129A, NEGR1, BOC, GHR, RAMP3, KCNMA1, RECK, AR, MAGI2, ACKR1, MRGPRF, COLEC12, FLNA, SLIT2, TNS2, CHRM2, ROR2, GUCY1B3, CLIP3, JAM2, JAM3, AOC3, PARVA, FXYD1, ABCA8, LIMS2, CYSLTR1, ARHGEF25, FHL1, GNG11, KCNA5, PLPP1, FXYD6, KCNMB1, RGMA, FAT3, PLIN4, PKD2, KLHL41, PRIMA1, EHD2, TRPC1, SELP, VSTM4, GNAO1, KLF9, EPM2A, MAP1B, NPR1, NPR2, ATP1A2, MAPK10, MCAM, PTGFR, ITPR1, ITGA9, ABCC9, TMEM47, PDE2A, SFRP1, PLSCR4, P2RY14, BNC2, ITGA7, SCN4B, APBB1, HTR2A, FEZ1 |
| GO:0030308~negative regulation of cell growth | 11 | $2.75 \times 10^{-5}$ | RERG, SFRP1, DACT3, CRYAB, FHL1, NPR1, WFDC1, FRZB, APBB1, SLIT2, SLIT3 |
| GO:0008285~negative regulation of cell proliferation | 19 | $8.51 \times 10^{-5}$ | AR, MAGI2, CYP1B1, PODN, ADARB1, NDN, IGFBP7, ZEB1, FRZB, PLPP1, KANK2, SLIT3, RERG, TNS2, SFRP1, SPEG, PKD2, ROR2, TGFB1I1 |

Table V. Continued.

| Term | Count | P-value | Genes |
|---|---|---|---|
| GO:0030336~negative regulation of cell migration | 8 | $8.89x10^{-4}$ | RECK, ADARB1, PODN, CYP1B1, MAGI2, SFRP1, RHOB, SLIT2 |
| GO:0030178~negative regulation of Wnt signaling pathway | 5 | 0.010 | BARX1, SFRP1, DACT3, NFATC4, FRZB |
| GO:0035385~roundabout signaling pathway | 3 | 0.016 | OGN, SLIT2, SLIT3 |
| GO:0007229~integrin-mediated signaling pathway | 6 | 0.022 | ITGA9, FBLN1, FERMT2, ITGA7, ILK, ADAM33 |
| GO:0007224~smoothened signaling pathway | 5 | 0.026 | EVC, FOXF1, CC2D2A, ROR2, BOC |

GO, gene ontology.

Table VI. Kyoto Encyclopedia of Genes and Genomes term enrichments of co-expressed genes of glutathione S-transferase Mu 5 in gastric cancer.

| Term | Count | P-value | Genes |
|---|---|---|---|
| hsa04022: cGMP-PKG signaling pathway | 17 | $3.04x10^{-9}$ | KCNMA1, ATP1B2, ADCY5, MRVI1, NPR1, NPR2, ATP1A2, KCNMB1, ITPR1, MYL9, EDNRA, PDE2A, PLN, PDE5A, GUCY1A3, NFATC4, GUCY1B3 |
| hsa04024: cAMP signaling pathway | 10 | 0.004 | EDNRA, FXYD1, ATP1B2, CHRM2, ADCY5, PLN, NPR1, MAPK10, ATP1A2, MYL9 |
| hsa04020: Calcium signaling pathway | 9 | 0.008 | EDNRA, CYSLTR1, TACR2, CHRM2, PLN, PDE1A, PTGFR, ITPR1, HTR2A |
| hsa04510: Focal adhesion | 9 | 0.017 | ITGA9, TLN1, ITGA7, ILK, PPP1R12C, MAPK10, FLNA, PARVA, MYL9 |
| hsa04514: Cell adhesion molecules (CAMs) | 7 | 0.026 | ITGA9, SELP, CADM3, NLGN2, JAM2, NEGR1, JAM3 |

Hsa, *Homo sapiens*.

biomarker. Despite previous studies revealing an association between GSTM5 and OS, associations between GSTM5 and survival of patients with GC have remained to be determined. The present study revealed that among all GSTM genes, only GSTM5 was independently associated with poor OS in patients with GC. According to the present nomogram, it was indicated that the contribution of the tumor stage increased with more advanced stages and higher expression levels of GSTM5 were associated with a less favorable survival prognosis. Compared with the tumor stage, age, sex and GSTM5 expression, the tumor stage and age were more significantly associated with GSTM5 expression. Therefore, the OS rate of patients with GC may be predicted using this model. The nomogram indicated that the combination of GSTM5 and other indicators may be considered a novel method to predict the prognosis for patients with GC.

In the stratified analyses with the KM plotter online database, higher levels of GSTM5 were associated with worse OS in female and male patients, clinical stages I-III, all Lauren classifications, treatment by surgery, administration of other adjuvant therapies, as well as a negative HER2 status. However, OS was not associated with female sex, age >65 years or clinical stage in the TCGA GC cohort. There were 375 cases of GC in the TCGA, but there were 875 cases of GC in the KM plotter online database. As TCGA appeared to have a shortage of GC samples, another prospective study should be performed in the future. The comprehensive survival analysis with stratification suggested that GSTM5 has value as an independent prognostic indicator for GC.

The mechanisms underlying the functions of GSTM5 in GC have remained largely elusive. In the present study, the outcomes of the GSEA analysis indicated that GSTM5 is involved in 'regulation of cell matrix adhesion', 'angiogenesis', 'regulation of cell growth', 'regulation of endothelial cell apoptotic process', 'Hedgehog signaling', 'MAPK signaling', 'TGF-β signaling' and other cancer-associated pathways. According to the results of the genome-wide co-expression matrix, co-expressed genes were enriched in 'cell adhesion', 'angiogenesis', 'apoptotic process involved in luteolysis', 'cGMP-PKG signaling pathway' and 'cAMP signaling

pathway'. In the GSEA analysis as well as the genome-wide co-expression matrix, enrichment in adhesion, angiogenesis and apoptosis was determined. During tumorigenesis, recurrence, invasion and metastasis are significantly affected by adhesion molecules (48). Aberrant expression of cell adhesion molecules may lead to abnormal proliferation of normal cells, and aberrant expression of these molecules is frequently associated with general carcinogenesis (51). Angiogenesis allows the tumor to grow, infiltrate and metastasize (52). Du *et al* (53) demonstrated that the synthesis of vascular endothelial growth factor is modulated by the cAMP-protein kinase A-cAMP response element-binding pathway. Zhang *et al* (54) reported that loss of the regulative effect of dimethylarginine dimethylaminohydrolase 1 in the nitric oxide-cGMP-PKG pathway may lead to decreased angiogenesis. These studies demonstrated that cAMP and cGMP biosynthesis are associated with angiogenesis.

The Hedgehog pathway serves a crucial role in cell proliferation and differentiation in adult organisms, and dysregulation of this pathway is associated with multiple cancer types. Saze *et al* (55) demonstrated that Hedgehog signaling is a prognostic indicator for patients with GC. Qin *et al* (56) revealed that Hedgehog signaling is associated with the apoptosis of GC cells. The MAPK signaling pathway serves a vital role in controlling cellular processes, including proliferation, differentiation and apoptosis (57). As a multifunctional growth factor, TGF-β regulates several cellular processes, including proliferation, development, homeostasis of stem cells, enhancing fibrosis and modulating the immune response through its downstream targets (58). TGF family of proteins are involved in tumor initiation and progression, cell proliferation, angiogenesis, epithelial to mesenchymal transition, invasion and inflammation (59). The results of the present GSEA analysis and genome-wide co-expression matrix demonstrated that high expression of GSTM5 was associated with the prognosis of GC. However, the exact mechanisms underlying the function of GSTM5 in the development of GC require to be elucidated.

The present study has certain limitations. First, since the clinical parameters were obtained from a public database, some clinical data were not available such as chemotherapy, smoking and infection with *Helicobacter pylori*, and therefore, a comprehensive analysis was not performed. Furthermore, the results were obtained with the dataset of the public TCGA database. Therefore, further experiments such as immunohistochemistry for GSTM5 should be designed and performed to validate the prognostic value in GC. In addition, the association between GSTM proteins and GC prognosis was not investigated, since GSTM protein expression data were not available. Despite these limitations, the present study was the first to systematically investigate the association between the expression of GSTM genes and OS in GC, to the best of our knowledge. The present results indicated that high expression of GSTM5 in GC is associated with poor prognosis, suggesting that GSTM5 may be a novel prognostic indicator for GC.

In conclusion, the present study indicated that GSTM5 mRNA expression is associated with unfavorable OS in patients with GC. This suggests that GSTM5 may be a prognostic marker and potential target for GC therapy. Furthermore, the potential mechanisms underlying GSTM5 function in GC were also explored using the GSEA and genome-wide co-expression analyses. To predict the OS of patients with GC, a nomogram composed of GSTM5 and tumor stage was also constructed. However, additional studies are required to further validate the results of the present study.

**Acknowledgements**

Not applicable.

**Funding**

The study was supported by the Natural Science Foundation of Guangxi Zhuang Autonomous Region (grant no. 2017AB45153), the Scientific Research and Technology Development Program of Guangxi (grant no. 1598011-4), the Natural Science Foundation of Guangxi (grant no. 2016GXNSFAA380180), the Guangxi Zhuang Autonomous Region Health and Family Planning Commission (grant no. Z2015526) and the Youth Science Foundation of Guangxi Medical University (grant no. GXMUYSF201502).

**Availability of data and materials**

The datasets generated and analyzed during the current study are available in TCGA repository, https://portal.gdc.cancer.gov.

**Authors' contributions**

JC, YC and BL conceived and designed the study. XL was responsible for the acquisition of data. YM performed the statistical analysis. JW, JL, ZW and SL, analysed and interpreted the data, drafted the manuscript and revised it critically for important intellectual content. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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