Integrated Bioinformatical Analysis Identified HTRA4 As An Immune-Related Prognostic Biomarker In The Gastric Cancer Microenvironment

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Research Article

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Abstract

HtrA serine peptidase 4 (HTRA4), which belongs to the AB family of proteases/chapterones, participates in multiple signal pathways and plays an important regulatory role in some malignancies; however, its role in the prognosis and immune infiltrates of gastric cancer (GC) remains unclear. HTRA4 expression was thus investigated in tumor tissues, and its association with immune infiltrates was assessed, to determine its prognostic roles in GC patients. Data for patients with GC was collected from three GEO datasets. This study has investigated if high expression levels of HTRA4 are associated with a poor prognosis in GC patients, especially in T2,T3,N1,NI&N2&N3,M1,stage3 and HER2+ subgroups. HTRA4 was positively correlated with CD8+ T cells, CD4+ memory activated T cells, gamma delta T cells, and M2 macrophages in the GC microenvironment. Gene Ontology and Gene set Enrichment Analysis showed that HTRA4 was enriched in some immune-related pathways. High expression levels of HTRA4 were significantly correlated with tumors in the T stage, N stage, and clinical stage, as well as those of histological grade. In summary, HTRA4 was found to play a vital role in the progression of GC and may be a predictive biomarker for the survival of GC patients.

Introduction

Gastric cancer (GC) is characterized by aggressively invasive tumors. Despite the considerable decreases in the mortality and incidence of GC during the last decade, it remains the third leading cause of cancer-related death, especially in China [1]. Most GC patients are diagnosed when the disease is at an advanced stage. Although there have been significant developments in multiple therapy strategies, almost 50% of GC patients will have metastases during their treatment [2], and the five-year survival rates for patients with stage II and stage IIIA is approximately 34% and 20%, respectively [3]. GC is a heterogeneous disease. To date, there are two widely applicable histological classification methods for GC: Lauren's classification and the World Health Organization (WHO) classification [4]. These two classifications are unable to satisfy the specific therapeutic methods for GC. The existing high level of microsatellite instability (MSI), deficient mismatch repair (dMMR), HER2 mutation, and gene amplification cannot completely explain the responses of GC to the different therapeutic treatments [5, 6]. At present, the GC patients who are insensitive to chemotherapy with MSI-high/dMMR are recommended to use immunotherapeutics. Of note, lung cancer and melanoma appear to markedly benefit from immunotherapies, such as with programmed death-1 (PD-1), programmed death ligand-1 (PD-L1) inhibitors, and cytotoxic T lymphocyte associated antigen-4 (CTLA4) inhibitors [7]. Thus, there is an urgent need to illuminate the immunophenotypes of tumor–immune interactions and identify the novel immune-related prognostic biomarkers in GC.

HtrA serine peptidase (HTRA) is a family of serine proteases, which includes four HTRA homologs (HTRA1- 4) [8–10]. In humans, these proteases are involved in numerous cellular processes including the maintenance of mitochondrial homeostasis and cell death. Among these proteases, HTRA4 is the least characterized human HTRA protease, both for structure and function. A previous study showed that the short N-terminal region of HTRA4 maintained its stability and oligomerization. Also, the PDZ domain

positively influenced the protease activity, which suggests that there is a requirement for allosteric and inter-molecular interaction network for HTRA4 functions [11]. HTRA4 has been found to be overexpressed in pre-eclampsia (PE), to and directly interact with HTRA1 and HTRA3, and to participate in the process of embryo implantation and decidualization [3, 6, 12, 13, 14]. In addition, HTRA4 can interact with anti-apoptotic XIAP, caspase 9, and executioner caspase 7 to influence apoptotic cell death by affecting cytoskeleton homeostasis. However, only some meta-analyses of available microarray data have shown that HTRA4 is upregulated in glioblastoma multiforme and breast cancer, and downregulated in metastatic prostate cancer, which suggests an association between HTRA4 and tumorigenesis [15, 16]. Recently, the role of HTRA4 in cell proliferation and cell cycle modulation was demonstrated [17], and HTRA4 was first reported to promote the death of cancer cells treated with chemotherapeutic drugs by reducing the survival, clonogenic potential, and motility of cancer cells [18]. Although HTRA4 is involved in important physiological, pathological, and cellular processes, its roles in different cancers are poorly understood.

In this study, we selected three GEO datasets for GC (GSE19826, GSE30727, and GSE79973) to identify differentially expressed genes (DEGs) in the GC tissues, when compared with normal gastric tissues. The HTRA4 was found to be extremely overexpressed in the intersections of the three GC datasets. RNA sequence data were downloaded from the Cancer Genome Atlas (TCGA) database to verify the high expression of HTRA4 in the GC tissues. Then the significance of HTRA4 in the GC was systematically analyzed using bioinformatical and statistical methods, including differentially expressed gene (DEG) analysis, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Gene set Enrichment Analysis (GSEA), CIBERSORT analysis, logistic regression, and Kaplan-Meier survival analysis.

The results suggested that HTRA4 was upregulated and associated with a poor GC prognosis, and could thus be considered a predictive biomarker for GC. GSEA analyses showed that HTRA4 expression was enriched in numerous immune-related pathways. Logistical regression analysis showed high HTRA4 expression was significantly correlated to tumors in the T stage, N stage (lymph node status), and clinical stage as well as those of histological grade. CIBERSORT analysis showed that high expression level of HTRA4 were positively infiltrated with different kinds of T cells. In summary, HTRA4 is important in the progression of GC and could be an ideal immune-related predictive biomarker for the survival for GC patients in the treatment process.

Methods

Data source and preprocessing

The Gene Expression Omnibus of National Coalition Building Institute (NCBI-GEO) is a free public database for microarray/gene profiles, and it was used to source the gene expression datasets analyzed in this study. They included three independent GC gene expression profiles (GSE19826, GSE30727, and GSE79973) that were screened and downloaded from the GEO as discovery datasets to identify the

DEGs. Gene expression data with clinical information from the STAD datasets (including 375 GC tumor tissues and 32 normal tissues, workflow type: HTSeq-FPKM) were downloaded from TCGA. Level 3 HTSeq-FPKM data were transformed into TPM (transcripts per million reads) for further analysis.

Analysis of DEGs

DEGs between the TCGA GC samples and normal samples were identified using the unpaired Student's t-test, with the DESeq2 package. Statistical ranking for HTRA4 expression above and below the median was defined as HTRA4-high and HTRA4-low groups. Futhermore, the DEGs between the HTRA4-high and HTRA4-low groups were also identified using the unpaired Student's t-test with the DESeq2 package. Genes with an adjusted P value < 0.05 and |log FC| > 2 were thought to be statistically significant. These findings and the values for the DEGs were plotted in volcano plots and on a heatmap ("limma" R package)

Gene set enrichment analysis

GO analysis is a commonly used method to define genes and the RNA or protein products that are used to identify the unique biological properties of high-throughput transcriptome or genome data, including Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF). The KEGG pathway analysis is a widely used database which stores large amount of genomic, biological pathway, disease, chemical substance, and drug data. The GO annotation and KEGG pathway enrichment analysis were performed using the Metascape online tool. We chose the top eight pathways according to the enrichment score in every analysis ($P < 0.01$).

GSEA (JAVA program gsea-3.0.jar and "ggplot2" R package) starts with the HTRA4 matrix that is differentially expressed and investigates the signal pathway variances among the low and high HTRA4 groups to estimate the signal pathways and associated phenotype. A permutation test was performed 1000 times to determine the altered pathways. Genes were regarded as related for FDR < 0.25 and $p < 0.01$. Statistical analysis was performed using R. To obtain the interactions of HTRA4 and immune cells and the relevance of this infiltration with various expression groups of HTRA4, Spearman correlation and Wilcoxon rank sum tests were applied. R package "ggplot2" was used for the lollipop graph, "corrplot" was used for the correlation analysis, and "vioplot" was used for the plot violin graphs.

To construct the protein-protein interaction (PPI) network, we used Search Tool for the Retrieval of Interacting Genes (STRING) database to integrate all DEGs. The PPI networks were extracted with a combined score > 0.4, and visualized by Cytoscape. CytoHubba is a plugin in Cytoscape, which can be used to measure the degree of each protein node. The nodes with higher degrees of connectivity tend to have more potential for maintaining the stability of the entire network, and we chose the top ten genes as hub genes.

Clinicopathological analysis and prognosis analysis

The association of the clinical pathologic parameters and HTRA4 were assessed with a regression analysis and displayed.The R package “tableone” was utilized for the clinical table. The cut off for the expression of HTRA4 was defined by the median value. In all analyses, a p value < 0.05 indicated statistical significance. The distinction in survival between the high HTRA4 groups and low HTRA4 groups was determined using the Kaplan-Meier online website (https://kmplot.com/).

Tumor infiltrating immune cells (TICs) analysis

To explore the correlation between HTRA4 and the infiltration levels of 22 immune cells, the R package: “Cibersort” (CIBERSORT) was applied to estimate the of TICs in GC patients. The signatures we used included a diverse set of adaptive and innate immune cell types. Only cases where $p \leq 0.05$ were used for further analysis. The R package “ggstatsplot” was used to construct the scatter plots.

Results

Identification of DEGs in GC

Three gene expression profiles (GSE19826, GSE30727, and GSE79973) were selected for use in this study (Fig. 1A, 1B, and 1C). Based on the criteria of adj P < 0.05 and |log FC| > 2, 178 DEGs were identified after the intersection of the three datasets (Fig. 1D; Supplemental table 1). To predict the functional enrichment information for these DEGs, GO function enrichment analysis was performed. The enriched GO terms were divided into BP, CC, and MF ontologies. These DEGs were involved in extracellular structure organization, collagen-containing extracellular matrix, and extracellular structural constituents. Moreover, extracellular matrix organization, endoplasmic reticulum lumen, and glycosaminoglycan binding were also involved in the GC progression (Fig. 1E). Detailed DEG volcano maps for each dataset are shown in Figs. 1F, 1G, and 1H.

Verification of the abnormally high expression of HTRA4 in GC

To identify an immune prognostic signature of GC, we analyzed three GEO datasets, and identified 178 differentially expressed genes (DEGs). HTRA4 was among the 178 DEGs and was found to play an important role in GC immune progression through a series of analysis. First, the pan-cancer analyses compared the expression of HTRA4 in different tumor samples of the TCGA and normal samples of GTEx using the Wilcoxon rank sum test statistical method (Fig. 2A). Second, the pan-cancer analyses of HTRA4 expression were also displayed in the TCGA tumor tissues and corresponding normal tissues (Fig. 2B). HTRA4 was upregulated in colon adenocarcinoma (COAD), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), and stomach adenocarcinoma (STAD) (P < 0.0001). Third, the high expression of HTRA4 was verified in 375 GC tissues and 32 normal tissues in the TCGA STAD dataset (Fig. 2C). The expression of HTRA4 between the 27 GC samples and matched para-cancerous samples was significantly different (Fig. 2D).

Functional enrichment and analyses of HTRA4 related genes in GC

We compared 187 HTRA4-high GC samples with 188 HTRA4-low GC samples and identified 178 HTRA4-related DEGs, including 177 upregulated genes and 1 downregulated gene (adjusted $p < 0.01$, $|\log FC| > 2$). The DEGs were then further analyzed using the DESeq2 package. Volcano and heat maps for the DEGs between the two cohorts are presented in Fig. 3A and Fig. 3B, respectively. GO enrichment analysis was used to show that HTRA4-related genes were involved in many immune-related BPs, CCs, and MFs, such as humoral immune response mediated by circulating immunoglobulin, immunoglobulin complex, and antigen binding (Fig. 3C). To determine the interactions between the 178 HTRA4-related DEGs in the GC samples, we analyzed the protein-protein interactions (PPIs) using the STRING database (Supplement Fig. 2A). We selected 100 proteins and 18 edges from the PPI network (Supplement Fig. 2B), and the top ten hub genes COL4A2, COL1A2, COL5A2, COL4A1, COL5A1, COL1A1, COL6A3, COL6A1, BGN and FN1 (Supplement Fig. 2C, Supplement table 2). GSEA identified significant pathways (adj $P < 0.05$) in the enrichment of the MSigDB collection (Supplement table 3). Six of the significantly enriched immune-related signaling pathways were selected based on their normalized enrichment score (HES), including antigen processing and presentation, toll-like receptor signaling pathway, intestinal immune network for IgA production, natural killer cell mediated cytotoxicity, T-cell receptor signaling pathway and primary immunodeficiency (Fig. 4A and 4B).

The correlation between HTRA4 expression and clinicopathological characteristics in the GC patients from TCGA with complete clinical information were analyzed to clarify the significance and roles of HTRA4 expression. The expression of HTRA4 was positively correlated with tumors in T stage (T1 vs T2, $P = 0.0049$; T1 vs T3, $P = 0.0052$; T1 vs T4, $P = 0.00099$), N stage (N1 vs N2, $P = 0.04$), clinical stage (stage I vs stage II, $P = 0.004$; stage I vs stage III, $P = 0.047$), and clinical grade (G1 vs G3, $P = 0.012$; G2 vs G3, $P = 1.1e\text{-}09$) (Fig. 5A–D). The univariate analysis with logistic regression demonstrated that HTRA4 expression was a dependent factor related to poor prognostic clinicopathological variables (Table 1). Increased HTRA4 expression in GC was positively associated with pathological T ($P = 0.025$), pathological N ($P = 0.045$), neoplasm histological grade ($P < 0.001$), and helicobacter pylori infection ($P = 0.033$). The results showed that higher HTRA4 expression levels were associated with more serious and advantaged stage in GC.

The association between HTRA4 expression and immune infiltration in GC

The correlation between the expression level (TPM) of the HTRA4 and the relative abundance of 24 immune cells and their infiltration levels was quantified by CIBERSORT and analyzed with the spearmen correlation (Fig. 6A, Supplement table 4). The expression of HTRA4 was positively correlated with the abundance of the top four immune cells, CD8 + T cells ($P = 0.01$), CD4 + memory activated T cells ($P = 0.00$), gamma delta T cells ($P = 0.00$) and M2 macrophages ($P = 0.00$) (Fig. 6B–E).

Overexpression of HTRA4 was strongly associated with a poor GC prognosis

The overall survival (OS) rates were extremely high among GC patients with low HTRA4 expression levels, when compared with those with high HTRA4 expression (HR = 1.43, P = 0.0011) (Fig. 7A), and progression-free survival (PFS) rates were also significantly higher in the low HTRA4 expression groups than the HTRA4 high groups (HR = 1.38, P = 0.013) (Supplement Fig. 2A).

The subgroup survival analyses of the OS and PFS, suggested that the prognosis of patients with HTRA4-high was poorer in T2, T3, N1, N1&2&3, M1, stage 3 and HER2 + subgroups of OS (Fig. 7B-H). Among these subgroups, the survival time of the patients with HTRA4-high was also shorter in stage 3, T3, N1&2&3, and HER2 + subgroups of PFS (Supplement Fig. 2B-E). In conclusion, the survival data suggested that the high expression of HTRA4 in GC patients was associated with a worse prognosis and shorter survival time, and may accelerate disease progression.

Discussion

Gastric cancer is the leading cause of cancer-related mortality worldwide, and its rapid progression and invasive metastasis often contributes to its poor prognosis. Both Lauren's classification and the World Health Organizations (WHO) classification [19, 20] recommend surgical resection, neoadjuvant, and adjuvant therapies as the primary treatment options for GC, but these have unfavorable prognosis. In recent years, cancer immunotherapies have received considerable attention for the treatment of various tumor types. Consequently, studies into the immunological regulation of GC have been increasing [21, 22, 23]. Among these immunotherapies, the blocking of immune checkpoints to target PD-L1 and CTLA4, has shown promising inhibitory effects. Whereas, in GC patients, the anti-CTLA4 drug tremelimumab has had insufficient therapeutic efficacy in clinical treatments [24], and PD-1 and PD-L1 inhibitors have had incomplete responses in most advanced GC patients [25, 26, 27]. Despite the substantial improvement in the survival of GC patients, identifying the population who can benefit from immunotherapy is still a challenge. Thus, there is an urgent need to identify more immune-related prognostic biomarkers for GC treatments.

The HTRA4 proteins are important members a of complicated protein network designed to promote cellular processes in stressful environments, and they are well conserved in the evolution of the HTRA (High-temperature requirement A) family of serine proteases [28, 29, 30]. There are four human HtrA proteins (HTRA1, HTRA2, HTRA3, and HTRA4), and of these, HTRA1, HTRA3, and HTRA4 have similar domain organizations. The HTRA 1, 2, and 3 proteases have been widely reported to function in many processes, such as extracellular matrix proteins, the protection of mitochondrial homeostasis, and cell apoptosis to promote cell death [31, 32, 33]. Moreover, HTRA1, HTRA2, and HTRA3 proteins are involved in neurodegenerative disorders and cancers. HTRA1 was the first sequenced protein of the human AB family [34], and its expression is regulated during the proliferation of the endometrium during pregnancy [35]. HTRA2 is expressed ubiquitously in human tissue, and its expression is extremely variable according to the type of tumor [36, 37, 38]. Like HTRA2, the expression of HTRA3 is also different according to the type of tumor [39], and previous research has described the functional mechanisms and role of HTRA1–3

proteins. However, the cellular functions of the HTRA4 protease are still poorly understood, especially in cancers.

HTRA4 can be secreted into the extracellular matrix to act as a similar signal secretory peptide as HTRA1 and HTRA3 [40.41]. It was previously suggested that HTRA4 plays an important role in the implantation of the embryo and could a biomarker with which to predict pre-eclampsia [6, 42, 43, 44]. In some malignancies, HTRA4 has been found to promote tumor progression. Analysis of microarray studies shows that HTRA4 is upregulated in glioblastoma multiforme, when compared to brain tissues in epilepsy patients [45]. Similarly, it has been indicated that HTRA4 is overexpressed in breast cancer tissues, when compared to normal breast tissues [46]. Conversely, HTRA4 has been found to have anti-tumor effects in other malignancies. HTRA4 is downregulated in hormone refractory metastatic prostate cancer when compared to primary prostate cancer [47]. Maser et al. indicated that the allelic ratio of HTRA4 is lower in glioblastoma and pancreatic cancer, when compared to normal DNA [48]. Recently, Wenta et al. suggested that HTRA4 stimulates the drug-induced death of cancer cells, and that this protease is a promising anti-cancer therapeutic target [18]. Nevertheless, knowledge regarding the HTRA4 biological features and its roles in cancers is currently limited. At present, there have been no previous studies on the role of HTRA4 in GC, and consequently this was the focus of this investigation.

Bioinformatics analysis using high throughput RNA-sequence data from the GEO and TCGA database shows obvious individual heterogeneity in the RNA data, and HTRA4 may be a potential identification biomarker for GC tissues. Increased expression of HTRA4 in GC tissues was accompanied by advanced clinical pathological characteristics, shorter survival time, poor prognosis, and more powerful T cell immune levels. To further investigate the functions of HTRA4 in GC prognosis, we conducted GO, KEGG, and GSEA analyses using the TCGA data. The results revealed that most immune-related pathways, including antigen processing and presentation, toll-like receptor signaling pathway, intestinal immune network for IgA production, natural killer cell mediated cytotoxicity, T-cell receptor signaling pathway, and primary immunodeficiency were differentially enriched in the HTRA4 high expression phenotype. CIBERSORT analysis shows that high HTRA4 expression levels are associated with higher infiltration levels of various T cells and macrophages. Therefore, these data suggested that HTRA4 might act as potential prognostic marker and immune-related therapeutic target in GC.

Tumor-infiltration lymphocytes (TILs) among stromal cells in the tumor microenvironment play an important role in the occurrence and development of tumors, which can influence the carcinogenic characteristics of adjacent tumor cells [49, 50]. TILs establish a complex interaction network, helping to improve and maintain the immune microenvironment, to promote immune effects, and thus promote tumor progression [51]. Immune infiltrates in tumors like tumor associated macrophages, tumor-infiltrating neutrophils, and various kinds of T cells are significantly relevant to patient prognosis and the efficacy of therapeutics [52, 53]. Our results demonstrated that there were four kinds of T cells in the HTRA4-high GC group than in the HTRA4-low GC group, suggesting that high expression of HTRA4 is associated with high immune cell levels. The CD8 + and CD4 + T cells in the tumor microenvironment have strong immunoenhancement activity. Therefore, our data elucidated that the immune effects

induced by the T cells in the primary tumor microenvironment might result in a lower overall survival rate in HTRA4-high GC patients.

High HTRA4 expression levels were shown to correlate to the poor prognosis of GC in T2, T3, N1, N1&2&3, M1, stage 3 and HER2 + subgroups when HTRA4 was highly expressed in the GC tissues. The expression of HTRA4 remains a powerful forecaster of the prognosis within these subgroups, showing that HTRA4 was independent of these important clinicopathological parameters.

These results have improved our understanding of the relationship between HTRA4 and GC, but the study had some limitations. First, to discover the specific role of HTRA4 in the development of GC comprehensively and completely, all clinical information should be included, such as the details on the treatment methods received by the patients and successive survival follow-up times, but the information for the treatments and follow-up times was lacking or inconsistent as the public database experiments were performed in different cancer centers. Second, the number of normal gastric tissue samples in the current study was too small, and this should be improved in the future to ensure balanced sample sizes. Third, retrospective studies overall have their own limits, and thus a prospective study should be conducted in the future to avoid analysis bias. Fourth, the data from the current study were analyzed using the sequencing data from the GEO and TCGA databases only, it is necessary to conduct further research to prove the direct mechanisms and role of HTRA4 in GC.

In the current study, we have reported that high HTRA4 expression levels were significantly related to the progression, poor survival, and immune infiltration of GC. HTRA4 may promote tumorigenesis through abnormal inflammation and immune responses and may have the potential to become a new immune biomarker of GC. Thus, HTRA4 was identified as a reliable predictor of GC prognosis. The mechanisms by which HTRA4 accelerates the progression and metastasis of GC will be investigated further. This study provides a new and promising immunotherapeutic target, HTRA4, that has clinicopathological significance and will improve molecular pathogenesis assessments.

Declarations

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Conflicts

All the authors declared no competing interests.

Availability of data and material

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Ce Ji contributed to concept and design of this article. Jing Wei Dai participated in manuscript writing. Zhen Wang and Xiangchen Hu participated in data collection and data analysis. Youwei Kou analyzed and interpreted data. All authors contributed to the article and approved the submitted version.

Ethics approval

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

References

1. Jeremy C, Mara C, Viji S, Alfonso B. HtrA Serine Proteases as Potential Therapeutic Targets in Cancer. Curr Cancer Drug Targets. 2009;9(4):451–68. doi:10.2174/156800909788486704.
2. Smyth EC, Verheij M, Allum W, Cunningham D, Cervantes A, Arnold D. Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2016;27:v38–49. doi:10.1093/annonc/mdw350.
3. Brew O, Sullivan MHF, Woodman A. Comparison of Normal and Pre-Eclamptic Placental Gene Expression: A Systematic Review with Meta-Analysis. PLoS ONE (2016) 11(8): e0161504. doi:10.1371/ journal.pone.0161504.
4. Lauren P. The two histological main types of gastric carcinoma: diffuse and so called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49. doi:10.1111/apm.1965.64.1.31.
5. Yueh HC, Hungwen C. GATA3 inhibits GCM1 activity and trophoblast cell invasion. Sci Rep. 2016;6:21630. doi:10.1038/srep21630.
6. Inagaki A, Nishizawa A, Ota S, Suzuki M, Inuzuka H, Miyamura H, et al. Upregulation of HtrA4 in the placentas of patients with severe pre-eclampsia. Placenta. 2012;33(11):919–26. doi:10.1016/j.placenta.2012.08.003.
7. Zhao Q, Cao L, Guan L, et al. Immunotherapy for gastric cancer: dilemmas and prospect. Brief Funct Genomics. 2018;18(2):1–6. doi:10.1093/bfgp/ely019.
8. Clausen T, Southan C, Ehrmann M. The HtrA family of proteases: implications for protein composition and cell fate. Mol Cell. 2002;10:443–55. doi:10.1016/s1097-2765(02)00658-5.
9. Gray CW, Ward RV, Karran E, Turconi S, Rowles A, Viglienghi D, et al. Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. Eur J Biochem. 2000;267(18):5699–710. doi:10.1046/j.1432-1327.2000.01589.x.
10. Nie GY, Hampton A, Li Y, Findlay JK, Salamonsen LA. Identification and cloning of two isoforms of human high-temperature requirement factor A3 (HtrA3), characterization of its genomic structure and

comparison of its tissue distribution with HtrA1 and HtrA2. Biochem. J (2003) 371, 39–48. doi: 10.1042/BJ20021569.

11. Raghupathi K, Shubhankar D, Lalith KC, Kakoli B. Discerning the Mechanism of Action of HtrA4: a Serine Protease Implicated in the Cell Death Pathway. Biochem J. 2019;476(10):1–30. doi: org/10.1042/BCJ20190224.
12. Tea K, Alejandra C, Anjuska K, Krista L, Juha K, Hannele L. Gene expression profiling of pre-eclamptic placentae by RNA sequencing. Sci Rep. 2016;5:14107. doi:10.1038/srep14107.
13. Esther T, Sonia SYT, Yao W, Gui NY. Elevated protease HtrA4 in the maternal circulation of preeclampsia may contribute to endothelial barrier disruption by cleaving key junctional protein VE-cadherin. Placenta. 2019;15:76. doi:https://doi.org/10.1016/j.placenta.2019.01.001.
14. Yao W, Rebecca L, Guiying N. HtrA4 may play a major role in inhibiting endothelial repair in pregnancy complication preeclampsia. Sci Rep. 2019;9:2728. Doi:https://doi.org/10.1038/s41598-019-39565-9.
15. Chien J, Campioni M, Shridhar V, Baldi A. HtrA serine proteases as potential therapeutic targets in cancer. Curr Cancer Drug Targets. 2009;9:451–68. doi:10.2174/156800909788486704.
16. Zurawa JD, Skorko GJ, Lipinska B. HtrA proteins as targets in therapy of cancer and other diseases. Expert Opin Ther Targets Taylor Francis. 2010;14:665–79. doi:10.2174/1381612811319060003.
17. Staff AC, Johnsen GM, Dechend R, Redman CW. Preeclampsia and uteroplacental acute atherosis: immune and inflammatory factors. J Reprod Immunol (2014). 101–2, 120–126. doi:10.1016/j.jri.2013.09.001.
18. Tomasz W, Michal R, Miroslaw J, Barbara L. HtrA4 Protease Promotes Chemotherapeutic-Dependent Cancer Cell Death. Cells. 2019;8:1112. doi:10.3390/cells8101112.
19. Lauren P. The two histological main types of gastric carcinoma: diffuse and socalled intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49. doi:10.1111/apm.1965.64.1.31.
20. Nagtegaal ID, Odze RD, Klimstra D, Paradis V, Rugge M, Schirmacher P, et al. The 2019 WHO classification of tumours of the digestive system. Histopathology. 2020;76:182–8. doi:10.1111/his.13975.
21. Bizzaro N, Antico A, Villalta D. Autoimmunity and gastric cancer. Int J Mol Sci. 2018;19:377. doi:10.3390/ijms19020377.
22. Murata M. Inflammation and cancer. Environ. Health Prev Med. 2018;23:50. doi:10.1186/s12199-018-0740-1.
23. Wang F, Meng W, Wang B, Qiao L. Helicobacter pyloriinduced gastric inflammation and gastric cancer. Cancer Lett. 2014;345:196–202. doi:10.1016/j.canlet.2013.08.016.
24. Ralph C, Elkord E, Burt DJ, Dwyer JF, Austin EB, Stern PL, et al. Modulation of lymphocyte regulation for cancer therapy: a phase II trial of tremelimumab in advanced gastric and esophageal adenocarcinoma. Clin Cancer Res. 2010;16:1662–72. doi:10.1158/1078-0432.CCR09-2870.

25. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015;372:2509–20. doi:10.1056/NEJMoa1500596.
26. Muro K, Chung HC, Shankaran V, Geva R, Catenacci D, Gupta S, et al. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. Lancet Oncol. 2016;17:717–26. doi:10.1016/S1470-2045(16)00175-3.
27. Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate142): an open-label, multicentre, phase 2 study. Lancet Oncol. 2017;18:1182–91. doi:10.1016/S1470-2045(17)30422-9.
28. Clausen T, Kaiser M, Huber R, Ehrmann M. HTRA proteases: regulated proteolysis in protein quality control. Nat Rev Mol Cell Biol. 2011;12:152–62. https://doi.org/10.1038/nrm3065.
29. Hansen G, Hilgenfeld R. Architecture and regulation of HtrA family proteins involved in protein quality control and stress response. Cell Mol Life Sci. 2013;70:761–75. https://doi.org/10.1007/s00018-012-1076-4.
30. Lipinska B, Zylicz M, Georgopoulos C. The HtrA (DegP) protein, essential for Escherichia coli survival at high temperatures, is an endopeptidase. J Bacteriol. 1990;172:1791–7. doi:10.1128/jb.172.4.1791-1797.1990.
31. Bao W, Zhu F, Duan Y, Yang Y, Cai H. HtrA1 resensitizes multidrug-resistant hepatocellular carcinoma cells by targeting XIAP, Biomed. Pharmacother Biomed Pharmacother. 2015;70:97–102. doi:https://doi.org/10.1016/j.biopha.2014.12.044.
32. He X, Khurana A, Maguire JL, Chien J, Shridhar V. HtrA1 sensitizes ovarian cancer cells to cisplatin-induced cytotoxicity by targeting XIAP for degradation. Int J Cancer. 2012;130:1029–35. doi:https://doi.org/10.1002/ijc.26044.
33. Wenta T, Glaza P, Jarząb M, Zarzecka U, Żurawa-Janicka D, Lesner A, et al. The role of the LB structural loop and its interactions with the PDZ domain of the human HtrA3 protease. Biochim Biophys Acta Prot Proteom. 2017;1865:1141–51. doi:https://doi.org/10.1016/j.bbapap.2017.06.013.
34. Zumbrunn J, Trueb B. Primary structure of a putative serine protease specific for IGF- binding proteins. FEBS Lett. 1996;398(2–3):187–92. doi:10.1016/s0014-5793(96)01229-x.
35. De LA, De FM, Fedele V, Cobellis L, Mastrogiacomo A, Laforgia V, et al. The serine protease HtrA1 is upregulated in the human placenta during pregnancy. J Histochem Cytochem. 2004;52(7):885–92. doi:10.1369/jhc.3A6186.2004.
36. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869–74. doi:10.1073/pnas.191367098.
37. Hummel M, Bentink S, Berger H, Klapper W, Wessendorf S, Barth TF, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med. 2006;354(23):2419–30. doi:10.1056/NEJMoa055351.

38. Gnanapragasam VJ, Lophatananon A, Wright KA, Muir KR, Gavin A, Greenberg DC. Delineation of prognostic biomarkers in prostate cancer. Nature. 2001;412(6849):822–6. doi:10.1371/journal.pmed.1002063.
39. Korkola JE, Houldsworth J, Chadalavada RS, Olshen AB, Dobrzynski D, Reuter VE, et al. Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. Cancer Res. 2006;66(2):820–7. doi:10.1158/0008-5472.CAN-05-2445.
40. Clausen T, Kaiser M. Huber R, Ehrmann M. HTRA proteases: Regulated proteolysis in protein quality control. Nat Rev Mol Cell Biol. 2011;12:152–62. doi:10.1038/nrm3065.
41. Zurawa-Janicka D, Wenta T, Jarzab M, Skorko-Glonek J, Glaza P, Gieldon A, et al. Structural insights into the activation mechanisms of human HtrA serine proteases. Arch Biochem Biophys. 2017;621:6–23. doi:10.1016/j.abb.2017.04.004.
42. Singh H, Zhao M, Chen Q, Wang Y, Li Y, Kaitu LTJ, et al. Human HtrA4 Expression is Restricted to the Placenta, is Significantly Up-Regulated in Early-Onset Preeclampsia, and High Levels of HtrA4 Cause Endothelial Dysfunction. J Clin Endocrinol Metab. 2015;100:E936–45. doi:10.1210/jc.2014–3969.
43. Chen YY, Chuang PY, Chen CP, Chiu YH, Lo HF, Cheong ML, et al. Functional antagonism between high temperature requirement protein A (HtrA) family members regulates trophoblast invasion. J Biol Chem. 2014;289:22958–68. doi:10.1074/jbc.M114.576744.
44. Dynon K, Heng S, Puryer M, Li Y, Walton K, Endo Y, et al. HtrA3 as an early marker for preeclampsia: Specific monoclonal antibodies and sensitive high-throughput assays for serum screening. PLoS ONE. 2012;7:e45956. doi:10.1371/journal.pone.0045956.
45. Sun L, Hui AM, Su Q, Vortmeyer A, Kotliarov Y, et al Pastorino S, et al. Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. Cancer Cell. 2006;9(4):287–300. doi:10.1016/j.ccr.2006.03.003.
46. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, et al. X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell. 2006;9(2):121–32. doi:10.1016/j.ccr.2006.01.013.
47. Yu YP, Landsittel D, Jing L, Nelson J, Ren B, Liu L, McDonald C, et al. Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J Clin Oncol. 2004;22(14):2790–9. doi:10.1200/JCO.2004.05.158.
48. Maser RS, Choudhury B, Campbell PJ, Feng B, Wong KK, Protopopov A, et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature. 2007;447(7147):966–71. doi:10.1038/nature05886.
49. Demaria O, Vivier E. Immuno-Oncology beyond TILs: Unleashing TILCs. Cancer cell. 2020;37(4):428–30. doi:10.1016/j.ccell.2020.03.021.
50. Callahan MK, Wolchok JD. Recruit or Reboot? How Does Anti-PD-1 Therapy Change Tumor-Infiltrating Lymphocytes? Cancer cell (2019) 36(3):215–7. doi: 10.1016/j.ccell.2019.08.009.
51. Aiming TILs at Melanoma, Cervical Cancer. Cancer discovery. 2019;9(8):990. doi:10.1158/2159-8290.cd-nb2019-072.

52. Waniczek D, Lorenc Z, Snietura M, Wesecki M, Kopec A, Muc-Wierzgon M. Tumor-associated macrophages and regulatory t cells infiltration and the clinical outcome in colorectal cancer. Arch Immunol Ther Exp. 2017;65:445–54. doi:10.1007/s00005-017-0463-9.
53. Zhang H, Liu H, Shen Z, Lin C, Wang X, Qin J, et al. Tumor-infiltrating neutrophils is prognostic and predictive for postoperative adjuvant chemotherapy benefit in patients with gastric cancer. Ann Surg. 2018;267:311–8. doi:10.1097/SLA.0000000000002058.

Tables

Table 1: The association between HTRA4 expression and clinicopathological variables.

	level	high	low	p
n		187	188	
vital_status (%)	Alive	105 (56.1)	109 (58.0)	0.209
	Dead	73 (39.0)	76 (40.4)	
	unknown	9 (4.8)	3 (1.6)	
pathologic_stage (%)	blank	13 (7.0)	10 (5.3)	0.259
	Stage I	22 (11.8)	31 (16.5)	
	Stage II	64 (34.2)	47 (25.0)	
	Stage III	70 (37.4)	80 (42.6)	
	Stage IV	18 (9.6)	20 (10.6)	
pathologic_T (%)	T1	4 (2.1)	15 (8.0)	0.025
	T2	41 (21.9)	39 (20.7)	
	T3	83 (44.4)	85 (45.2)	
	T4	52 (27.8)	48 (25.5)	
	TX	7 (3.7)	1 (0.5)	
pathologic_N (%)	N0	54 (28.9)	57 (30.3)	0.045
	N1	51 (27.3)	46 (24.5)	
	N2	27 (14.4)	48 (25.5)	
	N3	44 (23.5)	30 (16.0)	
	NX	11 (5.9)	7 (3.7)	
pathologic_M (%)	M0	163 (87.2)	167 (88.8)	0.867
	M1	13 (7.0)	12 (6.4)	
	MX	11 (5.9)	9 (4.8)	
neoplasm_histologic_grade (%)	G1	2(1.1)	8(4.3)	<0.001
	G2	46 (24.6)	91 (48.4)	
	G3	135 (72.2)	84 (44.7)	
	GX	4 (2.1)	5 (2.7)	
h_pylori_infection (%)	blank	117 (62.6)	95 (50.5)	0.033
	No	60 (32.1)	85 (45.2)	
	Yes	10 (5.3)	8 (4.3)	
family_history_of_stomach_cancer (%)	blank	50 (26.7)	38 (20.2)	0.312
	NO	130 (69.5)	141 (75.0)	
	YES	7 (3.7)	9 (4.8)	
anatomic_neoplasm_subdivision (%)	Antrum/Distal	65 (34.8)	73 (38.8)	0.247
	blank	5 (2.7)	3 (1.6)	
	Cardia/Proximal	26 (13.9)	22 (11.7)	
	Fundus/Body	73 (39.0)	57 (30.3)	
	Gastroesophageal Junction	15 (8.0)	26 (13.8)	
	Other (please specify)	1 (0.5)	3 (1.6)	
	Stomach (NOS)	2 (1.1)	4 (2.1)	
measure_of_response (%)	blank	137 (73.3)	118 (62.8)	0.098
	Clinical Progressive Disease	7 (3.7)	20 (10.6)	
	Complete Response	31 (16.6)	37 (19.7)	
	Partial Response	1 (0.5)	3 (1.6)	
	Radiographic Progressive Disease	6 (3.2)	5 (2.7)	
	Stable Disease	5 (2.7)	5 (2.7)	

Figures

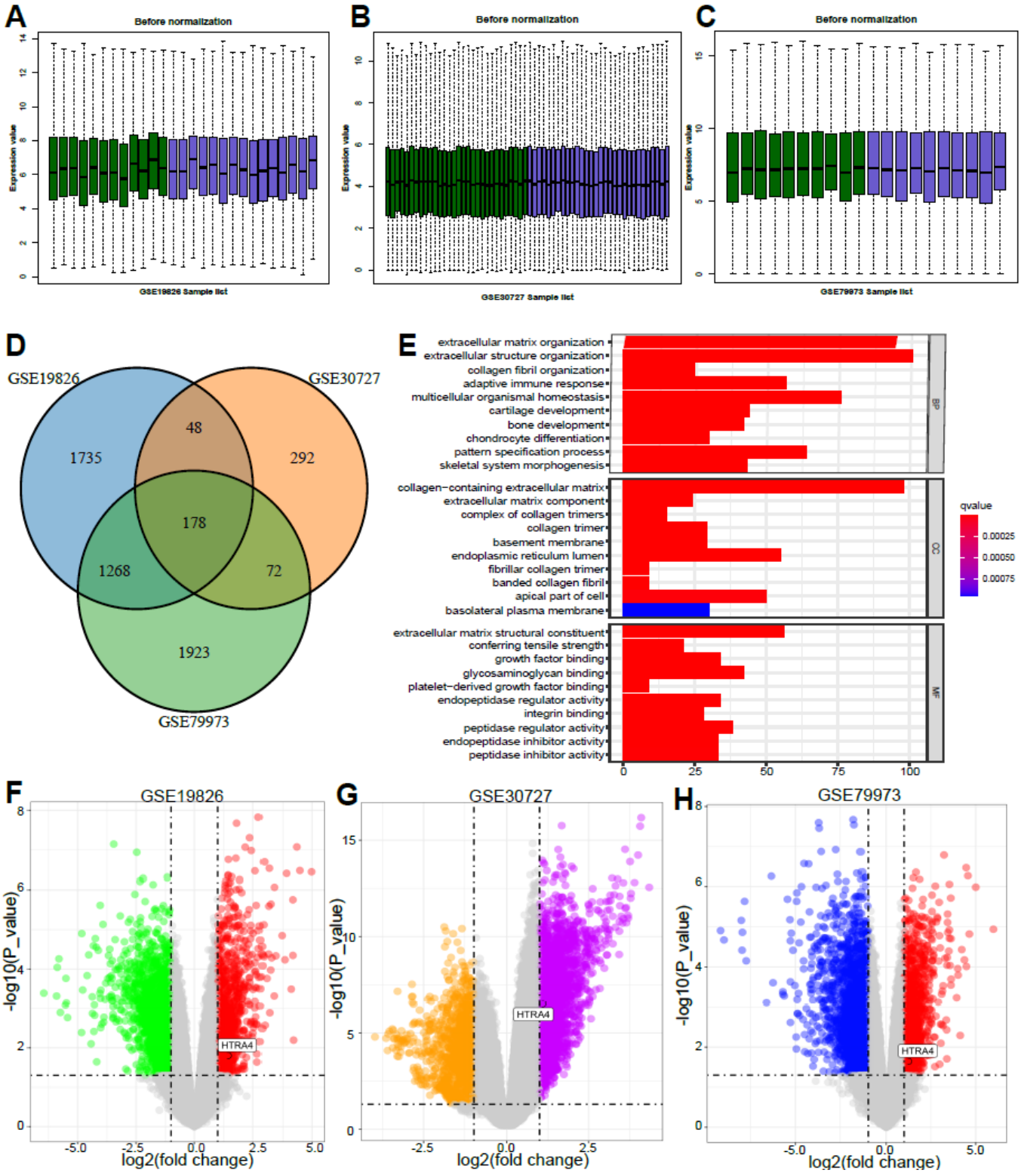

Figure 1

HTRA4 up-regulated in GC tissues in GEO datasets. (A-C) GC tissues and normal gastric tissues information from three GEO datasets GSE19826, GSE30727, and GSE79973. (D) 178 DEGs from three GEO datasets intersection between GC samples and normal tissues. (E). Top 11 of biological processes, molecular functions and cellular components enrichment related to 178 DEGs with bar graph. (F-H) Volcano ploys of the DEGs for three GEO datasets.

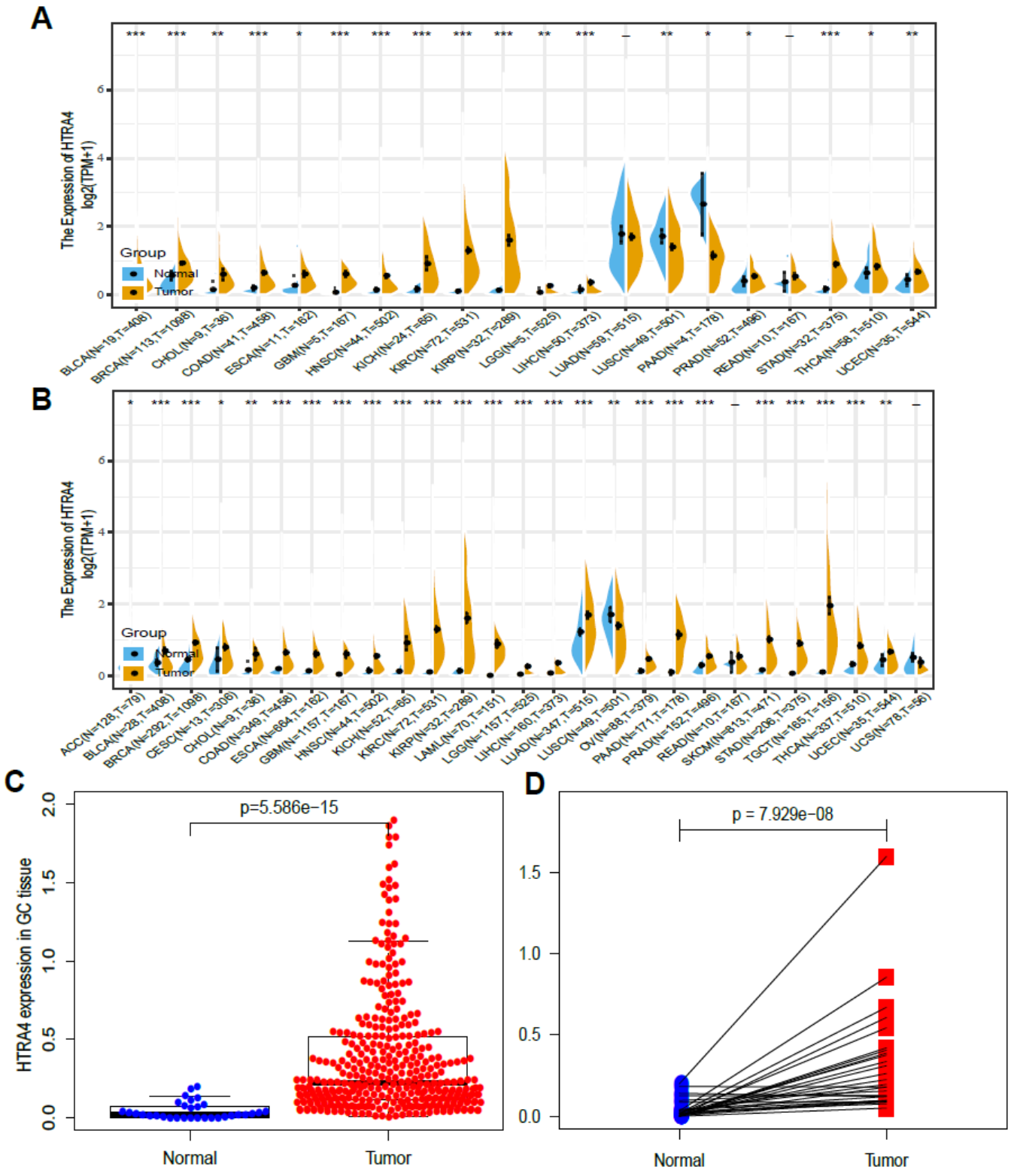

Figure 2

Verification the high expression of HTRA4 in TCGA-STAD. (A) The expression of HTRA4 of different cancers in TCGA cancer samples compared with normal samples in GTEx database. (B) The expression of HTRA4 of different cancers samples compared with normal samples in TCGA database. (C) Differentially expression level of HTRA4 in GC tissues and normal gastric tissues. (D) Differentially expression level of HTRA4 in paired GC tissues and normal gastric tissues.

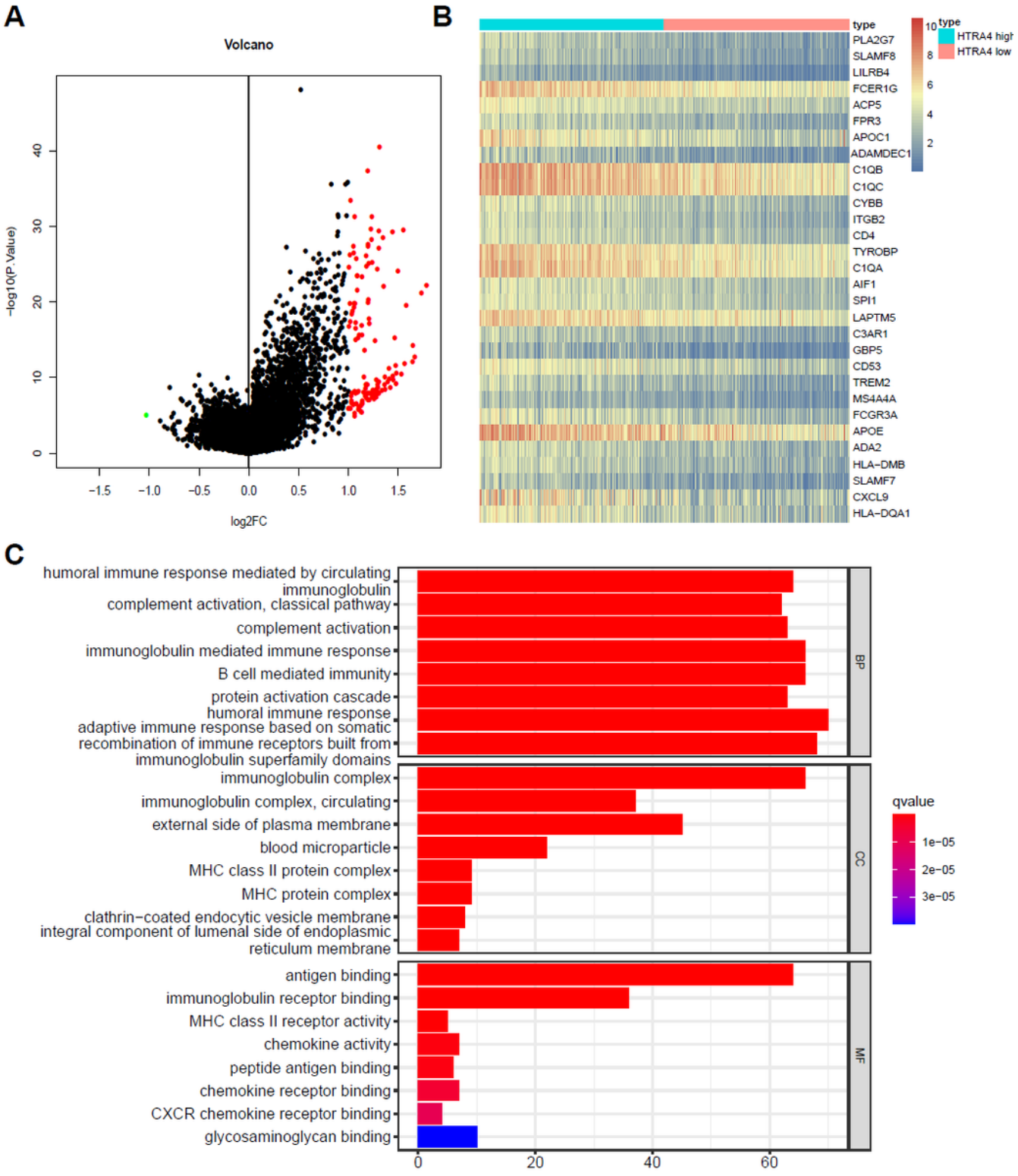

Figure 3

Enriched pathways of HTRA4 related genes in GC. (A) The volcano plot of HTRA4-related DEGs in GC. (B) The heat map of HTRA4-related DEGs in GC. (C) The molecular functions, cellular components, biological processes enrichment related to HTRA4 related genes with bar graph.

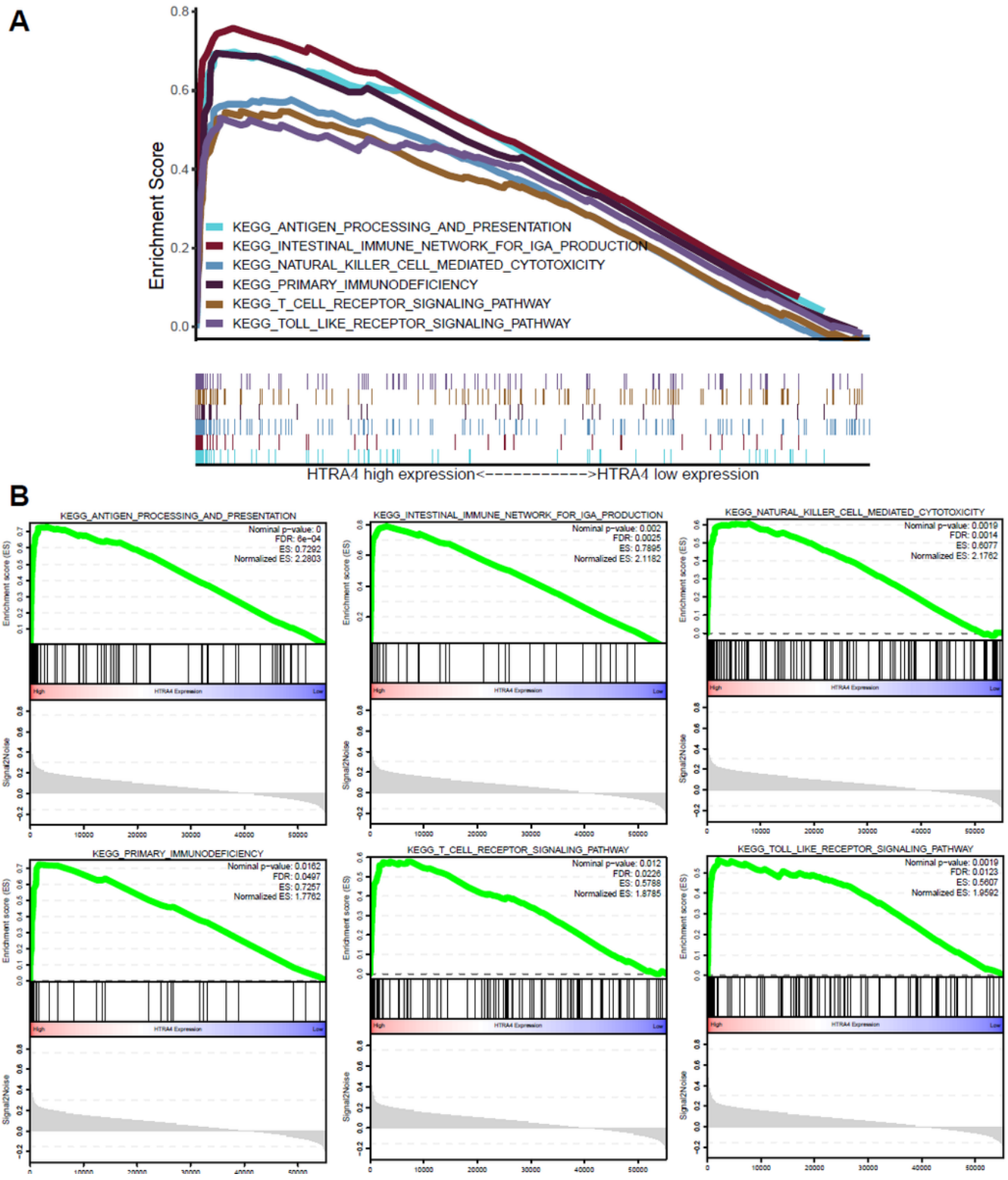

Figure 4

Significantly enriched GSEA pathways of HTRA4 related genes in GC. (A) Multiple 6 immune related GSEA pathways with line graph. (B) 6 immune related pathways enriched in HTRA4 related GC, including Antigen processing and presentation, intestinal immune network for IgA production, natural killer cell mediated cytotoxicity, T-cell receptor signaling pathway, primary immunodeficiency and Toll-like receptor signaling pathway.

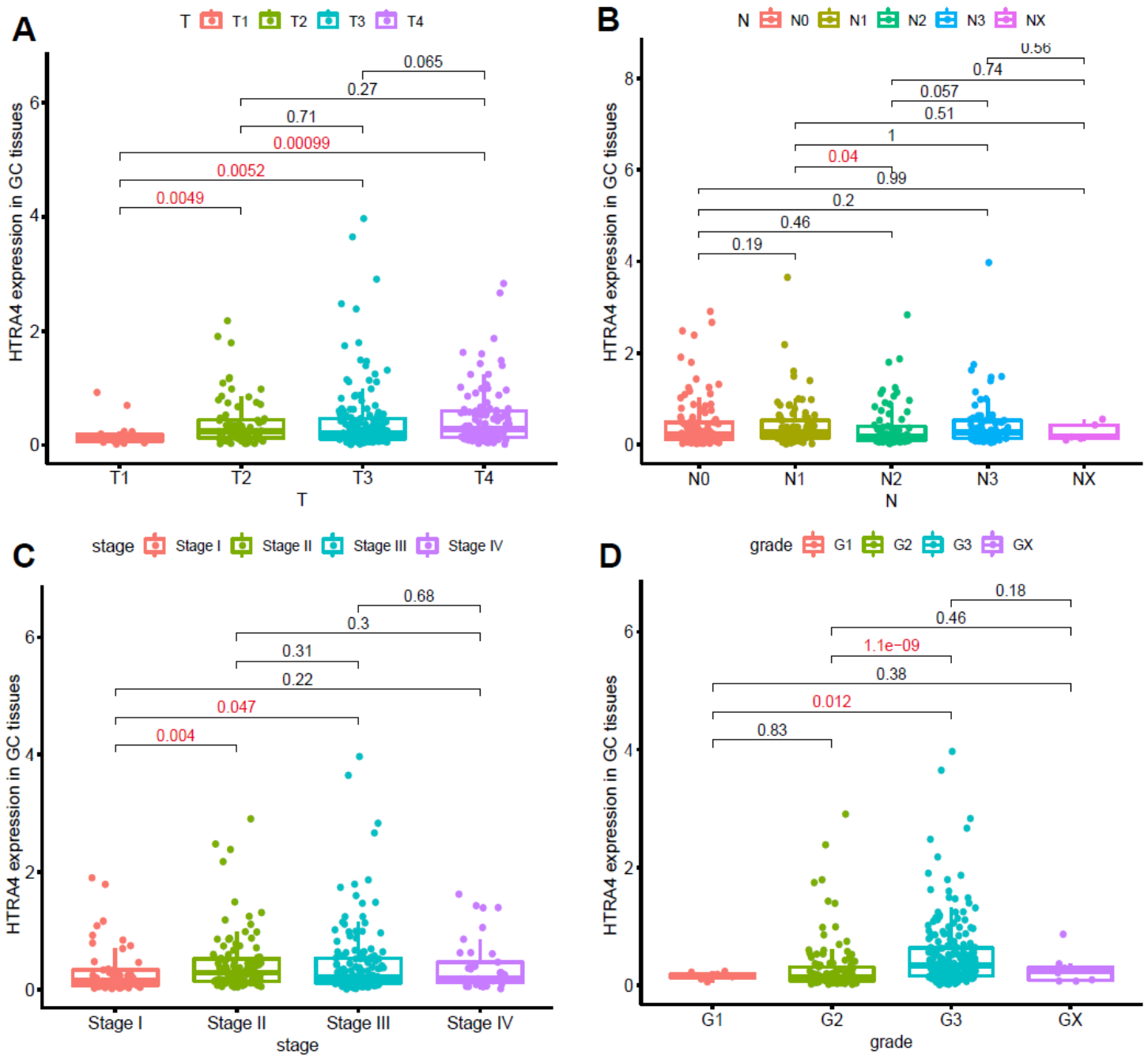

Figure 5

Relationship with HTRA4 expression and clinicopathological characteristics of GC patients. (A) Relationship between expression of HTRA4 and clinical T stage. (B) Relationship between expression of HTRA4 and clinical N stage. (C) Relationship between expression of HTRA4 and clinical pathological stage. (D) Relationship between expression of HTRA4 and clinical histological grade.

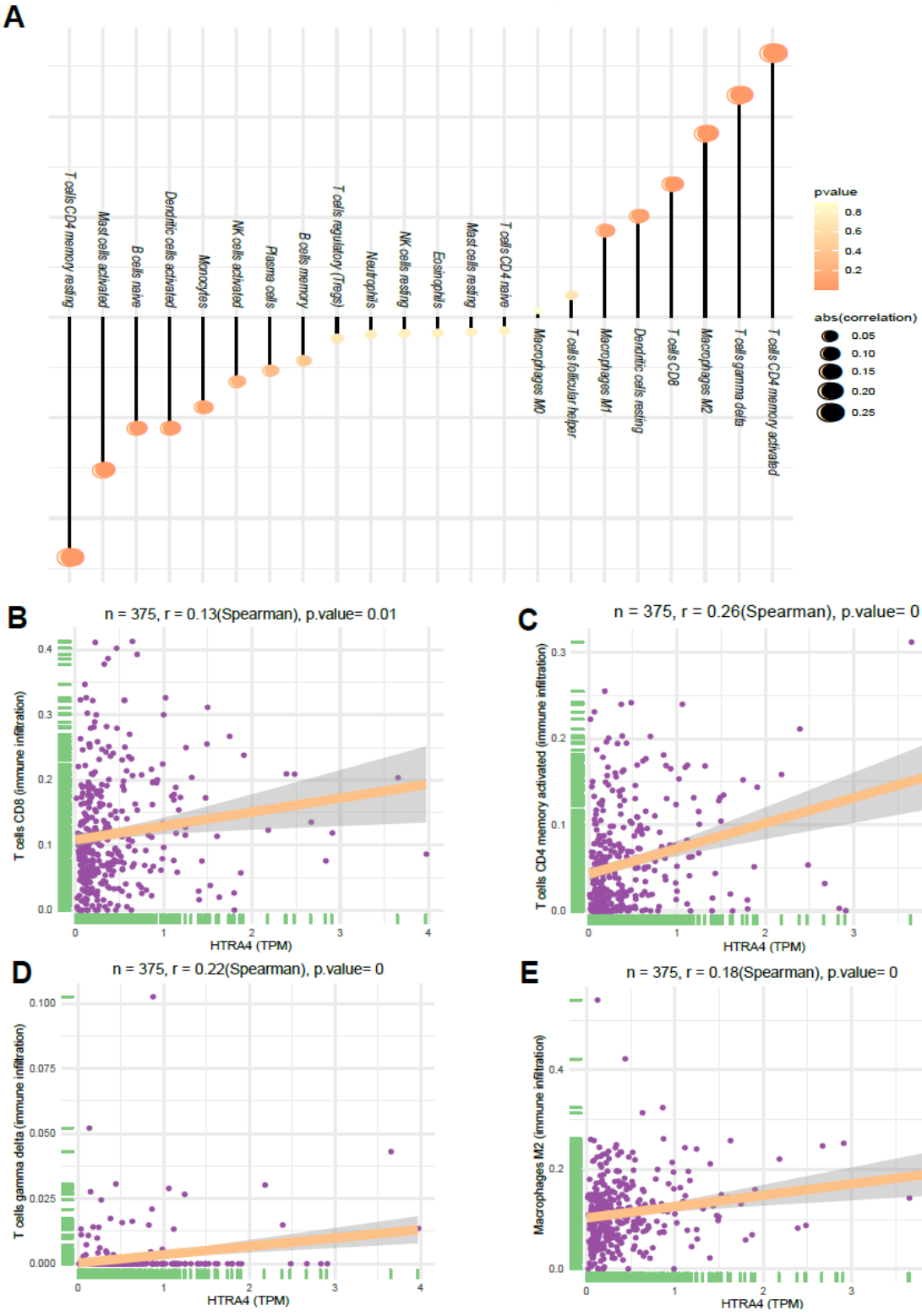

Figure 6

The expression level of HTRA4 correlated with the immune cells' infiltration in GC microenvironment. (A) Correlation between the relative abundances of 24 immune cells in GC microenvironment and HTRA4 expression level. The size of dots shows the absolute value of Spearman R. The color of dots shows the P values. (B-E) Scatter plots and correlation diagrams shows the association between expression of HTRA4 and CD8+ T cells, CD4+ memory activated T cells, gamma delta T cells and M2 macrophages.

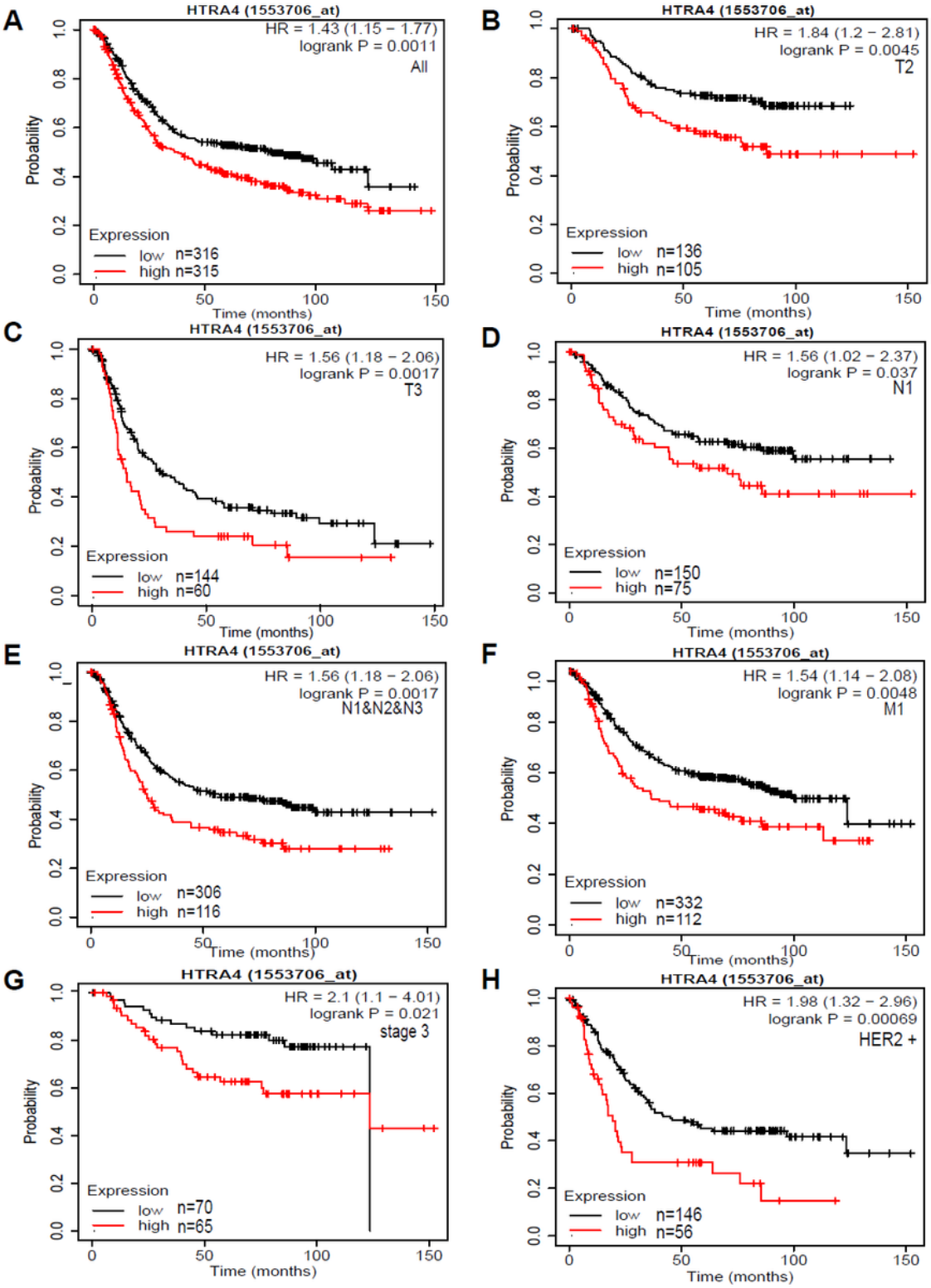

Figure 7

Kaplan-Meier survival curves suggesting the prognosis value of HTRA4 in GC. (A) Survival curves of OS between HTRA4-high and HTRA4-low patients in GC. (B-H) OS survival curves of T2,T3,N1,NI&N2&N3,M1,stage3 and HER2+ subgroups between HTRA4-high and HTRA4-low patients in GC.

Supplementary Files

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