CST1, as a prognostic biomarker for gastric cancer, is associated with tumor immune cells infiltration, invasion, and cell migration

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CST1, as a prognostic biomarker for gastric cancer, is associated with tumor immune cells infiltration, invasion, and cell migration

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Abstract

Background: *CST1*, acting as an oncogene, plays a critical role in tumor microenvironment (TME) remodeling. However, the function of *CST1* in gastric cancer (GC) remains elusive. The purpose of this study was to explore the prognostic significance, tumor immune cell infiltration and biological functions of *CST1* as a novel therapeutic candidate biomarker for GC.

Methods: Differentially expressed genes (DEGs) were identified using the library ("limma") package of R software. Gene Set Enrichment Analysis (GSEA) was applied to explore the molecular function of *CST1*. *CST1* expression in GC cell lines was detected using quantitative real-time PCR (RT-qPCR) in vitro. Cell proliferation, migration, and invasion were measured using the cell counting kit-8 assay and

Transwell assay.

Results: A total of 142 common DEGs were screened out in four datasets. Four overlapped DEGs (*CST1*, *CLDN4*, *CLDN7* and *CLDN1*) were identified and validated in TCGA database. *CST1* was the most significant to be selected for further study. Bioinformatics analysis revealed that *CST1* expression was associated with in tumor immune cell infiltration and clinical factors in GC. The survival analysis showed that *CST1* overexpression predicted poor outcome in GC patients. In Cancer Cell Line Encyclopedia (CCLE) database, the expression of *SULT1C4*, *PAGE4* and *FPR3* was significant positively related to *CST1*. *CST1* was also upregulated in GC cells AGS by qRT-PCR. Finally, we found that *CST1* promoted cell proliferation, invasion, and migration in GC.

Conclusion: This study reveals that *CST1* is a promising candidate biomarker for prognosis and potential therapy target for GC.

Keywords: gastric cancer, *CST1*, tumor immune cell infiltration, prognosis, invasion

1. Introduction

Gastric cancer (GC) is a heterogeneous disease and one of the most common cancers worldwide[1]. The five-year survival rate remains low for most GC patients are diagnosed at an advanced stage[2,3]. Furthermore, the prognosis of advanced GC remains unappealing despite the use of standard therapies such as chemotherapy and biological agents. Thus, it's essential to find effective biomarkers for GC patients. Increasing evidence indicated that novel GC biomarkers including different non-coding RNAs and genes have emerged[4-7]. Although some biomarkers are validated in clinical practice, the

ability in predicting prognosis in GC is still elusive[8].

Immune-related mechanisms play an important role in GC development[9,10]. For instance, some reports showed that PD-L1 positivity, high CD3+ T cell and high CD8+ T cell were independent prognostic factors for overall survival in GC[11]. Recently, immunotherapeutic strategies are considered as a promising treatment of GC[12]. Therefore, the biomarkers associated with tumor immune cells infiltration may be novel approaches for immunotherapy[13].

In the current study, to identify potential biomarkers of GC, the DEGs of GC were investigated and validated by integrating GC gene expression data from Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA) database. *CST1* was selected to be a gene of interest. Recently, *CST1* has been reported to be involved in the development of several cancers. Tumor immune cells infiltration levels were calculated using CIBERSORT algorithm. *CST1* overexpression was associated with tumor immune cells infiltration and prognosis. And subsequently in vitro experiments, we found that high expression of *CST1* promoted cell proliferation, migration, and invasion in GC.

2. Materials and methods

2.1 Data downloaded from the GEO and TCGA cohort

GSE19826, GSE56807, GSE63809 and GSE33335 genes expression microarray datasets including 78 pairs of GC tissues and non-tumorous tissues samples were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). RNA-seq data and survival information about 375

GC tissues and 32 adjacent gastric tissues samples were collected from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov).

2.2 Identification and integration of differentially expressed genes

DEGs were identified using edgeR package. DEGs from downloaded GEO and TCGA datasets were analyzed by the limma software package. Samples with a corrected *p* value < 0.05 and log|(FC)|≥2 were identified DEGs. Common DEGs of GSE19826, GSE56807, GSE63809 and GSE33335 were screened out in Venn diagram were constructed using online VENNY2.1 database. The RRA package, an openly approach available in the comprehensive R network, was downloaded to analyse the DEGs of GEO datasets (*p*-value of 0.05). The RRA method is based on the hypothesis that each gene is randomly ordered in each experiment.

2.4 GSEA enrichment analysis

Samples from TCGA were divided into two groups based on the expression of *CST1* (median value), and gene set enrichment analysis (GSEA) software[14] was applied to explore the enrichment pathways of *CST1* in the two groups. P-value <0.05 and false discovery rate (FDR) <0.25 were the cut-off criteria for GSEA.

2.5 Correlation of CST1 expression with genes in CCLE databse

The correlation of *CST1* expression with other genes were determined and validated in 881 samples from Cancer Cell Line Encyclopedia (CCLE) database (Broad Institute, Novartis Institutes for Biomedical Research)[15]. R software of biocLite ("limma") was performed to analyze the correlation between the expression of *CST1* and other genes with the standard of corFilter=0.5 and pFilter=0.001.

2.6 CIBERSORT

Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT), is a computational approach characterizing tumor immune cells composition from their gene expression profiles[16]. We obtained the infiltration levels of 22 immune cell types. The correlation of CST1 expression with tumor immune cells infiltration was analyzed using R software.

2.7 Kaplan Meier plotter

The Kaplan Meier plotter[17] (http://kmplot.com/analysis/) is capable to evaluate the effect of 54k genes (mRNA, miRNA, protein) on survival in 21 cancer types including breast (n=7,830), ovarian (n=2,190), lung (n=3,452), and gastric (n=1,440) cancer. Sources for the databases include GEO, EGA, and TCGA. We investigated the prognostic value of *CST1* with clinical factors using Kaplan Meier plotter.

2.8 Cell culture and transfection

GC cells AGS and normal cells GES-1 were purchased from the Tianjin Cosmo Biotechnology Co., Ltd. (Tianjin, China). AGS cells were cultured in RPMI 1640 medium (GIBCO, USA). All cells were cultured at 37 °C for 18-24 hours in a humidified incubator containing 5% CO2. AGS cells were transfected with plasmids using Lipofectamine 2000 Reagent (Invitrogen, USA) based on the manufacturer's instructions. The expression of *CST1* in the transfected cells was detected by quantitative PCR (qPCR). Target sequences for siRNAs were CAGAGAAAGCAAUG UUUAAGA (si-*CST1*-F), UUAAACAUUGCUUUCUCUGUG (si-*CST1*-R), UUCUC CGAACGUGUCACGUTT (si-NC-F) and ACGUGACACGUUCGGAGAATT (si-

NC-R).

2.9 Quantitative real-time PCR (RT-qPCR) analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and synthesized into cDNA using M-MLV reverse transcriptase (Takara, Japan) following the manufacturer's instructions. qRT-PCR was performed using SYBR Green assay (Roche). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as controls. The primers sequences were as follows:

TGCGCCAAGAGACAGACAGAGAA (*CST1*-F),

TGCGCCAAGAGACAGACAGAGAA (*CST1*-R),

GGTGAAGGTCGGTGTGAACG (*GAPDH*-F),

CTCGCTCCTGGAAGATGGTG (*GAPDH*-R).

2.10 Cell proliferation, migration, and invasion analyses

The transfected cells were cultured to 5×10^3 cells / well in 96-well plate. Then the mixture of CCK8 solution and DMEM with 100ul ratio of 1:10 was added to each well and cultured for 2.5 hours. And the absorbance of the sample was determined by enzyme labeling instrument (wavelength=450mn). For migration analysis, the cells were washed with PBS, digested (0.02%EDTA, 0.25% trypsin) and underwent the Transwell experiment. In each group, 4×10^5 AGS cells were resuspended in 200 μL serum-free culture medium and added into Transwell chamber. After incubation at 37 ℃ for 72 hours, the cells inside the well were washed off. The reverse side of Transwell chamber was fixed with a mixture of methanol and glacial acetic acid at 3:1 for 30 min and dyed with violet dye solution for 15min for final observation under the microscope. And for cell invasion analysis, 50 μL dissolved matrigel glue was added to each well,

and after digestion (0.02%EDTA, 0.25% trypsin), 4×10^5 AGS cells were resuspended in 200 μL serum-free culture medium and added into Transwell chamber. The culture medium containing 20% Gibico serum was added to the lower chamber. And after incubation at 37 ℃ for 24 hours, the cells inside the well were washed off. The reverse side of Transwell chamber was fixed with a mixture of methanol and glacial acetic acid at 3:1 for 30 min and dyed with violet dye solution for 15min for final observation under the microscope.

2.11 Statistical analysis

Survival R package and Kaplan-Meier curve were used for overall survival analysis. P values <0.05 were considered as statistically significant. All analyses were conducted using R software (version 3.3.2). Statistical significance was described as follows: ns, not significant; *p < .05; **p ≤ .01; ***p ≤ .001.

3. Results

3.1 Identification of DEGs with integrated GEO datasets in GC

GC expression microarray datasets GSE19826, GSE56807, GSE63809 and GSE33335 were standardized, and the results were shown in Figure S1. As the volcano plots showed, gene expression profiles from GSE19826 identified 210 DEGs with 72 genes upregulated and 138 genes downregulated in GC samples compared with the expression in normal control tissues (Figure. 1A). From GSE56807 data, we recognized 126 DEGs, of which 104 genes were upregulated and 22 genes were downregulated in GC (Figure 1B). 47 DEGs were ascertained in GSE63809, comprising 18 genes upregulated and 29 genes downregulated in GC (Figure 1C). There were 49 DEGs identified in GSE33335

with 6 genes upregulated and 43 genes downregulated (Figure 1D). Integrating four GEO datasets, we identified 142 common DEGs with 51 upregulated genes and 91 downregulated genes using RobustRankAggreg packages of R software. The top 20 upregulated and the top 20 downregulated genes were revealed in Figure 1E.

3.2 Identification and validation of four common genes

We determined four overlapped DEGs (*CST1*, *CLDN4*, *CLDN7* and *CLDN11*) from common upregulated genes in four datasets, and the result was shown in Figure 2A. Gene expression profiles from paired adjacent and tumor samples in TCGA database were used to validate the expression of four common genes in GC samples (Figure 2B-2E). The result showed that *CST1*, *CLDN4*, *CLDN7* and *CLDN1* were differentially overexpressed in TCGA database, and this was consistent with the GEO database. Based on the result of validation, *CST1* was selected as the most significant DEG and potential biomarkers for subsequent analysis.

3.3 GSEA enrichment analysis and correlation of *CST1* in CCLE database

Additionally, the potential molecular functions of *CST1* were evaluated using GSEA software. In *CST1* overexpression group, results suggested that the top 3 most involved significant pathways of *CST1* included long term potentiation, phosphatidylinositol signaling system and calcium signaling pathway (Figure 3A). Results suggested that the top 3 most involved significant pathways of *CST1* included systemic lupus erythematosus (SLE), RNA polymerase and proteasome (Figure 3B).

To explore the correlation of *CST1* expression with other genes, CCLE database was applied to performed the next analysis. We found that *SULT1C4*, *PAGE4*, and *FPR3* were the top 3 genes which were positively related with *CST1* expression in CCLE database. The result was presented in Figure 3C-3F.

3.4 *CST1*-overexpression was associated with immune cell infiltration in GC

Using CIBERSORT algorithm in TCGA database, we subsequently investigated the relationship between *CST1* and tumor immune cell infiltration. High *CST1* mRNA expression had significant negative correlations with B cells memory (R= −0.27, p = 3.5e−5; Figure 4A), B cell naive (R = −0.16, p = 0.017; Figure 4B) and dendritic cells activated (R = −0.18, p = 0.0051; Figure 4C). *CST1* expression had a positive correlation with the infiltration of dendritic cells resting (R = 0.15, p = 0.026; Figure 4D), NK cells activated (R = 0.14, p = 0.035; Figure 4E), T cells memory activated (R = 0.17, p = 0.01; Figure 4F), macrophages M1 (Cor = 0.26, p = 5.7e−05; Figure 4G), macrophages M0 (Cor = 0.19, p = 0.0049; Figure 4H) and macrophages M2 (Cor = 0.14, p = 0.034; Figure 4I).

3.5 *CST1* overexpression was associated with poor prognosis in GC patients

The association of *CST1* expression with overall survival in GC was evaluated by using the Kaplan Meier plotter. A total of 876 samples of GC datasets included 601 low expression and 275 overexpression of *CST1*. Result revealed that GC patients with high expression of *CST1* had a markedly lower overall survival rate (Figure 5A). In 8 subgroups (female, male, lauren classification intestinal, lauren classification intestinal mix, HER-2(+), poorly differentiated group, moderately differentiated group and well differentiated), we found that high expression of *CST1* had poor prognosis (Figure 5B-5I). As shown in Figure S2A-2H, high expression of *CST1* was significant negatively associated with prognosis in M0, M1, surgery only, N1, N2, N3, T3, T4 groups. *CST1* upregulated was also associated with poor outcome in post-progression survival (PPS) in GC (Figure S2I).

3.6 *CST1* suppression inhibits cell proliferation, migration, and invasion

To elucidated the molecular function of *CST1* in GC, firstly, *CST1* expression in gastric

cancer cells and normal gastric cells were evaluated in this study. Next, to verify the results of above bioinformatics analysis, *CST1* was significantly overexpressed in GC cells line AGS than in GES-1 cells line (Figure 6A, 6B). CCK8 assay was applied to evaluate the effect of *CST1* on proliferation, and the results found that siRNA-*CST1* markedly inhibited cell proliferation abilities. The result was presented in Figure 6C. Transwell migration and invasion assays also showed that suppression of *CST1* significantly inhibited cell migration and invasion (Figure 6D, 6E). These results suggested that *CST1* played an important role in gastric cancer development.

4. Discussion

Gastric cancer ranks the fifth most frequently diagnosed cancer and the third leading cause of cancer death[18]. Despite gastric cancer (GC) has shown a decreasing incidence in the last decades, the median overall survival time remains 10-12 months[19]. Recently, thanks to advances in sequencing and bioinformatics techniques, novel molecular characterizations and approaches may facilitate diagnosis and prognosis in GC[8,20].

Resulting from emerging studies, novel biomarkers associated with the tumor immune reaction have been identified to explore the characteristics of tumor microenvironment and the effect of its alteration on immunotherapy. For instance, an immune-related gene signature consisting of 16 genes was found predicted prognosis of gastric cancer[21]. Increasing findings were also pointed out regarding the clinical prognostic and predictive role of PD-1, PD-L1, PD-L2 expression and microsatellite instability status in GC[22].

In the present study, we integrated four microarray datasets GSE19826, GSE56807, GSE63809 and GSE33335 from GEO database and obtained four common genes *CST1*, *CLDN4*, *CLDN7* and *CLDN1*. We then validated the expression of four common DEGs in TCGA database, and the result was consistent with the results in GEO databases.

Membranous overexpression of claudin-4 (encoded by CLDN4) was reported associated with better patient prognosis in GC[23,24]. *CLDN7* (claudin-7) was demonstrated to involve in the development of human lung cancer and renal cell carcinoma[25,26]. However, the exact roles of *CLDN7* in GC tumorigenesis are largely unknown. Additionally, *CLDN1* was reported to promote cancer cell proliferation and metastasis[27-29].

CST1 gene encodes a secretory protein called *CST1*, also known as Cystatin SN, comprises a class of cysteine peptidase inhibitor presumed to mediate protective functions at various locations[30]. Previous studies showed that *CST1* was associated with the development of cancers including esophageal squamous cell carcinoma[31], breast cancer[32].

In this study, we found that *CST1* was the most significantly DEG in GC. GSEA enrichment analysis revealed that the potential molecular functions of high *CST1* level were associated with long term potentiation, phosphatidylinositol signaling system and calcium signaling pathway in GC. Numerous studies have demonstrated that alteration of calcium signaling pathway can affect tumorigenesis in GC[33,34].

To further explore the molecular role of *CST1*, co-expressed genes of *CST1* were identified using CCLE database. We found that *SULT1C4*, *PAGE4* and *FPR3* were the top 3 genes which were positively related with *CST1* expression, but *PSMD5*, *ANXA5* and *SGSH* were the top 3 genes that were negatively related with *CST1* expression.

Increasing evidence suggested that immune cell infiltration could affect tumor progression, recurrence, and clinical outcome in GC[35]. Yan, B.et al. reported that epithelium-derived cystatin SN enhanced eosinophil activation and infiltration through IL-5 in patients with chronic rhinosinusitis with nasal polyps[36].

CST4 is a cysteine protease inhibitor that is a secreted protein composed of 141 amino

acids. Wang, S. et al indicated that *CST4* overexpression affected tumor immune cell infiltration and the survival of patients with ovarian cancer (OC)[37]. The relationship of *CST1* expression with tumor immune cell infiltration remains unclear.

This research suggested that *CST1* expression was significantly associated with immune cell infiltration. Therefore, *CST1* may participate in remodeling tumor microenvironment by regulating tumor immune cell infiltration. However, further studies are needed to elucidate the interaction network between *CST1* and infiltrating immune cells.

CST1, as a prognostic biomarker, could predict the outcome in cancer patients, such as in pancreatic cancer[38] and colorectal cancer[39]. In the current study, Kaplan-Meier analysis indicated that overexpression of *CST1* was associated with a poor prognosis in GC patients. *CST1* has been proven to regulate tumor cell migration and invasion. Kim J et al. showed that HOXC10 directly bounded to *CST1* promoter regions to promote cell proliferation and migration in gastric cancer. This study also revealed that *CST1* could promote cell proliferation and migration in GC cell line MGC803 [40]. But its role in tumor development remains to be verified. We also validated that *CST1* was overexpressed in GC cell line AGS by comparison with that in normal human gastric epithelial GES-1 cell line using qRT-PCR. In addition, in vitro experiments showed knock-down of *CST1* inhibited cell proliferation, migration and invasion, indicating that *CST1* could be utilized as a therapeutic target for GC patients.

Conclusion

CST1 as a potential biomarker was associated with tumor immune cell infiltration and prognosis promoting cell proliferation, migration and invasion. This study suggested that *CST1* may be a prognostic biomarker and therapeutic target of GC.

Declarations：

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jianming Wei and Xibo Gao analyzed four microarrays expression from the GEO database regarding GC and performed RT-qPCR cell culture, transfection, CCK-8-Cell proliferation assay, transwell migration and invasion assay. Jianming Wei and Zhiwang Wei were major contributors in writing the manuscript. Tong Liu and Li Lu conceived and designed the study. All authors read and approved the final manuscript.

References

1. Dang Y, Ouyang X, Zhang F, et al. Circular RNAs expression profiles in human gastric cancer. *Sci Rep.* 2017;7(1):9060.
2. Koh J, Lee KW, Nam SK, et al. Development and Validation of an Easy-to-Implement, Practical Algorithm for the Identification of Molecular Subtypes of Gastric Cancer: Prognostic and Therapeutic Implications. *Oncologist.* 2019.
3. Price TJ, Shapiro JD, Segelov E, et al. Management of advanced gastric cancer. *Expert Review of Gastroenterology & Hepatology.* 2012;6(2):199-209.

4. Wei J, Wang J, Gao X, Qi F. Identification of differentially expressed circRNAs and a novel hsa_circ_0000144 that promote tumor growth in gastric cancer. *Cancer Cell Int.* 2019;19:268.

5. Cao S, Lin L, Xia X, Wu H. lncRNA SPRY4-IT1 Regulates Cell Proliferation and Migration by Sponging miR-101-3p and Regulating AMPK Expression in Gastric Cancer. *Molecular therapy Nucleic acids.* 2019;17:455-464.

6. Martins MR, Almeida RS, Lucena-Silva N, et al. MicroRNA expression profiling provides novel insights into immune-related pathways involved in gastric cancer. *Med Oncol.* 2019;36(9):81.

7. Lu R, Zhao G, Yang Y, Jiang Z, Cai J, Hu H. Inhibition of CD133 Overcomes Cisplatin Resistance Through Inhibiting PI3K/AKT/mTOR Signaling Pathway and Autophagy in CD133-Positive Gastric Cancer Cells. *Technol Cancer Res Treat.* 2019;18:1533033819864311.

8. Necula L, Matei L, Dragu D, et al. Recent advances in gastric cancer early diagnosis. *World J Gastroenterol.* 2019;25(17):2029-2044.

9. Kwak Y, Seo AN, Lee HE, Lee HS. Tumor immune response and immunotherapy in gastric cancer. *J Pathol Transl Med.* 2020;54(1):20-33.

10. Chang W-J, Du Y, Zhao X, Ma L-Y, Cao G-W. Inflammation-related factors predicting prognosis of gastric cancer. *World J Gastroenterol.* 2014;20(16):4586-4596.

11. Angell HK, Lee J, Kim KM, et al. PD-L1 and immune infiltrates are differentially expressed in distinct subgroups of gastric cancer. *OncoImmunology.* 2019;8(2):e1544442.

12. Coutzac C, Pernot S, Chaput N, Zaanan A. Immunotherapy in advanced gastric cancer, is it the future? *Critical Reviews in Oncology/Hematology.* 2019;133:25-32.

13. Lazăr DC, Avram MF, Romoșan I, Cornianu M, Tăban S, Goldiș A. Prognostic significance of tumor immune microenvironment and immunotherapy: Novel insights and future perspectives in gastric cancer. *World J Gastroenterol.* 2018;24(32):3583-3616.

14. Yang Z, Liang X, Fu Y, et al. Identification of AUNIP as a candidate diagnostic and prognostic biomarker for oral squamous cell carcinoma. *EBioMedicine.* 2019;47:44-57.

15. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables

predictive modelling of anticancer drug sensitivity. *Nature.* 2012;483(7391):603-607.

16. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods.* 2015;12(5):453-457.

17. Nagy A, Lanczky A, Menyhart O, Gyorffy B. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. *Sci Rep.* 2018;8(1):9227.

18. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424.

19. Digklia A, Wagner AD. Advanced gastric cancer: Current treatment landscape and future perspectives. *World J Gastroenterol.* 2016;22(8):2403-2414.

20. Cancer Genome Atlas Research N. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature.* 2014;513(7517):202-209.

21. Jiang B, Sun Q, Tong Y, et al. An immune-related gene signature predicts prognosis of gastric cancer. *Medicine (Baltimore).* 2019;98(27):e16273.

22. Vrána D, Matzenauer M, Neoral Č, et al. From Tumor Immunology to Immunotherapy in Gastric and Esophageal Cancer. *Int J Mol Sci.* 2018;20(1):13.

23. Luo J, Wang H, Chen H, Gan G, Zheng Y. CLDN4 silencing promotes proliferation and reduces chemotherapy sensitivity of gastric cancer cells through activation of PI3K/Akt signaling pathway. *Exp Physiol.* 2019.

24. Kwon MJ, Kim SH, Jeong HM, et al. Claudin-4 overexpression is associated with epigenetic derepression in gastric carcinoma. *Lab Invest.* 2011;91(11):1652-1667.

25. Lu Z, Kim DH, Fan J, et al. A non-tight junction function of claudin-7-Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment. *Mol Cancer.* 2015;14:120.

26. Li Y, Gong Y, Ning X, et al. Downregulation of CLDN7 due to promoter hypermethylation is associated with human clear cell renal cell carcinoma progression and poor prognosis. *J Exp Clin Cancer Res.* 2018;37(1):276.

27. Wu J, Gao F, Xu T, et al. CLDN1 induces autophagy to promote proliferation and metastasis

of esophageal squamous carcinoma through AMPK/STAT1/ULK1 signaling. *J Cell Physiol.* 2020;235(3):2245-2259.

28. Mahati S, Bolati D, Yang Y, Mao R, Zhang H, Bao Y. TMPRSS4 promotes cancer stem cell traits by regulating CLDN1 in hepatocellular carcinoma. *Biochem Biophys Res Commun.* 2017;490(3):906-912.

29. Mahati S, Xiao L, Yang Y, Mao R, Bao Y. miR-29a suppresses growth and migration of hepatocellular carcinoma by regulating CLDN1. *Biochem Biophys Res Commun.* 2017;486(3):732-737.

30. Dickinson DP, Thiesse M, Hicks MJ. Expression of type 2 cystatin genes CST1-CST5 in adult human tissues and the developing submandibular gland. *DNA Cell Biol.* 2002;21(1):47-65.

31. Chen YF, Ma G, Cao X, et al. Overexpression of cystatin SN positively affects survival of patients with surgically resected esophageal squamous cell carcinoma. *BMC Surg.* 2013;13:15.

32. Dai DN, Li Y, Chen B, et al. Elevated expression of CST1 promotes breast cancer progression and predicts a poor prognosis. *Journal of molecular medicine (Berlin, Germany).* 2017;95(8):873-886.

33. Panneerpandian P, Rao DB, Ganesan K. Calcium channel blockers lercanidipine and amlodipine inhibit YY1/ERK/TGF-β mediated transcription and sensitize the gastric cancer cells to doxorubicin. *Toxicol In Vitro.* 2021;74:105152.

34. Gao N, Yang F, Chen S, Wan H, Zhao X, Dong H. The role of TRPV1 ion channels in the suppression of gastric cancer development. *#N/A.* 2020;39(1):206.

35. Lv Y, Zhao Y, Wang X, et al. Increased intratumoral mast cells foster immune suppression and gastric cancer progression through TNF-alpha-PD-L1 pathway. *Journal for immunotherapy of cancer.* 2019;7(1):54.

36. Yan B, Lou H, Wang Y, et al. Epithelium-derived cystatin SN enhances eosinophil activation and infiltration through IL-5 in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol.* 2019;144(2):455-469.

37. Wang S, Wang C, Liu O, Hu Y, Li X, Lin B. Prognostic value of immune-related cells and

genes in the tumor microenvironment of ovarian cancer, especially CST4. *Life sciences.* 2021;277:119461.

38. Jiang J, Liu HL, Liu ZH, Tan SW, Wu B. Identification of cystatin SN as a novel biomarker for pancreatic cancer. *Tumour Biol.* 2015;36(5):3903-3910.

39. Yoneda K, Iida H, Endo H, et al. Identification of Cystatin SN as a novel tumor marker for colorectal cancer. *Int J Oncol.* 2009;35(1):33-40.

40. Kim J, Bae DH, Kim JH, Song KS, Kim YS, Kim SY. HOXC10 overexpression promotes cell proliferation and migration in gastric cancer. *Oncol Rep.* 2019;42(1):202-212.

Figures legends

Figure 1. Differential expression of data in GEO database.

(A) GSE19826 data; (B) GSE56807 data; (C) GSE63089 data; (D) GSE33335 data (the red points represent upregulated DEGs. The green points represent downregulation of the expression of DEGs. The black points represent genes with no significant difference); (E) Heatmap of top 20 upregulated and downregulated DEGs. (Red represents logFC >0, blue represents logFC <0, and the values in the box represent the logFC values.)

Figure 2. Identification and validation of four overlapped DEGs from integrated datasets.

(A) The four common DEGs in the four GEO datasets. (B) *CST1* validation in TCGA database. (C) *CLDN4* validation in TCGA database. (D) *CLDN7* validation in TCGA database. (E) *CLDN1* validation in TCGA database.

Figure 3. GSEA enrichment analysis and co-expression with *CST1*

(A) GSEA analysis showed that KEGG pathways enriched in high *CST1* expression group, (B) GSEA enrichment analysis showed that KEGG pathways enriched in low

CST1 expression group, (C) The heatmap of genes co-expressed with *CST1*, (D) Correlation of *SULT1C4* with *CST1* expression, (E) Correlation of *PAGE4* with *CST1* expression, (F) Correlation of *FPR3* with *CST1* expression.

Figure 4. Evaluation of *CST1*-related tumor immune cells infiltration in GC

The correlation of CST1 with tumor immune cells infiltration using TCGA database. (A) B cells memory, (B) B cell naive, (C) dendritic cells activated, (D) dendritic cells resting, (E) NK cells activated, (F) T cells memory activated, (G) macrophages M1, (H) macrophages M0 and (I) macrophages M2.

Figure 5. *CST1* overexpression was associated with poor outcome in GC.

(A) *CST1* was significantly related with overall survival. Survival curves also showed patients with high *CST1* expression significantly had poor outcome in subgroups. (B) Female, (C) Male, (D) lauren classification intestinal, (E) lauren classification intestinal mix, (F) HER-2(+), (G) poorly differentiated group, (H) moderately differentiated group and (I) well differentiated.

Figure 6. Reduced expression of *CST1* decreased cell proliferation, migration, and invasion in GC cell line.

(A) *CST1* expression in AGS and GES cell lines, (B) *CST1* expression in AGS cell line after transfection of si-*CST1* and negative control, (C) *CST1* promoted cell proliferation, Transwell chambers assay with Matrigel showed *CST1* overexpression promoted (D) cell invasion and (E) cell invasion in AGS cell line. (NC, negative control; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

Figure S1 Standardization of four GEO datasets for GC.

(A) The standardization of GSE19826 data, (B) the standardization of GSE56807 data, (C) the standardization of GSE63089 data, (D) the standardization of GSE33335 data. (The blue bar represents the data before normalization, and the red bar represents the normalized data.)

Figure S2 High expression of CST1 predicted poor prognosis in GC.

High expression of *CST1* was significant negatively associated with prognosis in subgroups (A) M0, (B) M1, (C) surgery only, (D) N1, (E) N2, (F) N3, (G) T3, (H) T4 groups. (I) CST1 upregulated was also associated with poor outcome in post-progression survival (PPS) in GC.

Figures

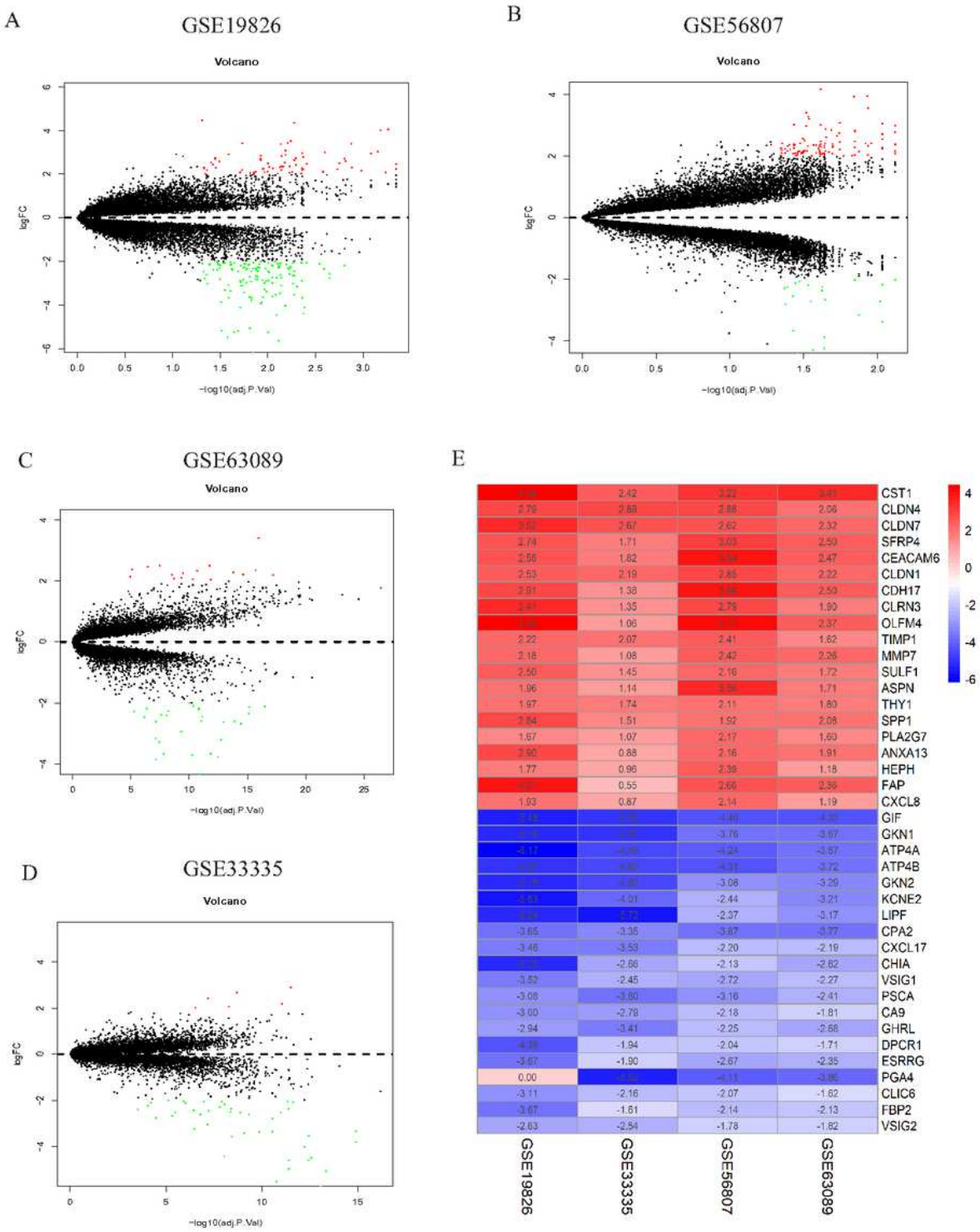

Figure 1

Differential expression of data in GEO database. (A) GSE19826 data; (B) GSE56807 data; (C) GSE63089 data; (D) GSE33335 data (the red points represent upregulated DEGs. The green points represent downregulation of the expression of DEGs. The black points represent genes with no significant

difference); (E) Heatmap of top 20 upregulated and downregulated DEGs. (Red represents logFC >0, blue represents logFC <0, and the values in the box represent the logFC values.)

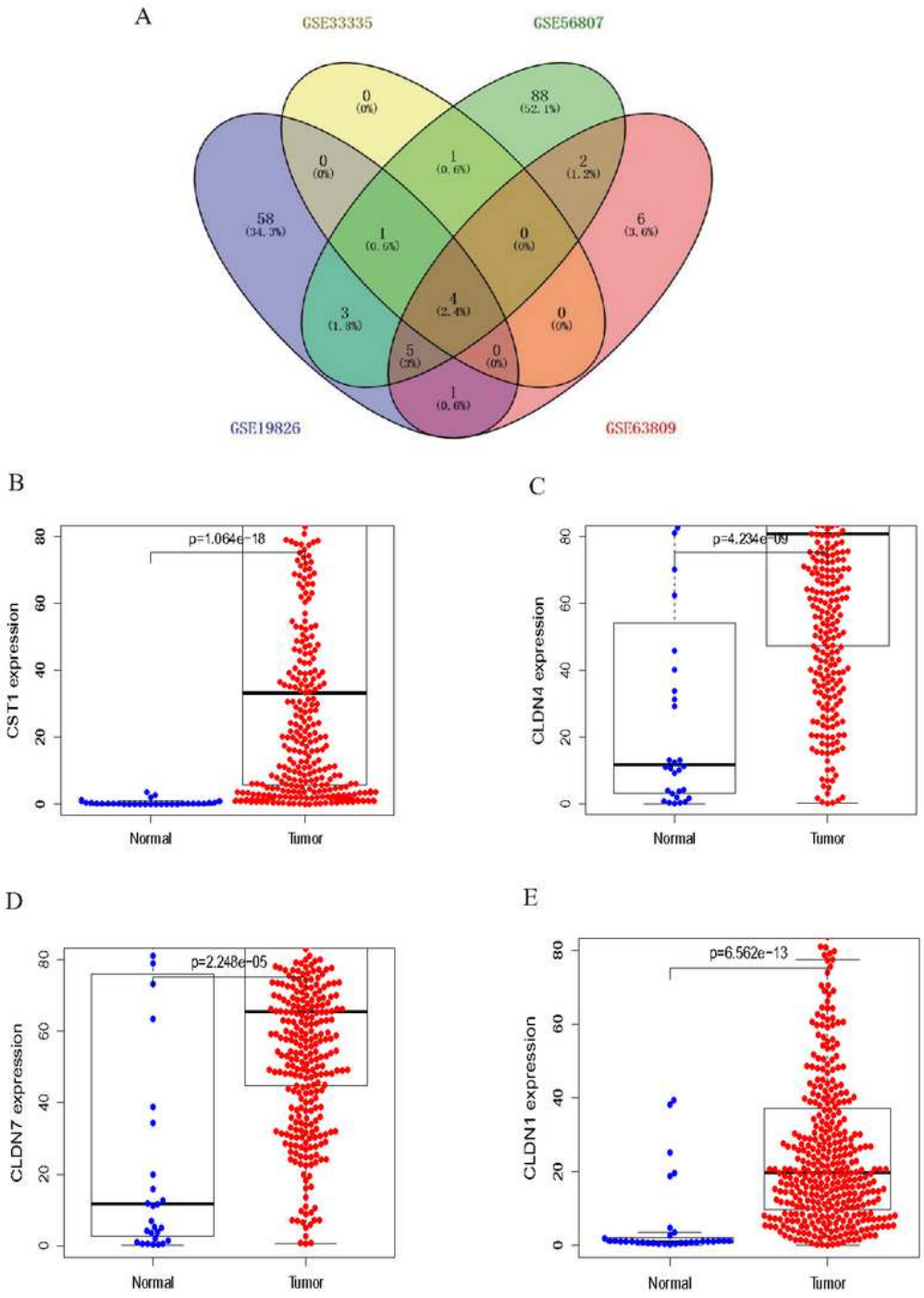

Figure 2

Identification and validation of four overlapped DEGs from integrated datasets. (A) The four common DEGs in the four GEO datasets. (B) CST1 validation in TCGA database. (C) CLDN4 validation in TCGA

database. (D) CLDN7 validation in TCGA database. (E) CLDN1 validation in TCGA database.

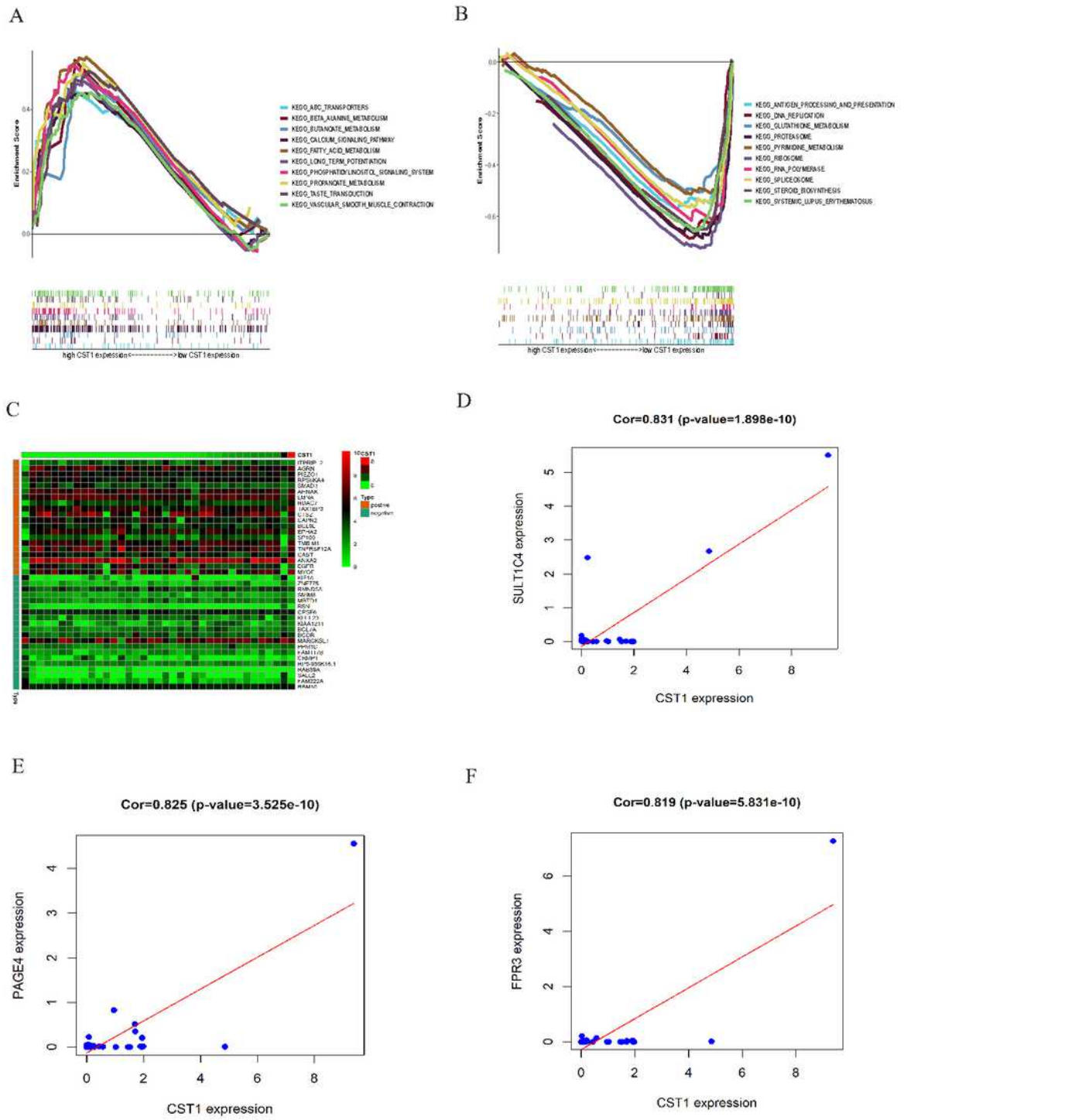

Figure 3

GSEA enrichment analysis and co-expression with CST1 (A) GSEA analysis showed that KEGG pathways enriched in high CST1 expression group, (B) GSEA enrichment analysis showed that KEGG pathways enriched in low CST1 expression group, (C) The heatmap of genes co-expressed with CST1, (D)

Correlation of SULT1C4 with CST1 expression, (E) Correlation of PAGE4 with CST1 expression, (F) Correlation of FPR3 with CST1 expression.

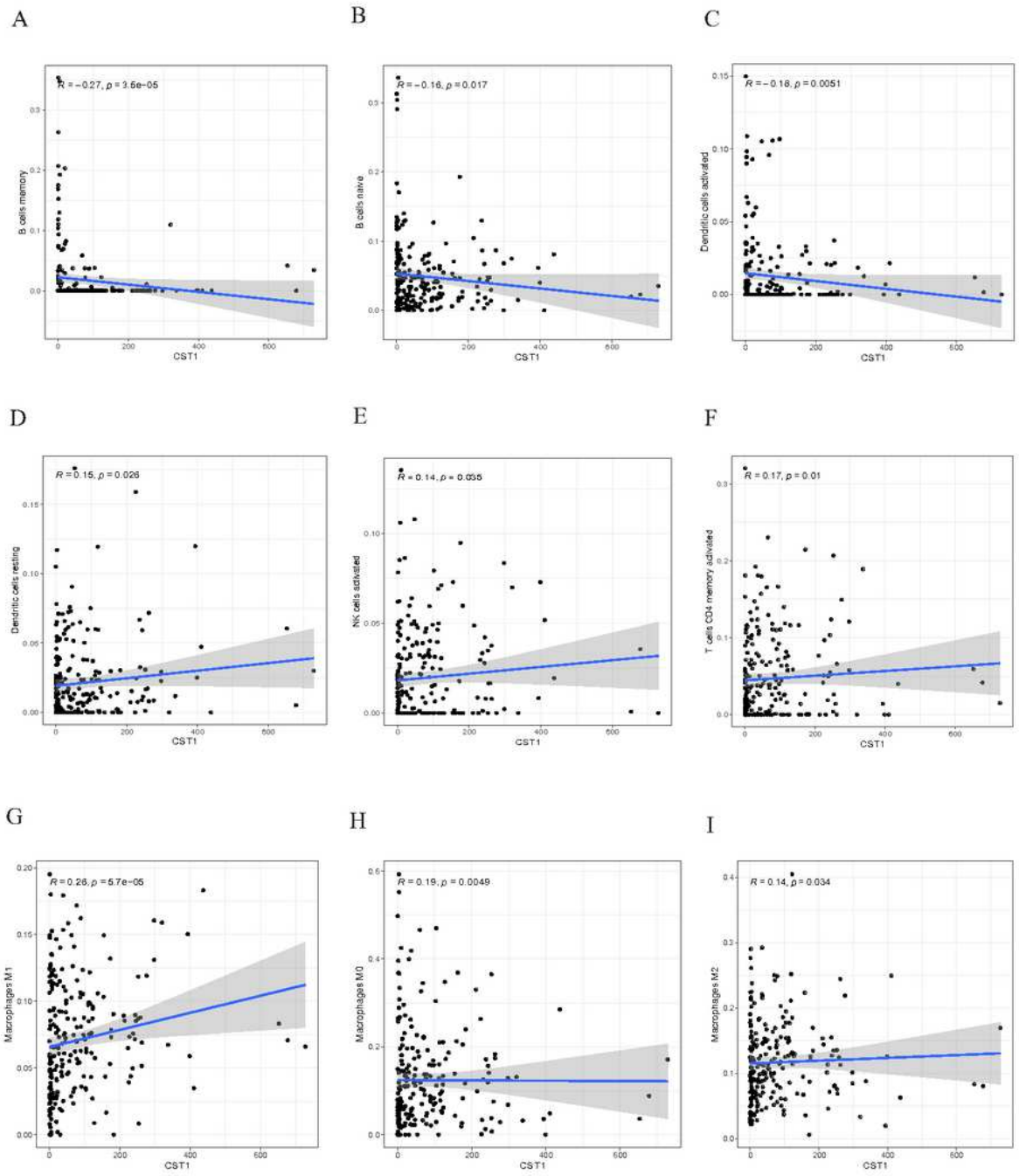

Figure 4

Evaluation of CST1-related tumor immune cells infiltration in GC The correlation of CST1 with tumor immune cells infiltration using TCGA database. (A) B cells memory, (B) B cell naive, (C) dendritic cells

activated, (D) dendritic cells resting, (E) NK cells activated, (F) T cells memory activated, (G) macrophages M1, (H) macrophages M0 and (I) macrophages M2.

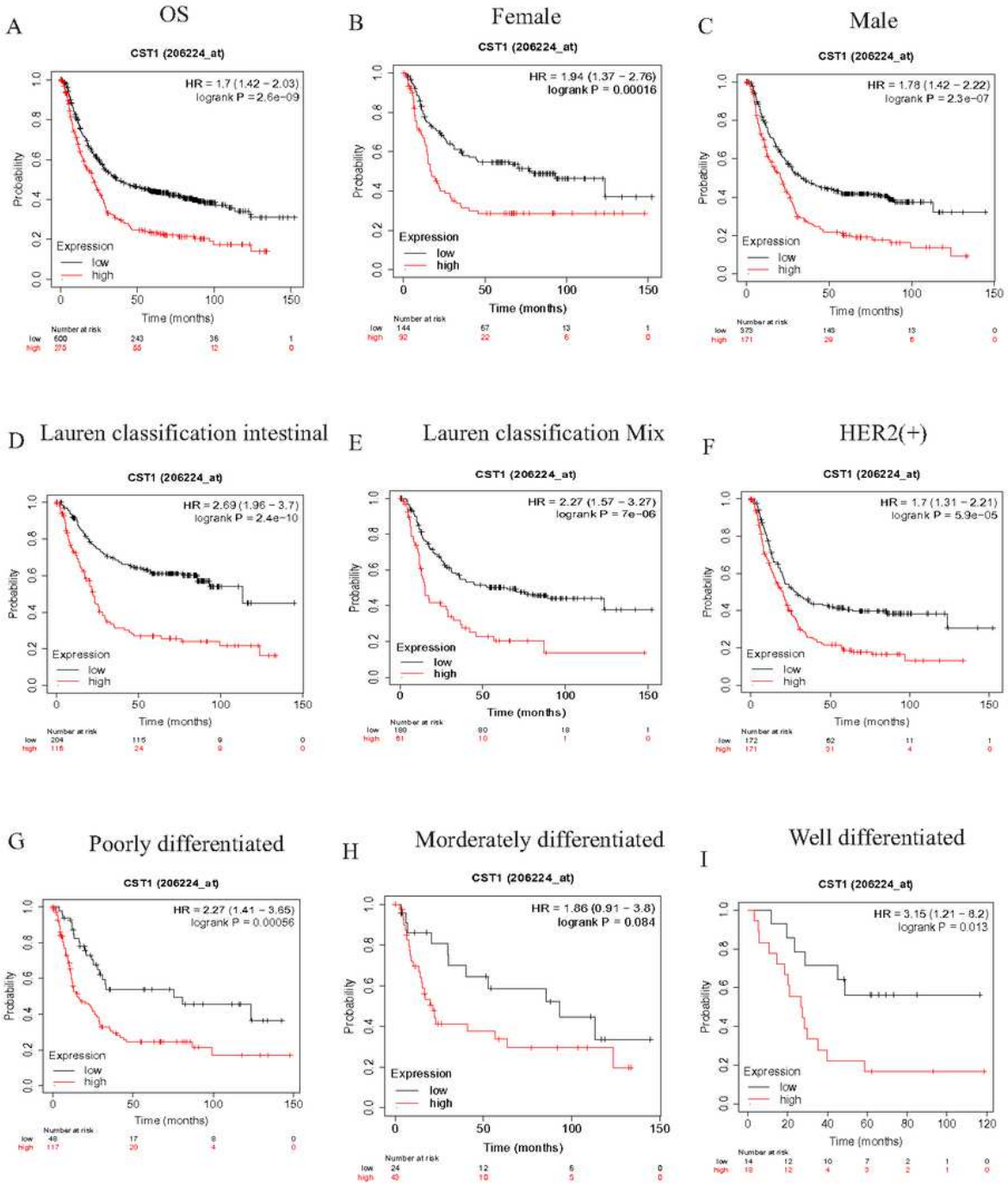

Figure 5

CST1 overexpression was associated with poor outcome in GC. (A) CST1 was significantly related with overall survival. Survival curves also showed patients with high CST1 expression significantly had poor

outcome in subgroups. (B) Female, (C) Male, (D) lauren classification intestinal, (E) lauren classification intestinal mix, (F) HER-2(+), (G) poorly differentiated group, (H) moderately differentiated group and (I) well differentiated.

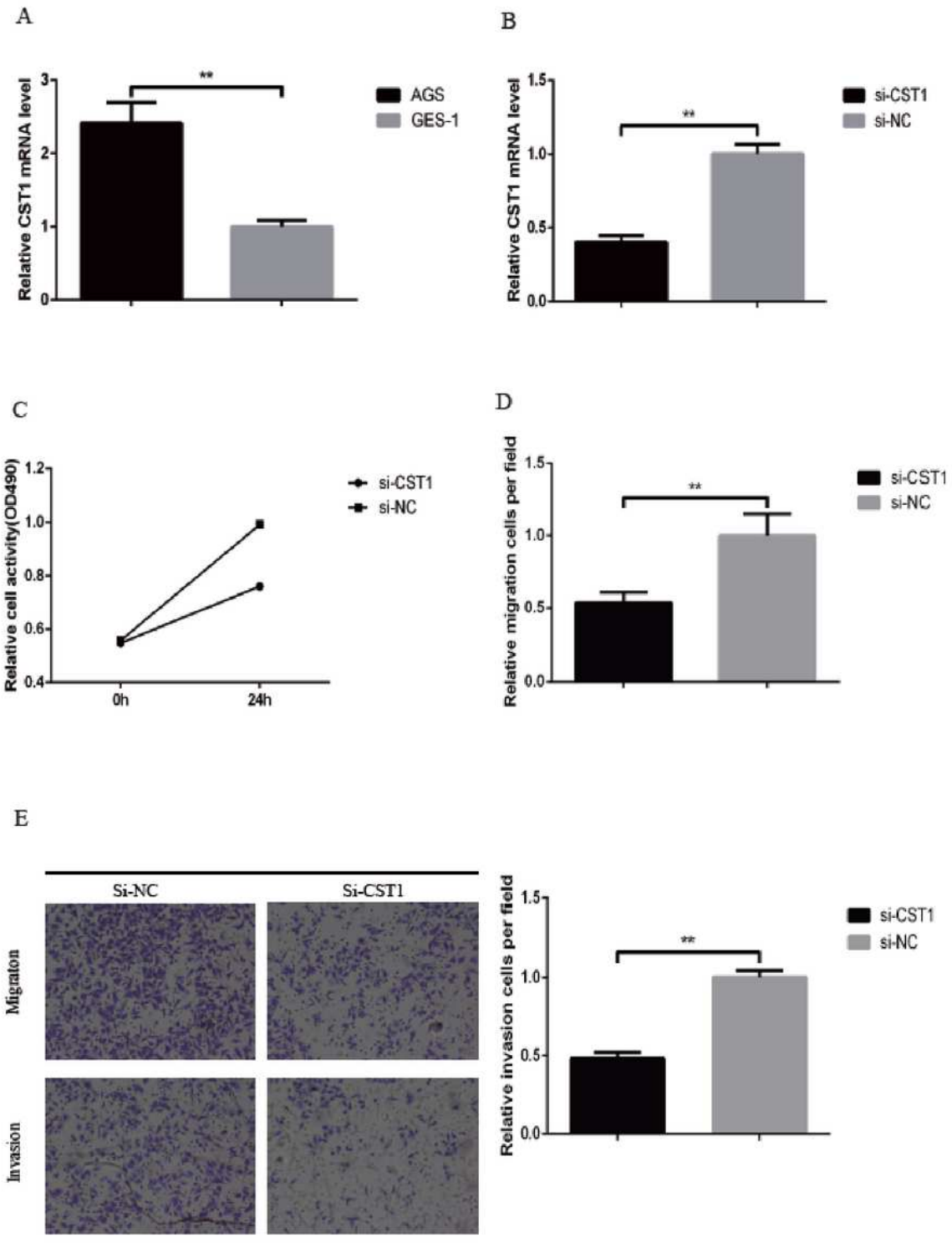

Figure 6

Reduced expression of CST1 decreased cell proliferation, migration, and invasion in GC cell line. (A) CST1 expression in AGS and GES cell lines, (B) CST1 expression in AGS cell line after transfection of si-CST1 and negative control, (C) CST1 promoted cell proliferation, Transwell chambers assay with Matrigel showed CST1 overexpression promoted (D) cell invasion and (E) cell invasion in AGS cell line. (NC, negative control; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FS1.pdf
- FS2.pdf