Upregulation of ECT2 is associated with transcriptional program of cancer stem cells and predicts poor clinical outcome in gastric cancer

DAO-YIN GONG[1], XIAN CHEN[1], TIAN-LIN YANG[1], YI WANG[1], YU GUO[2], JIN-HAO ZENG[2] and SHAN-ZE CHEN[3]

[1]Department of Pathology, [2]Geriatric Department, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan 610072; [3]Department of Pathyphysiology, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, P.R. China

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Abstract. Gastric cancer remains the third leading cause of cancer-associated mortality worldwide. The identification of prognostic indicators that are associated with clinical characteristics is urgently required. The aim of the present study was to determine the involvement of epithelial cell transforming 2 (ECT2) in gastric cancer. The results of the present study demonstrated that ECT2 expression was upregulated in human gastric cancer samples. Furthermore, high ECT2 expression was associated with advanced Tumor-Node-Metastasis stage and deeper tumor invasion. ECT2 upregulation was further confirmed in several independent publicly available clinical cohorts from the Gene Expression Omnibus database. In addition, patients with gastric cancer, with high ECT2 expression exhibited a significantly shorter overall survival time than those with low ECT2 expression, and Cox regression analysis demonstrated that ECT2 expression was an independent prognostic marker for overall survival time. Characterization of the transcriptome profiles of ECT2 upregulated gastric tumors indicated that ECT2 upregulation may be associated with transcriptional features of cancer stem cells (CSCs). Additionally, BUB1 mitotic checkpoint serine/threonine kinase and E2F transcription factor 7, two genes previously reported to account for the functionality of CSCs, were strongly enriched in $ECT2^{High}$ gastric cancer samples. Taken together, the results of the present study suggest that ECT2 may serve as a novel marker for CSCs and may be a potential prognostic indicator in gastric cancer.

Correspondence to: Dr Jin-Hao Zeng, Geriatric Department, Hospital of Chengdu University of Traditional Chinese Medicine, 39 Shi-er-qiao Road, Chengdu, Sichuan 610072, P.R. China
E-mail: zengjinhao0018@126.com

Dr Shan-Ze Chen, Department of Pathyphysiology, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, 17 People's South Road, Chengdu, Sichuan 610041, P.R. China
E-mail: 474076613@qq.com

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Introduction

Cancer stem cells (CSCs), also known as tumor-initiating cells, are characterized by stem-like properties, including self-renewal and ability to generate daughter cells. Cancer initiation, dissemination and recurrence are closely associated with CSCs (1). CSCs were first detected in acute myeloid leukemia and have since been identified in several solid tumors, including gastric cancer (1). Furthermore, certain populations of gastric CSCs abilities to self-renewal and undergo multipotent differentiation have been detected in gastric cancer (2). $Villin^+$ and $Lgr5^+$ gastric stem cells have been detected in the antrum, while $Troy^+$ chief cells have been found in the corpus (3). Additionally, $Sox2^+$ gastric stem cells are present in both the antrum and the corpus (3).

Gastric cancer is the seventh most common cancer and the third leading cause of cancer-associated mortality worldwide (4). In 2018, 1,033,701 new gastric cancer cases and 782,685 mortalities were reported worldwide (4). Gastric cancer has been extensively investigated in the biomedical field due to its high morbidity and mortality rates (4). It is speculated that gastric carcinogenesis may be associated with *Helicobacter pylori* infection, inherited susceptibilities, and environmental and dietary factors (5,6). In recent years, the prevailing hypothesis that the occurrence and progression of gastric cancer is associated with CSCs has been partially proven (7).

Epithelial cell transforming 2 (ECT2) is a proto-oncogene gene encoding a guanine nucleotide exchange factor for the Rho GTPases (8). When expressed in NIH/3T3 fibroblasts, ECT2 promotes their malignant transformation (9). Increased ECT2 expression has been detected in several types of human tumor, including glioma and liver, pancreatic and lung cancer (10-13). ECT2 upregulation significantly enhances the activity of RhoGPase, prevents cell apoptosis and induces cancer cell metastasis (10). Conversely, ECT2 downregulation suppresses activation of the ERK signaling pathway and impairs the

migration of cancer cells (10). However, whether and how ECT2 contributes to gastric cancer malignancy remains elusive.

The present study aimed to investigate the association between ECT2 expression and the clinicopathological characteristics of patients with gastric cancer. The expression levels of ECT2 were investigated using immunohistochemical analysis, combined with Gene Expression Omnibus database and gene set enrichment analysis, and it was revealed that gastric tumors with elevated ECT2 levels exhibited transcriptional traits of CSCs. In addition, high ECT2 expression predicted poor clinical outcome, suggesting its use as a novel prognostic indicator for gastric carcinoma. Further investigation into the role of ECT2 may provide alternative therapeutic targets for the treatment of gastric cancer.

Materials and methods

Clinical tissue samples. A total of 130 primary gastric cancer tissues and 108 paired adjacent normal tissues (some paired adjacent normal tissues were not harvested during the operation due to patients' clinical conditions) were collected from patients who underwent surgery at the Hospital of Chengdu University of TCM (Chengdu, China) between March 2012 and December 2015, and retrospectively analyzed. Paraffin-embedded tissue samples were stored at room temperature. None of the patients had received anticancer treatment prior to diagnosis and no additional malignancies were present. Pathological staging was based on the Union for International Cancer Control/American Joint Committee on Cancer Tumor-Node-Metastasis (TNM) Classification (8th edition of 2016) (14). The present study was approved by the Institutional Review Board of the Teaching Hospital of Chengdu University of TCM (Chengdu, China) (approval no. 2018KL-023) and written informed consent was provided by all patients prior to the study start.

Immunohistochemistry (IHC). The tissue samples were fixed in 4% paraformaldehyde >24 h at room temperature, then dehydrated in graded ethanol series (30, 50, 70, 95 and 100%), and embedded in paraffin. For IHC analysis, paraffin-embedded samples were cut into 3-μm-thick sections, dewaxed with xylene at room temperature and rehydrated in a descending ethanol series (100, 95, 85 and 75%). For antigen retrieval, sections were heated at 97˚C for 20 min. Following a brief proteolytic digestion with 0.1% trypsin at 37˚C for 10 min and peroxidase blocking with 3% hydrogen peroxide solution at room temperature for 15 min, the sections were incubated with primary antibodies against: ECT2 (1:400; cat. no. 07-1364; Sigma-Aldrich, Merck KGaA), BUB1 (1:200; cat. no. DF6698; Affinity Biosciences) and E2F transcription factor 7 (E2F7; 1:200; cat. no. DF2444; Affinity Biosciences) overnight at 4˚C. Following the primary antibody incubation, the sections were incubated with a HRP/Fab secondary antibody at room temperature for 20 min (freshly prepared solution from the kit; cat. no. PV-6000-D; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Tissue sections were stained with diaminobenzidine substrate for 5 min and counterstained with hematoxylin for 20 sec at room temperature. Each slide was analyed using light microscopy (H-7650; Hitachi, Ltd.). The magnification used was x200.

A total of two independent investigators, without prior knowledge of the clinicopathological data, evaluated the ECT2 staining in a semiquantitative manner. The final immunoreactivity scores (IRS) were determined according to the sum total of the percentage of positive cells (0 points, 0-5% positive cells; 1 point, 6-25%; 2 points, 26-50%; 3 points, 51-75% and 4 points, 76-100%), and staining intensity scores (0 points, no staining; 1 point, weak staining; 2 points, moderate staining and 3 points, strong staining). A final IRS >4 indicated strong positivity, while scores <4 indicated weak positivity.

ECT2 analysis in the Gene Expression Omnibus (GEO) database. ECT2 expression was assessed in several independent gastric cancer clinical datasets (15-18) available from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The GSE13861 dataset (15) included a collection of 65 primary gastric adenocarcinoma and 19 surrounding normal tissues. The GSE29272 (16) dataset included a cohort of 134 gastric adenocarcinoma and paired surrounding normal tissues. The GSE51575 (17) dataset consisted of a cohort of 27 advanced gastric carcinoma and paired surrounding normal tissues. The GSE65801 (18) dataset consisted of a cohort of 32 gastric cancer tissues and paired surrounding noncancerous tissues. The normalization procedures employed for gene expression intensity data are stated in the individual datasets and related publications.

Gene set enrichment analysis (GSEA). In order to determine how biological processes and signaling pathways are differentially regulated in gastric cancer with low or high ECT2 expression, transcriptomic data of gastric cancer were retrieved from The Cancer Genome Atlas (TCGA) database and analyzed using GSEA. TCGA gastric cancer cohort, consisting of 407 samples and transcriptional profiles, was downloaded from TCGA Data Portal (https://tcga-data.nci.nih.gov/docs/publications/tcga). GSEA was performed using GSEA software (v2.2.2; www.broadinstitute.org/gsea). The median ECT2 expression level (cut-off value=11.13) was used to dichotomize samples into low and high expression groups. A total of 1,000 permutations were used to calculate the P-values. All other parameters were set based on their default values.

Survival analysis. The prognostic value of ECT2 in gastric cancer was assessed using the Kaplan-Meier (KM) Plotter database (http://kmplot.com/analysis/index.php?p=service&cancer=gastric), which consists of a pool of gene expression and clinical data (19). The median time to first progression (FP) was 18.3 months and the median overall survival (OS) was 28.9 months. Overall survival time was assessed. The patient samples were divided into two groups according to the median gene expression value ($ECT2^{High}$/$ECT2^{Low}$, 437 cases/438 cases). A KM survival plot was used to compare the two groups. The hazard ratio (HR) with 95% confidence intervals (CIs) and log rank P-values were calculated.

Statistical analysis. Data are presented as the mean ± standard error of the mean from three independent experiments and were analyzed with SPSS v.22.0 software (IBM, Corp.). Pearson's χ^2 test and Fisher's exact test were used to assess the

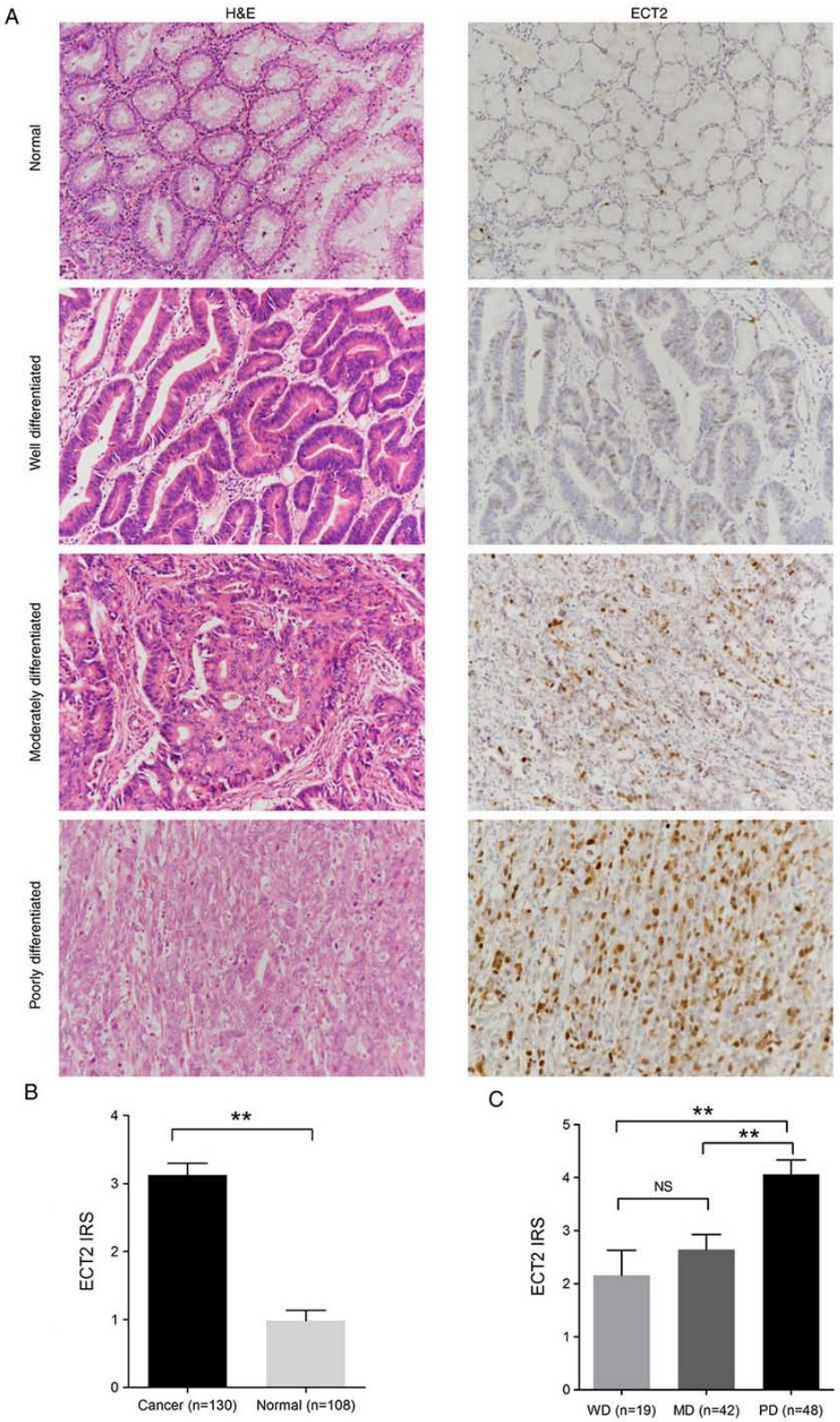

Figure 1. ECT2 expression is upregulated in human gastric cancer tissues. (A) Representative images depicting H&E staining (left panel), and immunohistochemical staining (right panel) of ECT2 in normal tissues and WD, MD and PD gastric cancer tissues. Magnification, x200. (B) ECT2 IRS in gastric cancer and adjacent normal tissue samples. (C) ECT2 IRS in WD, MD and PD gastric cancer tissues. Data are presented as the mean ± standard error of the mean. **P<0.01. ECT2, epithelial cell transforming 2; H&E, hematoxylin and eosin; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; IRS, immunoreactivity score; NS, no significance.

association between ECT2 expression and clinicopathological characteristics of patients with gastric cancer. Unpaired Student's t-test was used to assess the differences in ECT2, BUB1 and E2F7 expression levels between gastric cancer and control tissues. Spearman's correlation test was performed between BUB1 and ECT2 expression levels, and E2F7 and

Table I. Correlation between ECT2 positivity and clinicopathological characteristics of patients with gastric cancer (n=130).

Characteristics	Total n=130	ECT2 strong positivity, n=35	ECT2 weak positivity/absent, n=95	P-value
Age, years				0.241
<60	67	21	46	
≥60	63	14	49	
Sex				0.077
Male	93	21	72	
Female	37	14	23	
Tumor localization				0.091
Cardias	20	7	13	
Body	40	15	25	
Antrum	52	8	44	
Whole/Multiple	18	5	13	
Histology				0.216
ADC, WD	19	5	14	
ADC, MD	42	7	35	
ADC, PD	48	19	29	
Signet ring cell	14	3	11	
Mucinous adenocarcinoma	6	1	5	
Neuroendocrine carcinoma	1	0	1	
TNM stage				<0.001
I + II	67	8	59	
III + IV	63	27	36	
pT (Tumor invasion)				0.039
T1 + T2	22	2	20	
T3 + T4	108	33	75	
pN (Lymph node metastasis)				0.896
N0	42	11	31	
N1-N3	88	24	64	
pM (Distant metastasis)				0.473
M0	114	29	85	
M1	16	6	10	

ECT2, epithelial cell transforming 2; ADC, adenocarcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; TNM, tumor-node-metastasis.

ECT2 expression levels, respectively. Survival analysis was performed using the KM method and the log-rank test was used to assess statistical significance between the curves. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. P<0.05 was considered to indicate a statistically significant difference.

Results

ECT2 expression is upregulated in human gastric cancer. Following a routine H&E staining, IHC was performed to detect and grade ECT2 expression in gastric cancer and paired normal tissue sections. ECT2 positive staining was detected mainly in the nucleus and cytoplasm of gastric cancer cells. Weak staining was observed in the adjacent normal tissues. ECT2 expression was identified in 81.5% (106/130) of the gastric carcinoma tissues and 36.1% (39/108) of the adjacent normal tissues. The level of ECT2 protein was significantly higher in the gastric carcinoma tissues compared with the adjacent tissues (IRS, cancer=3.12±2.00 vs. normal=0.98±1.56; P<0.01; Fig. 1A and B).

Association between clinicopathological characteristics and ECT2 positivity in gastric cancer. The clinicopathological significance of ECT2 expression in gastric cancer was investigated. Analysis of the association between ECT2 expression levels (strong positivity vs. weak positivity/absent) and clinicopathological characteristics demonstrated that strong ECT2 positivity was significantly associated with advanced TNM stage (P<0.001) and higher pT stage (deeper tumor invasion; P=0.039). In the 130 gastric cancer cases assessed, high ECT2 expression was not associated with age, sex, tumor localization, pN stage and pM stage (all P>0.05; Table I; Table SI).

A significant association between strong ECT2 positivity and histological patterns of gastric cancer was not

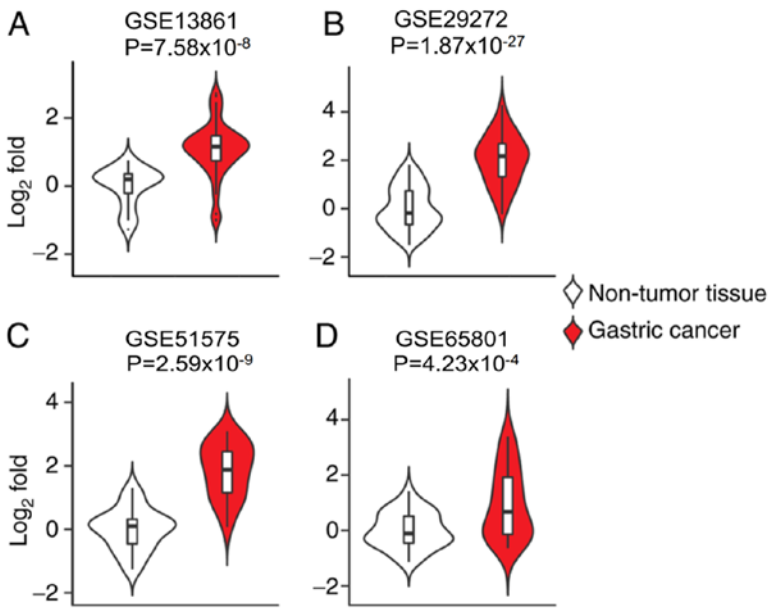

Figure 2. ECT2 mRNA expression is upregulated in gastric cancer. ECT2 expression pattern in gastric cancer and surrounding non-tumor samples based on transcriptomic data reported in the (A) GSE13861, (B) GSE29272, (C) GSE51575 and (D) GSE65801 datasets. ECT2, epithelial cell transforming 2.

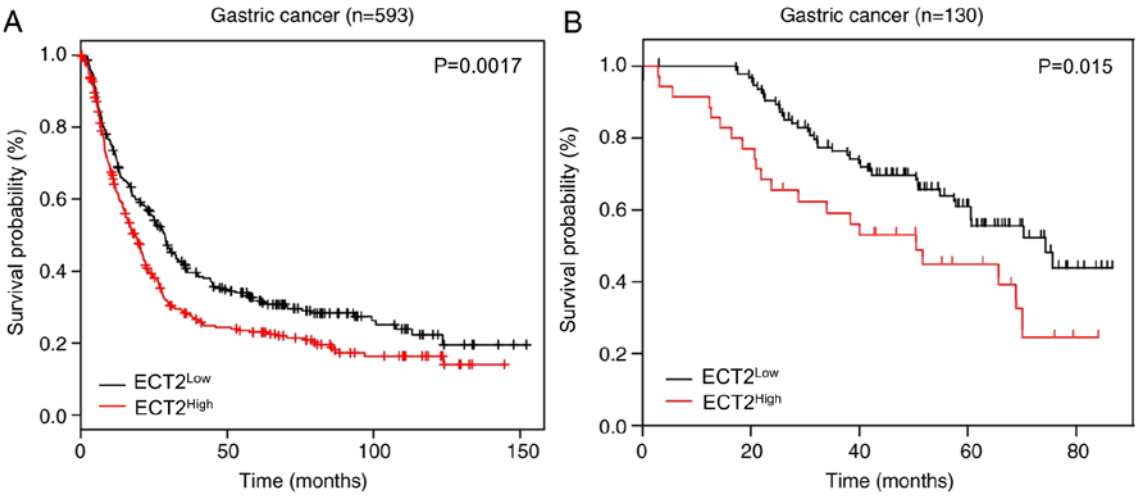

Figure 3. ECT2 upregulation predicts poor clinical outcome. (A) The Kaplan-Meier Plotter database was used to assess the difference in overall survival time between high and low ECT2 expression groups. (B) Difference in overall survival time between high and low ECT2 expression levels in gastric tissues. ECT2, epithelial cell transforming 2.

established (Table I; Table SI); however, ECT2 expression was closely associated with the histological differentiation degree (Fig. 1C). ECT2 expression escalated from well differentiated (WD), to moderately differentiated (MD) and to poorly differentiated (PD) variants (IRS, PD=4.06±1.87 vs. WD=2.16±2.06 or MD=2.64±1.83; P<0.01; Fig. 1C).

ECT2 mRNA upregulation in public datasets of gastric cancer. To further investigate the pathological role of ECT2 in the progression of gastric cancer, the ECT2 expression pattern in gastric cancer samples based on transcriptomic data from the GEO database was assessed. Consistent with IHC analysis, ECT2 mRNA expression levels were significantly increased in primary gastric adenocarcinoma tissues compared with surrounding normal tissues in the GSE13861 dataset (P<0.0001; Fig. 2A). Similar trends were observed in comparisons between paired samples of gastric carcinoma and adjacent normal tissues in the GSE29272 dataset (P<0.0001; Fig. 2B), advanced gastric carcinoma and adjacent normal tissues in the GSE51575 dataset (P<0.0001; Fig. 2C), and gastric cancer tissues and adjacent normal tissues in the GSE65801 dataset (P<0.0001; Fig. 2D). Collectively, these results indicate that ECT2 upregulation may play an important role in the malignant progression of human gastric cancer.

ECT2 upregulation predicts poor clinical outcome. The association between ECT2 expression and the prognosis of patients with gastric cancer was assessed using the KM plot database. High ECT2 expression was significantly associated with a shorter survival time in patients with gastric cancer,

Table II. Prognostic factors associated with overall survival as determined by univariate and multivariate analyses.

Variables	HR (95% CI)	P-value
Univariate analysis		
ECT2	1.905 (1.122-3.233)	0.017
Tumor localization (Body)	4.842 (1.436-16.328)	0.011
Tumor localization (Whole/Multiple)	14.106 (4.057-49.039)	<0.001
Histology (ADC, PD)	8.274 (2.538-26.971)	<0.001
Histology (signet ring cell)	5.511 (1.403-21.644)	0.014
Histology (mucinous adenocarcinoma)	5.319 (1.066-26.531)	0.042
TNM stage	4.992 (2.797-8.909)	<0.001
pT stage	18.481 (2.557-133.551)	0.004
pN stage	2.846 (1.510-5.367)	0.001
pM stage	3.013 (1.612-5.632)	0.001
Multivariate analysis		
ECT2	3.105 (1.567-6.153)	0.001
Tumor localization (Whole/Multiple)	13.301 (3.468-51.015)	<0.001
Histology (ADC, PD)	4.109 (1.246-13.550)	0.020
Histology (Mucinous adenocarcinoma)	6.186 (1.049-36.481)	0.044
pT stage	12.216 (1.445-103.281)	0.022
pN stage	3.967 (1.935-8.132)	<0.001

ECT2, epithelial cell transforming 2; ADC, adenocarcinoma; PD, poorly differentiated; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

stratified according to ECT2 expression levels. The median survival time for $ECT2^{High}$ patients was 16 months, which was significantly shorter than the 26 months observed for $ECT2^{Low}$ patients (P=0.0017; Fig. 3A). Survival analysis using KM curves was performed to verify these results. The results demonstrated that patients with gastric cancer, with low ECT2 expression exhibited a significantly longer overall survival time than those with high ECT2 expression (P=0.015; median OS, 74.360 vs. 50.430 months; Fig. 3B).

To determine whether ECT2 is an independent prognostic factor for the survival of patients with gastric cancer, univariate and multivariate Cox regression analyses were performed. As presented in Table II, the univariate analysis suggested that ECT2 was significantly associated with overall survival time in patients with gastric cancer [P=0.017; HR (95% CI), 1.905 (1.122-3.233)]. Tumor location (body, whole and multiple), histology (PD of adenocarcinoma, signet ring cell, mucinous adenocarcinoma) and TNM stage were all associated with overall survival time in patients with gastric cancer (all P<0.05). Multivariate analysis further demonstrated that high ECT2 expression was a significant independent prognostic marker for patients with gastric cancer [P=0.001; HR (95% CI), 3.105 (1.567-6.153)]. Taken together, these results suggest that ECT2 may serve as a prognostic biomarker for patients with gastric cancer.

Gastric tumors with higher ECT2 expression levels possess transcriptional traits of CSCs. To determine the ECT2-associated cellular processes and signaling pathways in gastric cancer, GSEA was performed using transcriptome data from TCGA. GSEA demonstrated highly significant enrichment of breast-cancer-progenitor-related genes in gastric cancer samples with higher ECT2 expression (Fig. 4A). Notably, BUB1 mitotic checkpoint serine/threonine kinase and E2F7, two genes previously reported to account for CSC functionality (20-22), were strongly enriched in $ECT2^{High}$ gastric cancer samples (Fig. 4B). A significant correlation was identified between ECT2 and BUB1 mRNA expression levels (r=0.63; P<0.0001; Fig. 5A), and between ECT2 and E2F7 mRNA expression levels in gastric cancer tissues (r=0.45; P<0.0001; Fig. 5B).

BUB1 and E2F7 IHC staining was performed in gastric cancer samples to verify these findings. The results demonstrated that BUB1 protein expression was significantly higher in gastric cancer tissues compared with adjacent normal tissues (IRS, cancer=4.61±1.89 vs. normal=1.94±0.89; P<0.01; Fig. 6A and B). Similarly, E2F7 protein expression was significantly higher in gastric cancer tissues compared with adjacent normal tissues (IRS, cancer=4.01±1.75 vs. normal=1.72±0.86; P<0.01; Fig. 6A and C). Collectively, these results suggest that gastric tumors with high ECT2 expression levels may possess transcriptional traits of CSCs.

Discussion

Gastric cancer remains the third leading cause of cancer-associated mortality worldwide (4). CSCs are implicated in different types of cancer, including gastric cancer (1). The identification of gastric CSCs has improved understanding of the molecular and cellular etiology of gastric cancer, and may

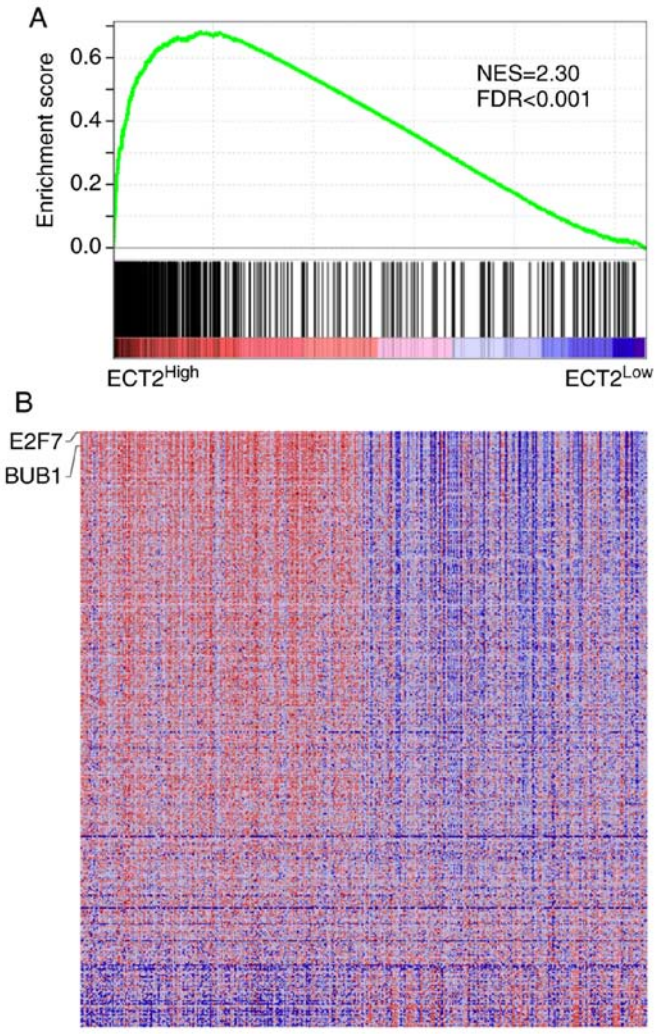

Figure 4. Gastric tumors with higher ECT2 levels possess transcriptional traits of cancer stem cells. (A) Gene set enrichment analysis enrichment score curve demonstrated higher ECT2 expression in gastric cancer samples with highly enriched breast-cancer-progenitor-related genes. (B) BUB1 and E2F7 are strongly enriched in ECT2High gastric cancer samples. ECT2, epithelial cell transforming 2; E2F7, E2F transcription factor 7; NES, normalized enrichment score; FDR, false discovery rate.

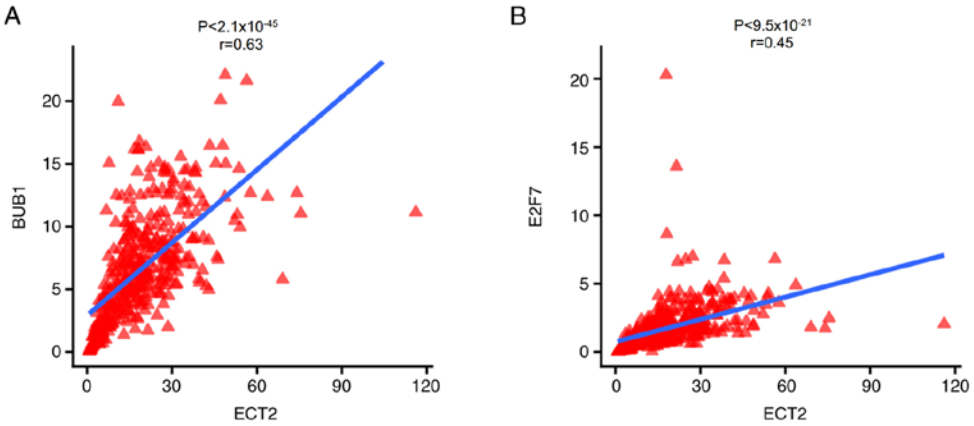

Figure 5. ECT2 expression correlates with BUB1 and E2F7 mRNA expression. A significant correlation was identified between (A) ECT2 and BUB1 mRNA expression levels and (B) ECT2 and E2F7 mRNA expression levels in gastric cancer tissues, respectively. ECT2, epithelial cell transforming 2; E2F7, E2F transcription factor 7.

aid the development of effective treatments. Experimentally, CSCs are characterized by their capacity for tumor propagation (2). CSCs are resistant to chemotherapy and radiotherapy and possess a quiescent nature (23). Thus, these cells play an important role in cancer recurrence (24). As a result, identification of specific gastric CSCs and the detection of their

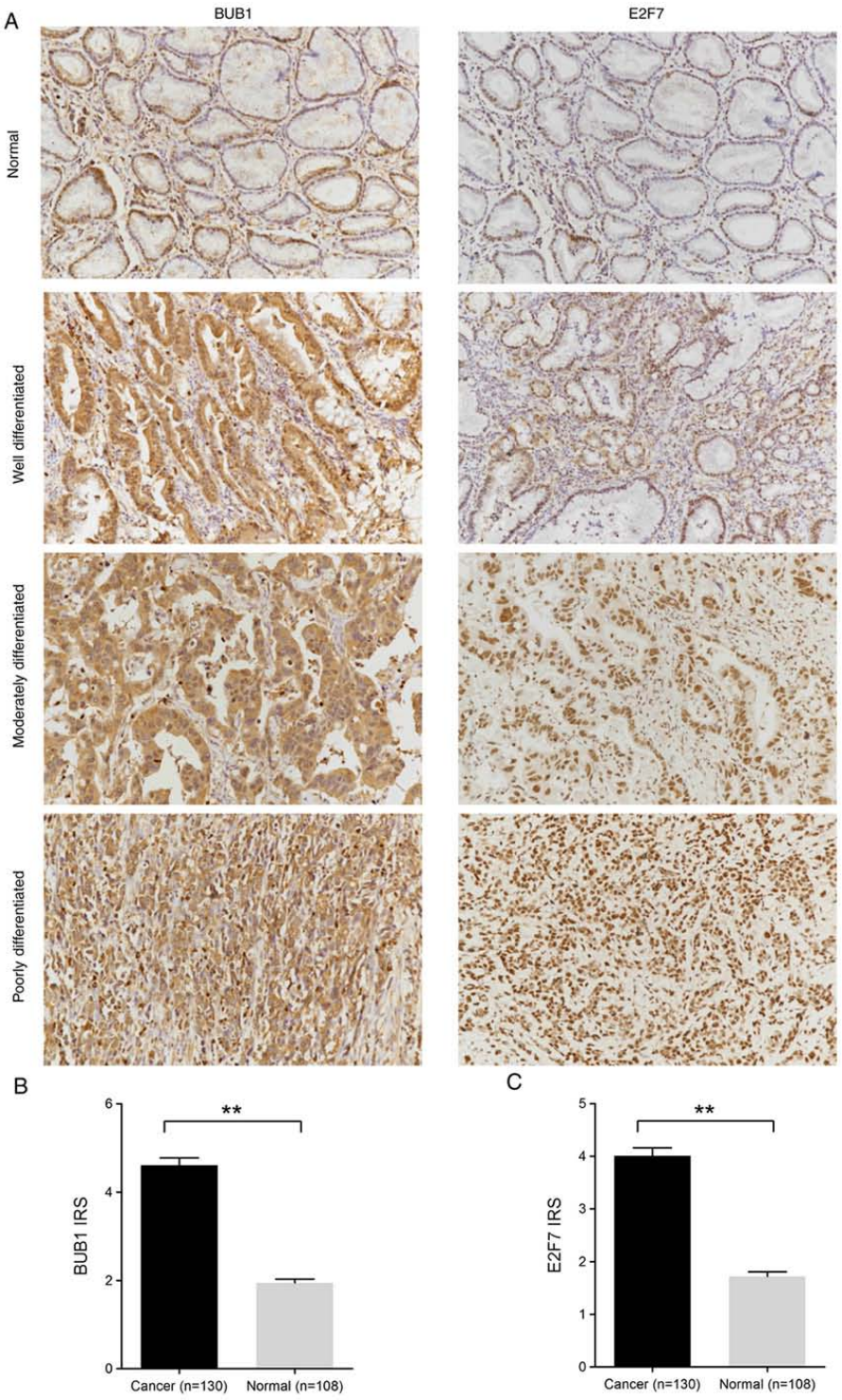

Figure 6. BUB1 and E2F7 protein expression in human gastric cancer tissues. (A) Representative images depicting immunohistochemical staining of BUB1 (left panel) and E2F7 (right panel) in normal tissues, and well differentiated, moderately differentiated and poorly differentiated gastric cancer tissues. Magnification, x200. IRS of (B) BUB1 and (C) E2F7 in gastric cancer and adjacent normal tissues. Data are presented as the mean ± standard error of the mean. **P<0.01. E2F7, E2F transcription factor 7; IRS, immunoreactivity score.

expression level will lead to the development of novel methods for the diagnosis and treatment of gastric cancer, which can further improve the survival rate of patients with the disease.

The results of the present study demonstrated that ECT2 expression was upregulated in gastric cancer tissues compared with adjacent normal tissues. This result was further verified based on the transcriptomic data from several independent clinical datasets. Consistent with the results of IHC analysis, ECT2 mRNA expression levels were significantly increased in gastric cancer tissues compared with adjacent normal tissues, suggesting that ECT2 upregulation may serve an important role in the malignant progression of human gastric cancer.

The present study also investigated the biological implications of ECT2 upregulation using GSEA. BUB1 and E2F7

upregulation have previously been demonstrated to play important roles in essential cellular processes, such as cell proliferation (25-27). It is speculated that BUB1 and E2F7 may be associated with transcriptional features of CSCs (20-22). A previous study revealed that BUB1 depletion using shRNAs reduces cancer stem cell potential of the MDA-MB-231 breast cancer cell line, resulting in inhibited formation of xenografts in immunocompromised mice (20). In addition, overexpression of E2F7 significantly enhanced the spheroid formation and growth rate of HepG2 and Huh7 cells (hepatocellular carcinoma cell lines), and also decreased their apoptosis (28). GSEA analysis indicated that ECT2 expression was notably associated with the transcriptional program of CSCs, with co-staining of BUB1 and E2F7 in gastric cancer tissues confirmed by IHC analysis.

The present study is not without limitations. First, only IHC analysis was performed to determine ECT2 protein expression, additional methods such as western blotting should be considered in future studies. However, IHC can simultaneously evaluate tissue expression localization, as well as morphology in cancer tissues, and thus is the preferred approach in analysis of clinical samples. Furthermore, future studies will focus on *in vitro* experiments to better understand the molecular mechanisms underlying ECT2 function in gastric cancer.

Carcinoembryonic antigen and cancer antigen 19-9 serve as the standard biomarkers for the diagnosis of gastric cancer; however, their use in clinical practice is limited due to low diagnostic sensitivity (29). Although emerging candidate biomarkers, such as microRNA and DNA methylation products have been extensively studied, several challenges hinder their application in a clinical setting (30). The results of the present study demonstrated that strong ECT2 positivity was significantly associated with advanced TNM stage and deeper tumor invasion. Furthermore, high ECT2 expression levels were associated with a shorter overall survival time. Thus, ECT2 expression may serve as an independent prognostic marker for the overall survival time of patients with gastric cancer. Similar results were reported by previous studies that demonstrated the prognostic value of ECT2 for gastric cancer (31,32). Taken together, the results of the present study suggest that upregulation of ECT2 predicts unfavorable clinical outcomes of patients with gastric cancer. Thus, ECT2 may serve as a potential prognostic marker and therapeutic target for the management of gastric cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JHZ and SZC conceived and designed the present study. TLY and YG collected and prepared the clinical samples. XC and YW performed H&E staining and confirmed pathological diagnosis. JHZ and DYG performed IHC and clinicopathological characteristics analyses. SZC acquired, interpreted and analyzed the GEO and GSEA data. DYG and SZC drafted the initial manuscript and critically revised it for important intellectual content. All authors have read and approved the final manuscript to be published.

Ethics approval and consent to participate

This retrospective study was approved by the Institutional Review Board of the Teaching Hospital of Chengdu University of TCM (Chengdu, China) (approval no. 2018KL-023) and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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