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

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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\textsuperscript{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.

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\textsuperscript{+} and Lgr5\textsuperscript{+} gastric stem cells have been detected in the antrum, while Troy\textsuperscript{+} chief cells have been found in the corpus (3). Additionally, Sox2\textsuperscript{+} 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 \textit{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 RhoGTPase, 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. DF6698; Affinity Biosciences) and E2F transcription factor 7 (E2F7; 1:200; cat. no. DF2444; Affinity Biosciences) and E2F transcription factor 28.9 months. Overall survival time was assessed. The patient median (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 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.

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 χ² test and Fisher's exact test were used to assess the
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

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.
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

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**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            |            |                             |                                  |         |
| <60                   | 67         | 21                          | 46                               | 0.241   |
| ≥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.
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 mRNA 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,
stratified according to ECT2 expression levels. The median survival time for ECT2$^{\text{High}}$ patients was 16 months, which was significantly shorter than the 26 months observed for ECT2$^{\text{Low}}$ 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.

**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

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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.
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 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. 
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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