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Original Article

Down-regulated CUL7 in gastric carcinoma (GC) correlates to GC histopathological features

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Abstract: As a key component of E3 ubiquitin ligase complex, CUL7 functions as a molecular scaffold to position the F-box protein bound substrate for ubiquitination, thereby participating in a variety of cellular processes. Recently, aberrant expression of CUL7 has been found in many malignancies, but the expression status of CUL7 in human gastric carcinoma (GC) remains unknown. In this study, we determined the CUL7 levels in GC tissues from a large clinical cohort by qRT-PCR, western blot and immunohistochemistry staining. Our results showed that CUL7 level was down-regulated in GC tissues and was associated with the histology and differentiation of GC, implying CUL7 may serve as a potential therapeutic target as well as a histopathological marker for GC patients.

Keywords: CUL7, gastric cancer, histological type, differentiation degree

Introduction

Gastric carcinoma (GC) is the fifth most commonly diagnosed malignant carcinoma, accounting for the third most common cancer-related death disease worldwide. Approximately 984,000 new cases of GC and 841,000 deaths were reported in 2013 [1]. In spite of the advents of new surgical techniques and chemotherapeutics in the past years, the overall outcome of GC remains poor primarily due to the late diagnosis [2]. The majority of GC patients in advanced stages experience recurrences with the median survival time and 2-year survival rates estimated to be only 12.5~13.0 months and 22.9~23.6%, respectively [3, 4]. Thus, there is an urgent need to discover novel prognostic and therapeutic targets that may help improve the clinical outcome for GC patients.

Cullin7 (CUL7) belongs to the cullin protein family, which consists of eight members sharing an evolutionarily conserved cullin domain at C-terminal [5]. As a scaffold protein, CUL7 assembles a SCF-like E3 complex with Skp1 (S-phase kinase associated protein 1), F-box protein Fbw8 (also known as Fbx29, Fbw6 or Fbxw8) and the RING finger protein Rbx1 (also termed Roc1 or Hrt1) [6, 7]. CUL7 E3 ligase plays important roles in various cellular processes, such as growth control, apoptosis and genome integrity maintenance [5, 8, 9]. Genetic mutations of CUL7 gene have been identified as a causal factor leading to 3-M syndrome, which is an autosomal recessive disorder characterized by severe pre- and postnatal growth retardation and minor skeletal changes [10]. Resembling the human hereditary syndromes, CUL7 knockout mice showed severe intrauterine growth retardation likely due to the dys-regulated turnover of insulin receptor substrate 1 (IRS-1), which has been identified as the substrate of CUL7 E3 ligase [11, 12]. In humans, the expression level of CUL7 varies at different developmental stages, with the highest levels found in kidney and placenta in fetus versus skeletal muscle, heart and pancreas in adults [10]. Recently, abnormal expression of CUL7 has been reported in various cancers [13-16]. However, the expression level as well as the diagnostic value of CUL7 in human GC remains elusive.

In this study, aiming to elucidate the role of CUL7 in pathobiology of GC, we determined the CUL7 levels in GC tissues and evaluated the

Table 1. Primer sequences of mRNA structural components used in qPCR

Components	Position	Sequence
Cullin7	Forward	AGTTTCGGCAGAGCAACAAC
	Reverse	TTCCTCAAAGCCCAAGATCTCC
GAPDH	Forward	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG

clinicopathological characteristics relative to CUL7 expression level in GC patients.

Materials and methods

Chemicals and antibodies

TRIzol reagent was obtained from Life technologies (USA), PrimeScript RT Master Mix and SYBR GREEN Premix reagents were obtained from TaKaRa (Japan), Antibodies against CUL7 were obtained from Abcam (USA), β-actin antibodies and corresponding secondary antibodies were obtained from Proteintech (USA).

Patients and tissues

Archived paraffin-embedded 158 GC tissues and 92 normal gastric tissues, including 60 GC and paired-adjacent normal gastric tissues, were obtained from GC patients undergoing curative surgery in Tianjin Medical University Cancer Hospital (TJMUCH) from 2003 to 2010. The 18 fresh GC and paired-adjacent normal gastric tissues, which were used for quantitative real-time PCR (qRT-PCR) and Western Blot analysis, were immediately frozen in liquid nitrogen after surgical removal in TJMUCH from 2014 to 2015 and stored in liquid nitrogen until use. None of above-mentioned patients had been given chemo- and radiotherapy before surgery. Research protocols were approved by the Hospital Ethics Committee of Tianjin Medical University Cancer Hospital, and informed consent was obtained from all individual participants included in the study.

RNA and quantitative real-time PCR

Total RNA from GC and paired normal gastric tissues were extracted using the TRIzol reagent. RNA was quantified using Nano-drop 1000 (Thermo Fisher Scientific, USA). The 2 μg RNA from each sample was used for cDNA synthesis with PrimeScript RT Master Mix reagent according to the manufacturer's protocol. By using the specific primer pairs (**Table 1**) and SYBR GREEN Premix reagent, qRT-PCR was performed on the QuantStudio 5 real-time PCR system (Thermo Fisher Scientific, USA). Expression data were normalized to the geometric mean of the housekeeping GAPDH gene to control the variability in mRNA expression levels.

Western blot analysis

Standard methods were used for western blot. GC and paired normal gastric tissues lysates were prepared by extraction with lysis buffer. The lysates were centrifuged at 12000 g at 4°C for 10 minutes. Then, the protein concentration was measured using the BCA protein assay reagent (Bio-Rad, USA) following the manufacturer's instructions. Equal amounts of protein were separated by 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel, and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk solution, and followed by incubation with primary antibody at 4°C overnight. After washing with PBST for three times, the membranes were incubated with specific secondary antibody. Antibody localization was visualized by ECL chemoluminescence.

Immunohistochemical analysis

The slides of tissue sections were fixed in cold 100% acetone for 5 minutes, air-dried, and they were incubated with the antibody against CUL7 (1:500) at 4°C overnight. After washing with PBS, the slides were incubated with Polymer Helper and Poly peroxidase-anti-mouse/rabbit IgG (PV-9000, ORIGENE, China), followed by further incubation with diaminobenzidine (DAB). Lastly, the tissue sections were counterstained with hematoxylin and mounted. Two independent pathologists evaluated the slides and the scores for CUL7 staining were calculated based on the staining intensity (0-below the level of detection, 1-weak, 2-moderate, and 3-strong) and the percentage of the stained cells at each intensity level (0%-100%). The final scores were calculated by multiplying the intensity score by the percentage.

Statistical analysis

The results were analyzed using SPSS17.0 software (USA). Data were represented as the mean ± standard deviation. The differences expressed were using the Student's *t*-test. *P* value of <0.05 was established to demonstrate significance in all statistical analyses.

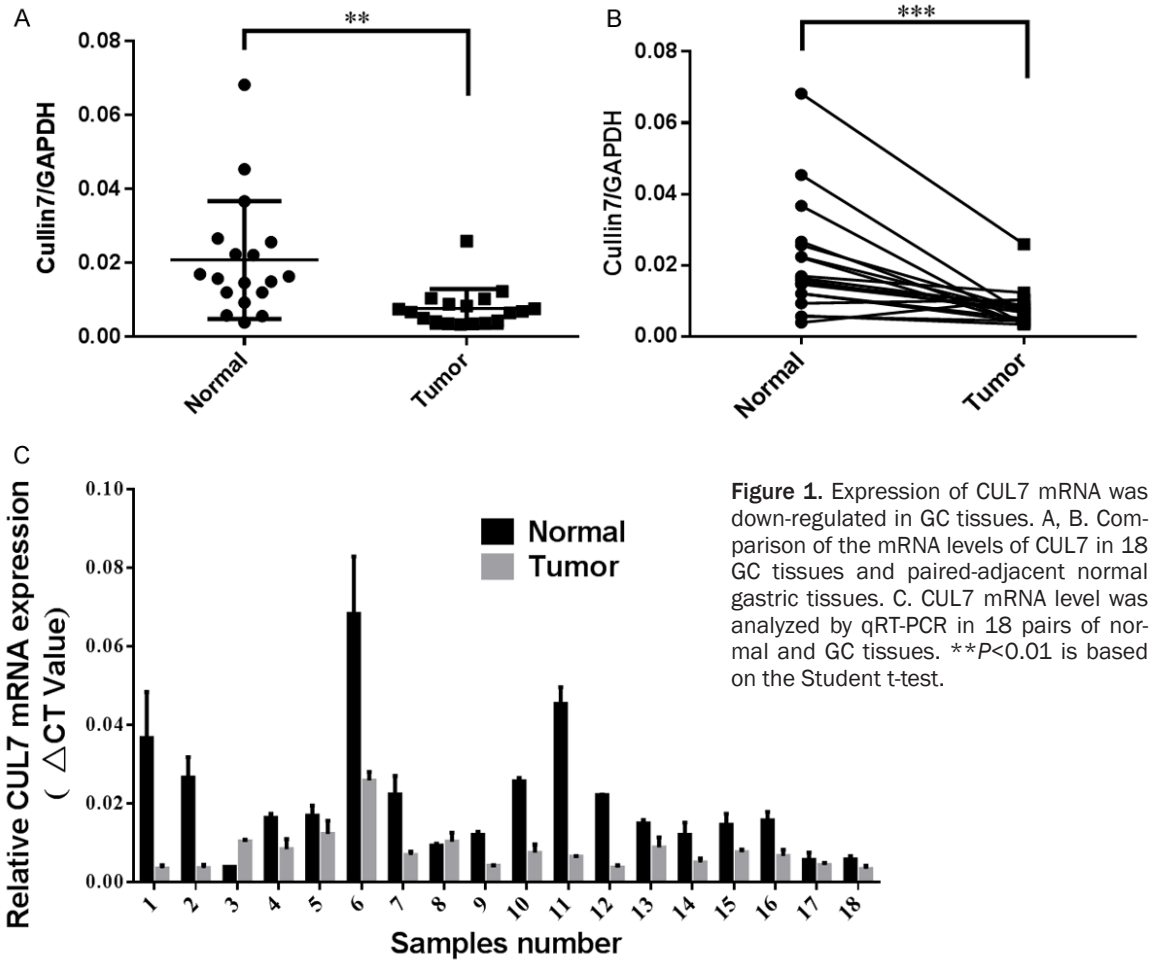

Figure 1. Expression of CUL7 mRNA was down-regulated in GC tissues. A, B. Comparison of the mRNA levels of CUL7 in 18 GC tissues and paired-adjacent normal gastric tissues. C. CUL7 mRNA level was analyzed by qRT-PCR in 18 pairs of normal and GC tissues. ***P*<0.01 is based on the Student t-test.

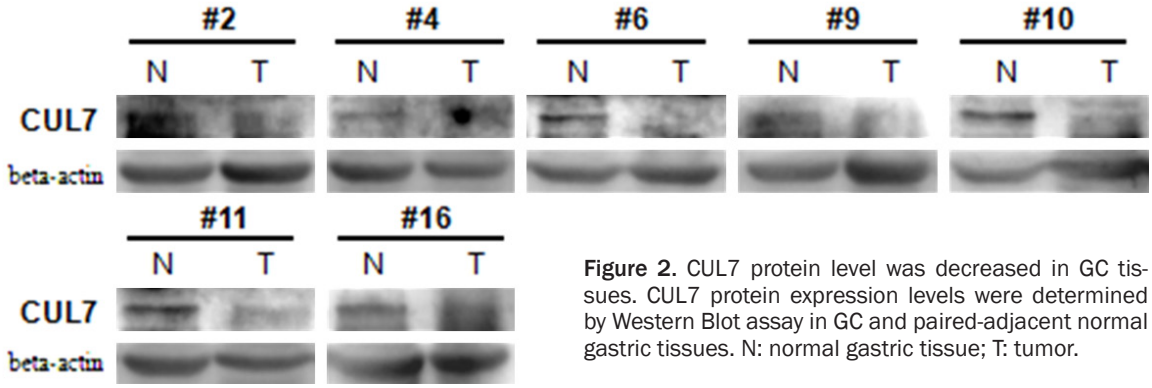

Figure 2. CUL7 protein level was decreased in GC tissues. CUL7 protein expression levels were determined by Western Blot assay in GC and paired-adjacent normal gastric tissues. N: normal gastric tissue; T: tumor.

Results

Expression level of CUL7 was down-regulated in GC tissues

We first determined the mRNA levels of CUL7 by qRT-PCR using the fresh specimens of human GC tissues and the paired-adjacent normal gastric tissues from 18 patients. The qRT-PCR data were analyzed using ΔCT values after normalized to GAPDH. Our results showed that CUL7 mRNA levels were significantly decreased in GC tissues, accounted for about 88.89%, compared to that of paired normal gastric tissues (P<0.01, **Figure 1A-C**). Consistently, as shown in **Figure 2**, the protein levels of CUL7 in the 18

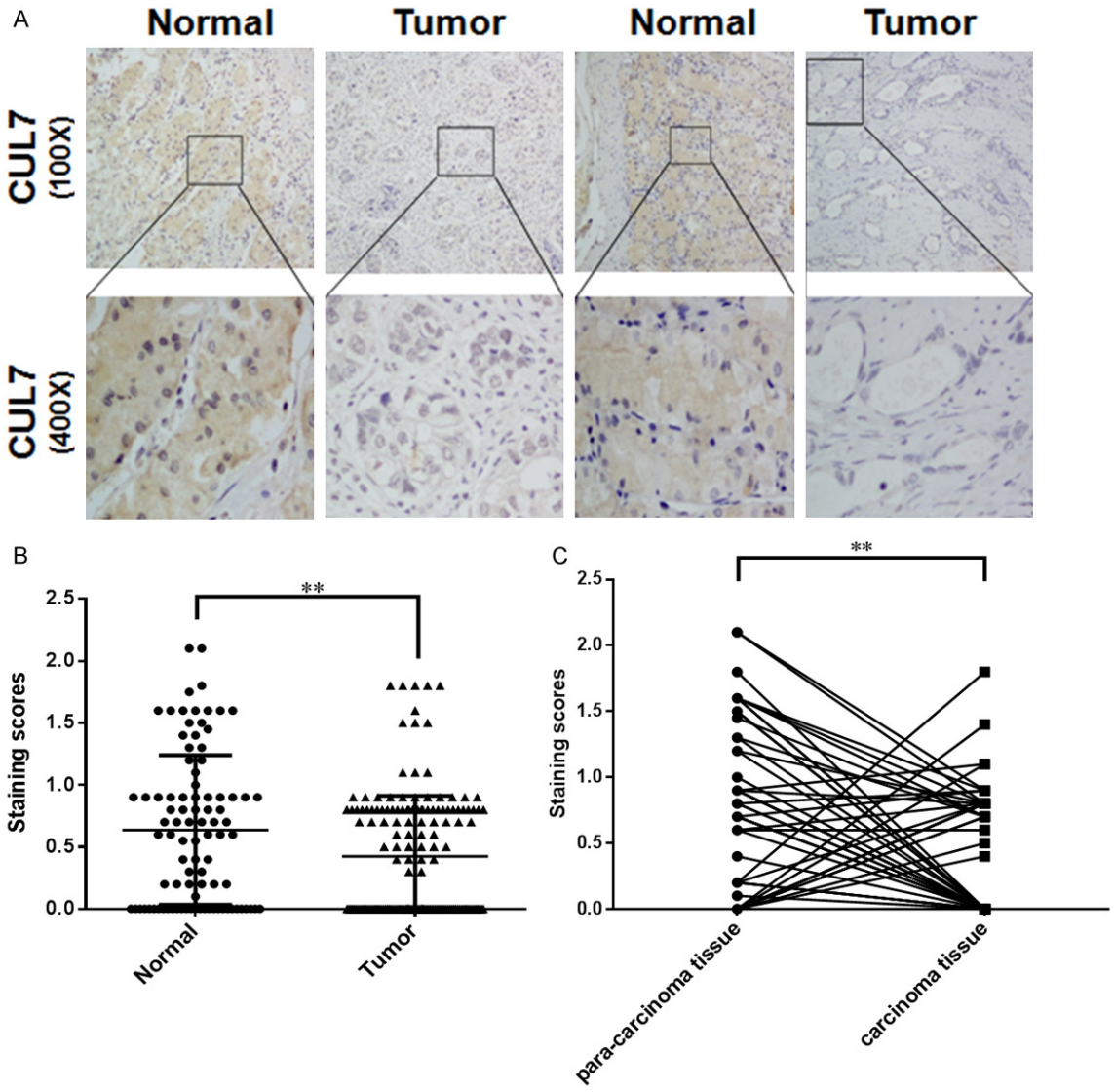

Figure 3. Low expression of CUL7 in human GC tissues. A. CUL7 protein level was evaluated by immunohistochemistry staining in normal and GC tissues. CUL7 are mainly located in the cytoplasm. B. Comparison of the CUL7 levels in GC tissues and normal gastric tissues based on the staining scores. C. Comparison of the CUL7 levels in 60 GC tissues and paired-adjacent normal gastric tissues based on the staining scores. $**P<0.01$ is based on the Student t-test.

GC tissues were also lower than that of adjacent normal tissues by western blot assay (**Figure 2**).

Down-regulation of CUL7 level was validated in a large GC cohort

To further evaluate the expression level of CUL7 in GC, we determine the CUL7 level by immunohistochemistry (IHC) assay using tissue microarray containing 158 GC specimens and 92 normal controls. IHC results showed that the CUL7 was primarily located in cytoplasm, consistent with the previously proposed role of CUL7 as a cytosol protein (**Figure 3A**). After evaluated by experienced pathologists, the statistical result based on the calculated scores showed that GC tissue exhibited lower CUL7 level than that of normal control tissue, which validated our previous results derived from the fresh tissues [17] (**Figure 3B**). This result was further statistically confirmed by the comparison between the 60 paired GC and the adjacent normal tissues (**Figure 3C**).

Table 2. Correlation of the clinicopathologic parameters with CUL7 expression in GC patients

	Characteristics	NO.	Cullin7		P
			High	Low/None	
Age (y)	≤60	76 (48.1%)	31 (40.8%)	45 (59.2%)	0.054
	>60	82 (51.9%)	46 (56.1%)	36 (43.9%)	
TNM stage	I-II	74 (46.8%)	36 (48.6%)	38 (51.4%)	0.984
	III-IV	84 (53.2%)	41 (48.8%)	43 (51.2%)	
Differentiation	Low	96 (60.8%)	38 (39.6%)	58 (60.4%)	0.004
	Medium/High	62 (39.2%)	39 (62.9%)	23 (38.1%)	
Tumor Size	≤5 cm	89 (56.3%)	42 (47.2%)	47 (52.8%)	0.659
	>5 cm	69 (43.7%)	35 (50.7%)	34 (49.3%)	
Histological type	Tubular adenocarcinoma	58 (36.7%)	35 (60.3%)	23 (39.7%)	0.024
(WHO)	Papillary adenocarcinoma	4 (2.5%)	1 (25%)	3 (75%)	
	Mucinous adenocarcinoma	23 (14.5%)	7 (30.4%)	16 (69.6%)	
	Mucous cell carcinoma	2 (1.3%)	0 (0%)	2 (100%)	
	Poorly differentiated adenocarcinoma	67 (42.5%)	34 (50.7%)	33 (49.3%)	
	Signet-ring cell carcinoma	4 (2.5%)	0 (0%)	4 (100%)	
LM metastasis	≤2	79 (50%)	37 (46.8%)	42 (53.2%)	0.633
	>2	79 (50%)	40 (50.6%)	39 (49.4%)	

Relationship between CUL7 expression level and clinicopathological parameters

Immunohistochemistry staining of CUL7 expression levels was statistically analyzed to determine their relationship with the clinical parameters of 158 GC patients. As shown in **Table 2**, we found that CUL7 expression was significantly correlated with histological type (P=0.024) and differentiation degree (P= 0.004). CUL7 expression level was general lower in GC patients with mucinous adenocarcinoma, mucous cell carcinoma and signet-ring cell carcinoma, which are largely comprised of the diffuse-type GC of the Lauren classification. However, tubular adenocarcinoma and poorly differentiated adenocarcinoma did not correlate with low CUL7 expression. Furthermore, CUL7 level was associated with the differentiation of GC. Low CUL7 level was corresponding to the low differentiated GC. Given both the diffuse-type and low differentiated GC are associated with poor prognosis, CUL7 level may serve as a potential prognostic marker for GC patients. There were no significant association between CUL7 expression and other clinicopathologic parameters, including patients' age (P=0.054), TNM stage (P=0.984), tumor size (P=0.659) and lymph nodes metastasis (P=0.633).

Discussion

Cullin proteins function as scaffold molecules and play pivotal roles in the ubiquitin modification of key proteins, thereby controlling a wide range of cellular processes, including cell growth, DNA repair, and apoptosis [18]. Accumulating evidence has shown that dysfunction of cullin proteins may contribute to oncogenesis [19].

CUL7 has been proposed as an oncogene in that it cooperates with Myc to promote transformation by inhibiting Myc-induced apoptosis [20]. In line with this, over-expression of CUL7 has been observed in various tumor types, including breast cancer, liver cancer, lung cancer and epithelial ovarian cancer [13-16]. However, the expression level of CUL7 as well as its possible roles in GC is yet to be determined. In this study, we evaluated the CUL7 levels in GC tissues from a large cohort by qRT-PCR, western blot, and immunohistochemistry staining. Surprisingly, our results showed significantly lower CUL7 levels at both mRNA and protein levels in GC tissues compared to the normal gastric mucosa, in disagreement with its well-known function as a pro-growth protein.

CUL7 was initially isolated as a cellular protein bound to simian virus 40 (SV40) large T antigen (T Ag) [17]. As a member of the polyomaviridae family of DNA viruses, SV40 is capable of im-

mortalizing and transforming rodent cells by disabling critical cellular tumor suppressive mechanisms via T Ag. Association between T Ag and CUL7 E3 complex (containing CUL7, SKP1, RBX1, and an F-box protein FBXW8) has been reported to be essential for SV40 induced transformation. The T Ag mutant (Δ69-83) lacking the CUL7 binding residues showed significantly reduced transformation potential, whereas this mutant enabled the transformation of mouse embryo fibroblasts in the absence of CUL7, suggesting a protective function of CUL7 against the cellular transforming activity mediated by proper T Ag binding.

CUL7 has been implicated in the proteasomal degradation of insulin receptor substrate 1 (IRS-1) [12], a key component of the IGF-1 receptor signaling pathway. Upon activated by upstream IGF-1 receptor, IRS-1 triggers the activation of its downstream Akt/PI3K and RAS/MEK/ERK cascades, driving cells into the pro-mitotic status [21, 22]. The CUL7-containing E3 ligase promoted ubiquitination and proteolytic turnover of IRS-1, thus turning off the excessive activities via IGF-1 receptor signaling pathway [21]. Indeed, accumulation of IRS-1 was observed in CUL7 knockout murine embryonic fibroblasts, exhibiting increased activation of Akt and MEK/ERK pathways [12]. Thus, diverting from its conventional growth-promoting function, CUL7 may also exert an anti-proliferative function in certain circumstances via negative regulation of extrinsic growth signals. Consistently, CUL7-Fbw8 complex has been found to be responsible for ubiquitination and degradation of the hominoid-specific TBC1D3 oncoprotein, which enhances growth factor receptor signaling and subsequently promotes cellular proliferation [23].

Furthermore, Yan et al. has demonstrated that CUL7 plays an important role in maintenance of genome integrity. Severe mitotic defects were observed upon siRNA knockdown of CUL7 in U2OS osteosarcoma cells [9]. Although mitotic entry was intact, depletion of CUL7 resulted in prometaphase failure of chromosome congression and segregation defects. These genome instability phenotypes due to CUL7 deficiency, manifested by polyploid cells and cells with excess centrosomes, suggested a tumor-suppressive role of CUL7 in those osteosarcoma cells.

Up to date, it remains an open question whether CUL7 is an oncogene or a tumor suppressor. Although further investigations are still needed to resolve the “oncogene or tumor suppressor” puzzle, it is not difficult to speculate CUL7 may play dual roles in tumorigenesis in a cell type- or context-dependent manner.

In this study, our data showed significantly down-regulated CUL7 levels in human GC tissues, implying that CUL7 may play an anti-oncogenic role in GC development and progression. Moreover, our results showed that CUL7 expression level was related to histological type and differentiation degree of GC, indicating it may serve as a potential biomarker to complement the traditional clinic-pathological prognostic factors and help guide the treatment strategy for GC patients.

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Disclosure of conflict of interest

None.

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