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ORIGINAL ARTICLE

LncRNA EGOT Promotes Tumorigenesis Via Hedgehog Pathway in Gastric Cancer

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Abstract

Gastric cancer (GC) is one of the mostly terminal malignancies with poor prognosis. Long noncoding RNA EGOT (EGOT) acts as a crucial regulator in the breast cancer. However, the function of EGOT in GC remains unknown. This work was to explore the clinical value and biological significance of EGOT in GC. EGOT levels in GC tissue and cell were analyzed by qRT–PCR. After knockdown of EGOT, GC cell growth and cycle progression were detected. The expression of EGOT was observably elevated in GC. Upregulation of EGOT was related with lymphatic metastasis and TNM stage. In addition, knockdown of EGOT by siRNA could significantly inhibit GC cell proliferation and arrest cycle progression in G1 phase. Moreover, EGOT mediated cyclin D1 expression in GC cells which was regulated by Hedgehog pathway. Further, loss of EGOT downregulated Hedgehog signaling pathway in GC cells. EGOT functions as an oncogene in GC, and may be useful as a conceivable diagnostic and prognostic biomarker for GC tumorigenesis.

Keywords Gastric cancer · EGOT · lncRNA · Tumorigenesis · Hedgehog

Introduction

Gastric cancer (GC) is one of deadly malignancies and is now becoming a major global health menace [1]. On account of aggressiveness, distant metastases have commonly occurred in most GC patients at the time of diagnosis [2]. Until now, there is no sensitive screening test or effective treatment for patients with GC [3]. These deficiencies lead GC to be a highly terminal illness with an overall 5–year survival rate under 30% [1, 4]. So identifying novel biomarkers and uncovering the mechanisms of GC development is urgent to improve the disease surveillance for patients with GC.

Emerging evidences showed that long noncoding RNA (lncRNA) plays a momentous functional part in the occurrence and evolution of malignancies [5]. Specially, some lncRNAs are reported to be dysregulated and involved in pathological processes of cancer via a variety of mechanisms [6]. LncRNA EGOT resides in 3p26.1 and plays a regulative role in various processes of bone marrow hematopoietic stem cell [7]. EGOT function as an oncogene in breast cancer [8]. Nevertheless, clinical implication of EGOT in GC has never been explored up to now.

In current study, we demonstrated that EGOT was signally up–regulated and positively related with the clinicopathological feathers of GC. Then, we found that EGOT knockdown abrogated cell growth and cycle progression of GC cells by downregulating cyclin D1 via Hedgehog pathway. These findings provided the first evidence that EGOT promotes GC.

Material and method

Patients and Samples

This work was approved by Jiangsu Cancer Hospital Ethnics Committee. Informed consents from all were obtained. Total 39 GC patients from 2007 to 2011 were selected and specimens from these patients were obtained from Jiangsu Cancer

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Hospital or First people′s hospital of Yunnan province. Clinical pathologic parameters of all participators were described in Table 1.

Cell Culture

The GC cell lines (MGC80–3, AGS, NCI–N87, MKN–45 and SGC–7901) and normal gastric cell (GES–1) were provided by American Type Culture Collection (Manassas, USA). Cells were foster in Roswell Park Memorial Institute – 1640 (Gibco, Grand Island, USA) harboring 15% fetal calf serum (Gibco) and 1% streptomycin– penicillin (Hyclone, Logan, USA). All cells were cultivated at 37 °C in an incubator with 5% CO_2.

Real–Time Quantitative Polymerase Chain Reaction (qRT–PCR)

Extraction of RNA in tissues or cells using the Trizol (Invitrogen, Carlsbad, USA) following manufacturer′s direction. 2 μg of RNA was conducted into the first chain cDNA, and gene expression was analyzed by qRT–PCR using One Step SYBR® PrimeScript™ RT–PCR Kits (Takara, Dalian, China). Gene–specific primers: EGOT [8] sense: 5′–CACTGCACAGGGAAACACAAA–3′ and antisense: 5′–ACCCTGTTCATAAGCCCTGATG–3′; GAPDH [9] sense: 5′–AGACTCGCTGATGATCCATGC–3′ and antisense: 5′–AGGTGACCACAGTGTTCTG–3′ using an Applied Biosystems Prism 7300 (Applied Biosystems, Foster City, USA). Levels of GAPDH expression were used to normalize the relative abundance of target genes.

Table 1 Clinical pathological parameters of GC patients and their correlation with EGOT expression

Characteristics	n	EGOT expression		*P*
		Low	High	
Gender				0.234
Male	21	11	10	
Female	18	10	8	
Age (years)				1.000
< 60	16	9	7	
≥ 60	23	12	11	
Location				0.751
Gastroesophageal	20	10	10	
Middle to distal	19	11	8	
Depth of invasion				0.528
T1, T2	22	13	9	
T3, T4	17	8	9	
Lymphatic metastasis				0.010*
Negative	20	15	5	
Positive	19	6	13	
TNM stage				0.000*
I, II	15	15	0	
III, IV	24	6	18	
Tumor differentiation				0.504
Low	14	9	5	
Medium, high	25	12	13	

*Statistically significant ($P < 0.05$)

Cell Transduction

Small interfering RNAs (siRNAs) against human EGOT vector with green fluorescent protein (GFP) were purchased from GenePharma (Shanghai, China). Sequences of siRNAs targeting EGOT were as follows: 5′–ATCGCAACAGCUTAUACACUC–3′ (siEGOT – 1), 5′–GUGCAAGAGUAGUTGCTTGAAA–3′ (siEGOT – 2), and 5′–UTGCUCCTCGAUCAUCAGUC–3′ (siEGOT – 3).

Cell Growth Assay

Cell growth was detected by cell count kit 8 (Dojindo Molecular Technologies, Japan) in accordance with the manufacturer′s instruction. Cells were cultured at 5×10^3 cells per well, and then grown in 96–well plates for 48 h. Next, CCK–8 liquor of 10 μl was accessed to each well, and hatched for 1 h. The absorbance (A) was analyzed at 450 nm by microplate reader (Thermo Fisher Scientific, USA). All assays repeated at least three times.

Flow Cytometer Detection

Cells were gathered and immobilized with ethanol for 1 h at 4 °C and storaged at −20 °C. Then cells were rinsed and redisplayed in 1 mL PBS with 50 μg/mL RNase A and ethidium bromide. After culturing at 37 °C for 20 min, cell cycles were evaluated using Flow Cytometer (Becton Dickinson, USA). Cycle interspersion was detected by cycle analysis software (ModFit LT).

Western Blot Assay

Cyclin D1, Shh, SUFU, GLI1 and GAPDH expression was determined as previous reports [10] using cyclin D1 antibody (ab134175, 1:1000 dilution, Abcam, Massachusetts, USA), GAPDH (ab9485,1:1000, Abcam), Shh (2207#, 1:1000, Cell Signaling Technology Beverly, USA), SUFU (2520#, 1:1000,Cell Signaling) and GLI1 (3538#, 1:1500, Cell Signaling).

Statistical Analysis

Data analysis was implemented with SPSS statistical analysis software (IBM, Armonk, USA). Data were reprehensive of

mean ± SD. Pearson chi–square inspection was performed to test the clinical pathologic factors, and Kaplan–Meier tests for comparing GC patient survival according to the expression of EGOT. Receiver operating characteristic (ROC) curve was employed to estimate the diagnostic value for GC patients. $P < 0.05$ by the test was determined to be statistical difference.

Results

Elevated Expression of EGOT Is Related with Progression in GC

Levels of EGOT expression were signally elevated than normal in GC tissues ($P < 0.001$, Fig.1a). Then, we analyzed the association between EGOT expression and clinicopathological feathers of gastric cancer patients (Table 1). EGOT was related with lymphatic metastasis (negative vs. positive, $P = 0.010$) and TNM stage (I, II vs. III, IV, $P = 0.000$) (Fig. 1b and c). EGOT expression in human GC cells (MKN–45, MGC80–3, SGC–7901, NCI–N87 and AGS), was also significantly higher than normal gastric cell GES–1 ($P < 0.01$, Fig. 1d). Cell line MKN–45 with highest level of EGOT expression was chosen for the follow–up studies.

Prognostic and Diagnostic Value of EGOT in GC

To uncover whether EGOT expression related with outcome of GC patients, Kaplan–Meier method was executed to analyze the follow-up data. We demonstrated that patients with low levels of EGOT expression had signally longer overall survival (OS) time than that in high-EGOT group ($P = 0.0393$, Fig. 2a).

To predicate whether EGOT can be used to be a biomarker to discriminate GC from normal, we established a ROC curve by conveying all cancer and normal samples into one class. Levels of EGOT expression were acquired from the group of 39 patients by qRT-PCR analysis. The area under the ROC curve was 0.8330 ($P < 0.001$) (Fig. 2b), revealing that EGOT has potential value in diagnosis of GC.

Loss of EGOT Suppressed GC Cell Cycle Progression and Proliferation

Next, we examined the influence of EGOT on gastric cancer cells, and EGOT siRNA (siEGOT – 1, 2 and 3) was used to suppress the endogenous expression of EGOT in MKN–45 cells ($P < 0.01$, Fig. 3a). To unearth the further impact of EGOT knockdown on GC cells, we tested FACS cycle distribution analysis 48 h after siEGOT – 1 transfection. FCM assays revealed that G1 phase arrest in MKN–45 cells after depletion of EGOT ($P < 0.05$, Fig. 3b). Then, western blot were carried out to detection of cyclin D1 expression. The result revealed that cyclin D1 in MKN–45 cells were dramatically abrogated after transfection of siEGOT – 1 compared with control (Fig. 3c). These results indicated that EGOT was engaged in cycle progression. Thus we deduced that EGOT was responsible for GC cell growth. CCK–8 assays indicated that cell growth was abrogated in MKN–45 cells than control ($P < 0.01$, Fig. 3d).

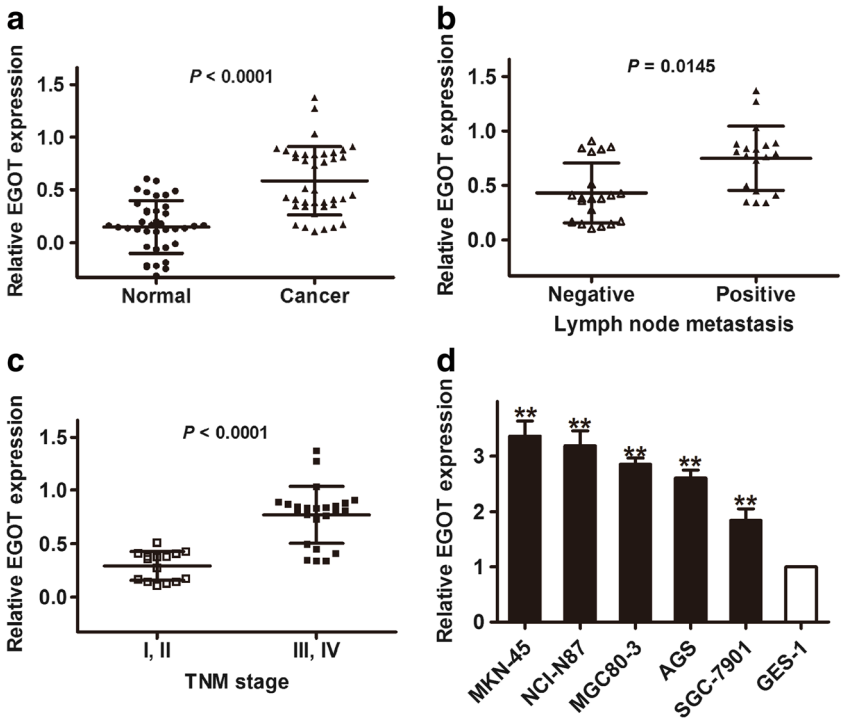

Fig. 1 Expression of EGOT in GC. **a** Expression of EGOT was elevated in GC tissues compared with normal by qRT–PCR analysis. **b**–**c** Correlation between EGOT expression and clinicopathological parameters (lymphatic metastasis and TNM stage) in GC patients. **d** EGOT expression was significantly increased in five GC cell lines relative to normal gastric epithelial cell (GES–1). *$P < 0.05$; **$P < 0.01$

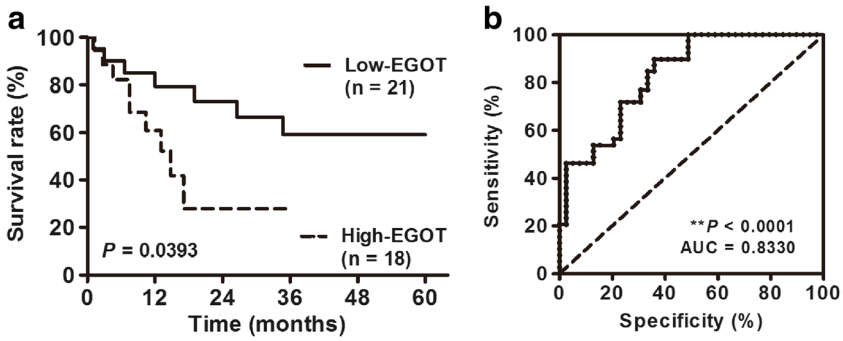

Fig. 2 Prognostic and diagnostic value of EGOT in GC

Effect of EGOT Expression on Hedgehog Signaling Pathway in GC Cells

To uncover the underlying mechanisms of EGOT in GC cells (MKN–45) and the potential signaling pathway, we analyzed the mRNA and protein levels of Shh, SUFU and GLI1 expression in MKN–45 cells. The levels of Shh, SUFU and Gli1 mRNA and proteins were down–regulated in GC cells transfected with siEGOT–1 in comparison with control ($P < 0.01$, Fig.4a and b).

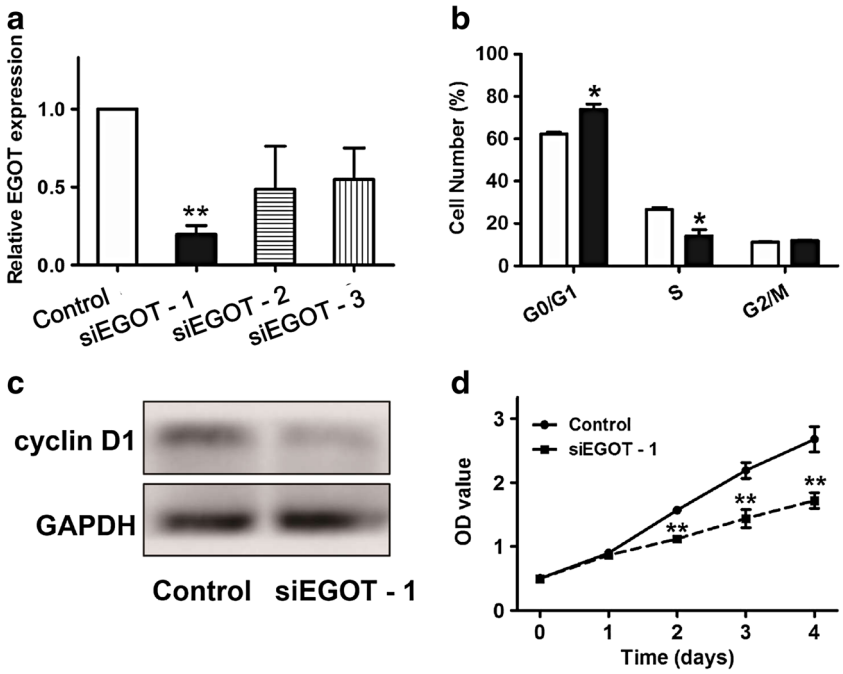

Fig. 3 Loss of EGOT regulated cell cycle progression and growth in GC cells. **a** Levels of EGOT expression was analyzed by qRT–PCR assays in MKN–45 cells treated with siEGOT – 1, siEGOT – 2 and siEGOT – 3. **b** Compared with control, G1 and S phase arrest were found in MKN–45 cells after transfection with siEGOT – 1 for 48 h. **c** Expression of cyclin D1 protein in MKN–45 cells was dramatically refrained after transfection with siEGOT – 1. **d** EGOT knockdown observably suppressed MKN–45 cell growth. Data are represented as mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$

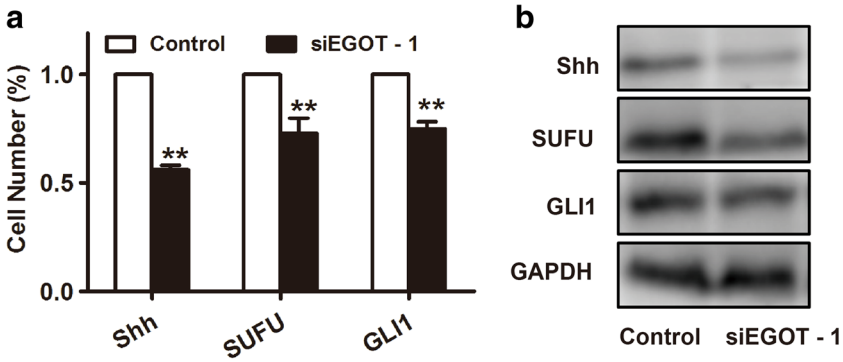

Fig. 4 Regulation of Hedgehog pathway in GC cells. (**a**) and (**b**) The mRNA and protein levels of Shh signaling expression (including Shh, SUFU and Gli1) were examined by qRT–PCR and western blot assays for 48 h after treatment. GAPDH employed as control. **$P < 0.01$

Discussion

Accumulating reports have showed that lncRNA is a sort of non–coding RNA transcript with no or little protein–coding capacity, and act as an important role in oncogenesis [11]. By modulating the expression of responsible genes, lncRNAs are participated in various courses of oncogenesis [12]. However, the function of lncRNA in GC is not well characterized. In current work, we revealed that EGOT is overexpressed by qRT–PCR in GC.

Previous studies identified EGOT as a potential oncogene [13], which was overexpressed in bone marrow [7] and breast cancer [8]. This work indicates that EGOT take participate in GC aggressiveness. EGOT was elevated in GC samples compared with the normal.

To further uncover the biological significance of EGOT in GC, assays in vitro were carried out. Our results proved that loss of EGOT led to remarkable arrest of cell proliferation, while inhibited cycle progression. These data verified that overexpression of EGOT was involved in the oncogenesis of GC and EGOT acts as oncogene in GC.

The Hedgehog pathway seems to be reactivated in several cancers [14]. Aberrant activation of Hedgehog signaling has been shown to be associated with digestive tract tumours [15]. Additionally, Hedgehog signaling acts in the homeostatic control of colorectal cancer [16]. However, lncRNAs have not been shown to directly regulate Hedgehog expression or activity until now. In present study, we first dig out the functions of EGOT in the control of GC cell growth through the Hedgehog signaling pathway.

This work supports the first evidence to show clinical value and pro–oncogenic character of EGOT in GC. The functional mechanism of EGOT impact on GC is related with the modulation of Hedgehog signaling pathway. EGOT can be used to be a candidate aim for GC therapeutic strategies. Although whether and how EGOT interacts with other regulator molecules are poorly understood, our findings extend our knowledge about the pathogenesis of GC and may assist in developing therapies for human GC.

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Compliance with Ethical Standards

Conflict of Interest None.

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