WITHDRAWN ARTICLE

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LncRNA EGOT regulates the proliferation and apoptosis of colorectal cancer by miR-33b-5p/CROT axis

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Abstract

Accumulating researches have proved that long noncoding RNAs (lncRNAs) regulate a variety of cellular processes during cancer progression. However, the detailed function of most lncRNAs in

colorectal cancer (CRC) remains mostly unknown. This study was aimed at exploring the specific role of lncRNA EGOT in CRC. Data from this study revealed that EGOT expression was obviously upregulated in CRC tissues and cell lines, and high EGOT expression indicated poor overall survival of CRC patients. Besides, functional assays proved that EGOT knockdown inhibited cell proliferation and promoted cell apoptosis in CRC. Then, subsequent molecular mechanism assays uncovered that EGOT could bind with miR-33b-5p and negatively regulate miR-33b-5p expression. Additionally, CROT was a downstream target of miR-33b-5p. Further, rescued-function assays suggested that the suppressive influence of EGOT depletion on CRC progression was reversed by miR-33b-5p inhibition or CROT overexpression. In conclusion, lncRNA EGOT mediates the tumor-facilitating part in CRC via miR-33b-5p/CROT pathway.

Key words: EGOT; CRC; miR-33b-5p; CROT.

Introduction

Colorectal cancer (CRC) is a kind of frequent malignant cancer with poor clinical outcome (1). The only hope of treating precancerous CRC might be the radical surgery (2). However, even after the surgery, radiation and chemotherapy still have little effect (3). Previous researches have indicated that some molecular markers are associated with prognosis, but

the pathogenesis of CRC is still unclear (4). Hence, it is an urgent thing to identify potential biomarkers related to cancer progression, which can help develop new treatments for CRC.

Long noncoding RNAs (lncRNAs) are recognized as a group of RNA molecules which are longer than 200 nucleotides and lack any open reading frame to encode protein. Besides, they have been previously manifested as crucial regulators in diverse tumors (5, 6). Specifically, lncRNA SPRY4-IT1 contributes to the tumorigenesis of gastric cancer (7); lncRNA PANDAR predicts poor prognosis and is involved in tumorigenesis in hepatocellular carcinoma (8); and lncRNA LUNAR1 facilitates cell proliferation and high expression of LUNAR1 leads to poor prognosis in diffuse large B-cell lymphoma (9). LncRNA EGOT is backward-looking at the level of nucleotide and was initially found to be implicated in the development of eosinophils and expressed in mature eosinophils (10). In addition, EGOT has been revealed to have a high expression in bone marrow and function in the development of bone marrow hematopoietic stem cells (11). In recent years, the close relationship between abnormally expressed EGOT and tumorigenesis has been clarified in many cancers (11-15). The findings of these researches imply that dysregulation of EGOT is tightly associated with prognosis and that EGOT plays significant role in the occurrence and development of tumors. However, the specific role of EGOT in CRC is largely

unknown.

This study was the first attempt to investigate the underlying function and mechanism of EGOT in CRC, aiming to shed new lights on CRC treatment.

Materials and methods

Clinical specimens

The 70 pairs CRC tissue specimens and matched non-tumor tissue specimens were collected from CRC patients from 2013 and 2018. The ethical approval of this study was obtained from the Ethics Committee of Harbin Medical University Cancer Hospital. All the participants didn't receive any treatment and they all signed the informed consents prior to this study. All clinical samples were instantly frozen in liquid nitrogen and further stored at -80℃.

Cell lines

Human normal cell line NCM460 and four CRC cell lines including SW480, SW620, HCT15 and HT29, were cultivated continuously in DMEM (Gibco, Grand Island, NY, USA) at 37℃ and 5% CO_2. All cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). Culture medium was added with 10% fetal bovine serum (FBS; Gibco) and antibiotics as supplements.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extraction of total RNAs from SW620 and HT29 cells or tissue samples were performed using TRIzol Regent (Invitrogen, Carlsbad, CA, USA). 1 μg of sample was reversely transcribed into cDNA for conducting qPCR in the presence of SYBR® Premix Ex Taq™ II (Takara, Tokyo, Japan) on the Step-One Plus Detection System (Applied Biosystems, Foster City, CA, USA). RNA level was calculated by the comparative $2^{-\Delta\Delta Ct}$ method. The sequences of primers were listed in Supplementary Table 1.

Cell transfection

CRC cells (SW620 and HT29) were seeded in the 6-well and grown in an incubator with CO_2 at 37°C. The two specific shRNAs to EGOT (sh-EGOT#1/2), CROT (sh-CROT#1/2) and control shRNAs (sh-NC), as well as pcDNA3.1/CROT (with empty pcDNA3.1 vector as control), were all produced at RiboBio (Guangzhou, China). Moreover, miR-33b-5p mimics and NC mimics, miR-33b-5p inhibitor and NC inhibitor, were bought from Genechem (Shanghai, China). Cell transfection was performed for 48 h in the presence of Lipofectamine 2000 reagent (Invitrogen). The shRNA sequences were shown in Supplementary Table 2.

Colony formation assay

Transfected CRC cell lines were plated into 6-well plates (1×10^3/100 μL) at 37℃ with 5% CO_2 for 2 weeks. Colonies were rinsed in phosphate-buffered saline (PBS), fixed in 5% paraformaldehyde and labeled with 1 mL of 0.1% crystal violet solution. After counting manually, visible colonies were recorded.

TUNEL assay

CRC cells were transfected with indicated plasmids and harvested for TUNEL assay. Fixed cells in 1% formaldehyde were subjected to 0.2% Triton X-100 for permeabilization and dUTP-end labeling kit (Clontech, Mountain View, CA, USA). Cells were counterstained with DAPI and analyzed by fluorescence microscope (NIKON, Tokyo, Japan).

Western blot

Cells were seeded into 6-well plates prior to being collected for western blot analysis. The total proteins were isolated from the cell lines using RIPA buffer reagent (Thermo Fisher Scientific, Rochester, New York, USA) separated with 10% SDS-PAGE, and transferred onto PVDF membranes on ice overnight. Bovine serum albumin (BSA, 5%; Beyotime, Shanghai, China) was used to block membranes for 2 h at room temperature. Afterwards, membranes were subjected to primary

antibodies and secondary antibodies in succession. Primary antibodies including anti-Bcl-2 (ab692), anti-Bax (ab182733), anti-cleaved caspase-3 (ab2302), anti-total caspase-3 (ab2302), anti-CROT (ab175450), anti-Tubulin (ab210797) and anti-GAPDH (ab22555), together with HRP-labeled secondary antibodies were all acquired from Abcam (Cambridge, MA, USA). Subsequent to washing in PBS, bands were visualized with enhanced chemiluminescence (ECL) system (Bio-Rad lab, Hercules, CA, USA).

Subcellular fractionation

In line with the manufacturer's recommendations of PARIS™ Kit (Invitrogen), subcellular fractionation was assayed in SW620 and HT29 cells. The cell lysates from cell fractionation buffer were first centrifuged and then mixed with cell disruption buffer. The levels of EGOT in cytoplasmic and nuclear fractions were determined by qRT-PCR, with GADPH and U6 as cytoplasmic and nuclear, respectively.

Dual-luciferase reporter vector analysis

For EGOT and CROT 3'UTR luciferase analysis, the pmirGLO Dual-Luciferase reporter constructs containing wild type or mutant miR-33b-5p interacting sequences were formed and termed as EGOT-WT/Mut and CROT-WT/Mut. SW620 or HT29 cells in 24-well

plates were co-transfected with above reporter constructs and indicated transfection plasmids for 48 h, followed by analysis by the Dual Luciferase Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity.

RNA immunoprecipitation (RIP)

Using EZ-Magna RIP Kit (Millipore, Billerica, MA, USA), RIP assay was carried out in CRC cells. SW620 and HT29 cells were washed in ice-cold PBS and lysed in RIP lysis buffer. Human anti-Ago2 antibody and nonspecific control normal IgG antibody (Millipore) were used for immunoprecipitation. RIP lysates were cultured with magnetic beads all night and then precipitated RNAs were purified for qRT-PCR analysis.

RNA pull-down assay

The wild type or mutant CROT sequences which contained the possible miR-33b-5p binding sites were obtained and biotinylated into Bio-CROT-WT/Mut. Cell extracts from RIPA lysis buffer were treated with Bio-CROT-WT/Mut or Bio-NC. After adding magnetic beads, the complex was centrifuged and evaluated by qRT-PCR.

Statistical analyses

Graph generation and data analyses in this study were both disposed via PRISM 6 (GraphPad, San Diego, CA, USA). Besides, data were showed as the mean ± SD of three independent assays. Significant differences between groups were assayed through Student's t test or ANOVA, with threshold of probability (p-value) less than 0.05.

Results

Downregulation of EGOT inhibited cell proliferation and promoted cell apoptosis in CRC

To make the exploration of the underlying role that EGOT played in CRC, we first detected its expression status in tissue samples obtained from CRC patients. And the data from qRT-PCR uncovered that EGOT was highly expressed in CRC tissue specimens versus peri-tumor specimens (Fig. 1A). Then clinical data revealed that high expression of EGOT resulted in low survival rate of CRC patients (Fig. 1B). Next, the expression of EGOT was examined in the normal NCM460 cells and four CRC cell lines by qRT-PCR. The result showed that EGOT expression was relatively high in CRC cell lines, particularly in SW620 and HT29 cells (Fig. 1C). Therefore, SW620 and HT29 cells were selected for the following experiments. After knocking down EGOT in SW620 and HT29 cells by transfection with sh-EGOT#1/2 (Fig. 1D), cell proliferation ability was weakened (Fig. 1E). Conversely, data from TUNEL assay

revealed that knockdown of EGOT facilitated the apoptosis of SW620 and HT29 cells (Fig. 1F). Besides, western blot analysis demonstrated that downregulation of EGOT decreased the expression of Bcl-2 whereas increased the expression of Bax and cleaved caspase-3, further validating that silenced EGOT could induce the apoptosis of SW620 and HT29 cells (Fig. 1G). All these data clarified that EGOT was overexpressed in CRC tissues and cells, and EGOT depletion suppressed cell proliferation but promoted cell apoptosis in CRC.

MiR-33b-5p combined with EGOT

Afterwards, we aimed to study the mechanism of EGOT in CRC, the subcellular fractionation assay was first implemented to find out the subcellular location of EGOT. Interestingly, the result showed that EGOT was mostly located in the cytoplasm of SW620 and HT29 cells (Fig. 2A), implying that EGOT was involved in the posttranscriptional regulation of gene expression. After utilizing online starBase, 7 miRNAs which could possibly bind with EGOT were predicted. Then, data from qRT-PCR revealed that only the expression of miR-33b-5p was found affected most by knockdown of EGOT (Fig. 2B). To further investigate the interaction between miR-33b-5p and EGOT, starBase was applied again to predict the binding sites between EGOT and miR-33b-5p (Fig. 2C). After elevating miR-33b-5p expression in SW620 and HT29 cells by transfection with miR-33b-5p mimics (Fig. 2D), the luciferase activity of

EGOT-WT reporter was decreased a lot. Nevertheless, when the binding sequences of EGOT were mutated, the luciferase activity of EGOT-Mut reporter showed no clear changes between two groups (Fig. 2E). This indicated that EGOT could interact with miR-33b-5p at predicted sites. Moreover, qRT-PCR analysis demonstrated that the expression of miR-33b-5p was signally elevated in CRC cell lines when compared with the normal cell line (Fig. 2F). Further, we observed that the proliferation ability of SW620 and HT29 cells was obviously decreased after miR-33b-5p was overexpressed (Fig. 2G). On the contrary, upregulation of miR-33b-5p could promote the apoptosis of SW620 and HT29 cells (Fig. 2H). Taken together, EGOT targeted the tumor-suppressive miR-33b-5p in CRC.

CROT was the target of miR-33b-5p

Based on the above findings, we proceeded to find the targets of miR-33b-5p by utilizing starBase. With the help of five bioinformatics tools (miRmap, PITA, TargetScan, PicTar and RNA22), two possible candidate targets of miR-33b-5p were screened out (Fig. 3A). Then, it was exhibited that under the condition of EGOT knockdown, CROT expression was downregulated while NAA15 expression was not influenced in SW620 and HT29 cells (Fig. 3B). Thus, CROT was chosen for further analysis. Later, the binding sites between CROT and miR-33b-5p were predicted by starBase (Fig. 3C). Afterwards, the

luciferase reporter assay suggested that elevating miR-33b-5p expression decreased the luciferase activity of CROT-WT reporter but not that of CROT-Mut reporter (Fig. 3D). Furthermore, RIP and RNA pull down assays further proved that CROT could bind with miR-33b-5p (Fig. 3E-F).More importantly, it was proved that the suppressive effect of silenced EGOT on the mRNA and protein levels of CROT was reversed by miR-33b-5p inhibition (Fig. 3G-H). These data showed that EGOT regulated the expression of CROT in CRC by competitively binding with miR-33b-5p. Then, the expression of CROT was found upregulated in CRC cell lines (Fig. 3I). Moreover, cell proliferation capability was attenuated whereas the apoptosis capability was enhanced in SW620 and HT29 cells with CROT depletion (Fig. 3J-L). Briefly, CROT was the target of miR-33b-5p and CROT functioned as a tumor promoter in CRC.

LncRNA EGOT regulated the proliferation and apoptosis of CRC by targeting miR-33b-5p/CROT axis

After validating the existence of EGOT/miR-33b-5p/CROT network in CRC, we further studied the influence of this competing endogenous RNA (ceRNA) mechanism on CRC cell proliferation and apoptosis. Prior to rescued-function assays, we conducted qRT-PCR assay and obtained a satisfactory efficiency of CROT overexpression (Fig. 4A). Expectedly, we unveiled that miR-33b-5p inhibition or CROT upregulation could rescue the suppressive influence of EGOT depletion on cell proliferation

(Fig. 4B). Likewise, the increased cell apoptosis rate caused by EGOT silence was reversed by inhibiting miR-33b-5p expression or overexpressing CROT (Fig. 4C), while such phenomenon was further testified by western blot data (Fig. 4D). To sum up, EGOT regulated CRC cell proliferation and apoptosis via miR-33b-5p/CROT axis.

Discussion

CRC is the leading cause of cancer-related mortality worldwide. Accumulating evidence has verified that lncRNA is implicated in the progression of many malignant tumors, including CRC (16-19). Disorder of EGOT is a common phenomenon in different kinds of cancers, such as breast cancer (11), gastric cancer (14) and renal cell carcinoma (12). As far as we know, few studies have focused on the specific role of lncRNA EGOT in CRC. Therefore, further study of EGOT in CRC may do some help for the treatment of this fatal disease. This study first revealed the expression pattern of EGOT in CRC and further explored the impact of EGOT on CRC cell proliferation and apoptosis, along with its underlying mechanism.

In our study, EGOT was expressed at high levels in CRC tissues and cells, and EGOT predicted poor prognosis of CRC patients, indicating that EGOT might be a possible biomarker for CRC diagnosis. Next, we studied the impact of EGOT on CRC cell proliferation and apoptosis.

Data from loss-of-function assays confirmed the tumor-accelerating effect of EGOT on CRC tumorigenesis. Moreover, Bcl-2 and Bax are essential regulators for the activity of cell apoptosis. Accumulating studies have shown that lncRNAs regulate the expression of Bcl-2 and Bax in various cancers. Yin Z et al uncovered that PPMD1 depletion could increase the protein level of Bax and decrease the protein level of Bcl-2 expression, thus inducing cell apoptosis in colon cancer (20). Wang AH et al elucidated that lncRNA PCAT-1 could regulate Bax and Bcl-2 expression and facilitate cell apoptosis in CRC (21). In this paper, we proved that EGOT depletion decreased Bcl-2 expression and enhanced Bax expression in CRC and induced cell apoptosis in CRC. Besides, several previous findings unveiled that EGOT promoted tumorigenesis in gastric cancer through activating Hedgehog pathway (22), which provided another way for the potential downstream pathway for EGOT in regulating CRC.

Since ceRNA is a typical post-transcriptional pattern and EGOT was mainly distributed in cytoplasm of CRC cells, we assumed that EGOT served as a ceRNA via sponging certain miRNA to regulate CRC cells. Via a range of molecular mechanism assays and rescue tests, we verified that EGOT competitively bound with miR-33b-5p to enhance CROT expression, thus facilitating CRC cell proliferation. Interestingly, Yang X et al. revealed that miR-33b-5p could sensitize gastric cancer cells to

chemotherapy drugs via targeting HMGA2 (23). Moreover, Zhao M et al. previously disclosed the existence of ceRNA network containing miR-33b-5p in prostate cancer, and clarified that CUL4B/miR-33b/C-MYC axis contributed to the progression of prostate cancer (24). However, CROT was scarcely researched in cancers. Previously, Chen X et al. suggested that FECH/CROT axis could predict recurrence of prostate cancer (25). Besides, Hage-Sleiman R et al. indicated that CROT was involved in oxidative stress response in leukemia cells of Molt-4 (26).

Further, lncRNA-associated ceRNA pattern in CRC has been widely reported. Yu C et al. unveiled that lncRNA AC009022.1 could promote CRC cell proliferation, migration and invasion via promotion on ACTR3B expression through miR-497-5p suppression (27). And Wang Y et al. suggested that lncRNA TTN-AS1 enhanced KLF15 expression via acting as a sponge of miR-376a-3p, therefore promoting CRC progression (28). In addition, Wang Y et al. manifested that upregulation of lncRNA CCAT5 could boost CRC cell growth, invasion and metastasis through activation of STAT3 (29). Consistently, our study further provided the evidence of the existence of ceRNA mechanism in CRC and EGOT/miR-33b-5p/CROT axis was firstly confirmed to influence CRC cell proliferation and apoptosis.

On the whole, present study unveiled a novel ceRNA pattern in which

EGOT competitively bound with miR-33b-5p to upregulate CROT expression, therefore promoting carcinogenesis in CRC. This finding might provide novel promising biomarkers for CRC treatment.

Ethical Standards

The ethical approval of tissue samples was obtained from the Ethics Committee of Harbin Medical University Cancer Hospital. All the participants signed the informed consents.

Funding

No funding was received.

Authors' contribution

This research was designed mainly by Yin Ni and most of the experiments were accomplished by Yin Ni and Chunbo Li; the experimental data was acquired by Chen Bo; the data was analyzed and interpreted by Bomiao Zhang; figures and tables was completed by Yanlong Liu and Xuefeng Bai; the writing and revision of the manuscript was finished by Bingbing Cui and Peng Han.

Conflict of interest

None.

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Figure legends

Figure 1 Down-regulation of EGOT inhibited cell proliferation and

promoted cell apoptosis in CRC.

A. EGOT expression CRC tissue samples and peri-tumor samples was detected via qRT-PCR.

B. The association between EGOT expression and overall survival of CRC patients was analyzed via Kaplan-Meier analysis.

C. The expression of EGOT was tested in the normal NCM460 cells and four CRC cell lines by qRT-PCR.

D. The efficiency of EGOT knockdown in SW620 and HT29 cells was examined via qRT-PCR.

E. Colony formation assay was done to test the effect of EGOT knockdown on cell proliferation.

F. TUNEL assay was implemented to examine the impact of EGOT depletion on cell apoptosis. Scale bar: 200 μm.

G. Western blot analysis was used to analyze the influence of EGOT deficiency on the expression of apoptosis-related proteins. $^{**}P < 0.01$.

Figure 2 MiR-33b-5p combined with EGOT.

A. The subcellular fractionation assay was used to determine the subcellular location of EGOT in SW620 and HT29 cells.

B. qRT-PCR was used to test the effect of silenced EGOT on the expression of seven candidate miRNAs in SW620 and HT29 cells.

C. The binding sites between EGOT and miR-33b-5p were predicted by starBase.

D. The efficiency of miR-33b-5p upregulation was examined by qRT-PCR.

E. Luciferase reporter assay was done to confirm the interaction between EGOT and miR-33b-5p.

F. The expression of miR-33b-5p in CRC cell lines compared to NCM460 cells was examined via qRT-PCR.

G. The proliferation of SW620 and HT29 cells transfected with miR-33b-5p mimics or NC mimics was evaluated via colony formation assay.

H. The apoptosis of transfected cells was analyzed via TUNEL assay. Scale bar: 200 μm. $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 3 CROT was the target of miR-33b-5p.

A. Target genes of miR-33b-5p were predicted by starBase.

B. The expression of CROT and NAA15 in SW620 and HT29 cells transfected with sh-NC and sh-EGOT#1 was detected by qRT-PCR.

C. The binding sites between CROT and miR-33b-5p were predicted by starBase.

D-F. The interaction between CROT and miR-33b-5p was validated via luciferase reporter, RIP and RNA pull down assays.

G. The efficiency of miR-33b-5p inhibition was examined by qRT-PCR.

H. The mRNA and protein levels of CROT in different groups were detected via qRT-PCR and western blot.

I. The expression of CROT in CRC cell lines and NCM460 cells were tested by qRT-PCR.

J. The expression of CROT in SW620 and HT29 cells transfected with sh-NC, sh-CROT#1 and sh-CROT#2 was detected by qRT-PCR.

K. The colony formation assay was done to test the effect of CROT knockdown on cell proliferation.

L. TUNEL assay was implemented to examine the impact of silenced CROT on cell apoptosis. Scale bar: 200 μm. $^{**}P < 0.01$.

Figure 4 LncRNA EGOT regulated the proliferation and apoptosis of CRC by targeting miR-33b-5p/CROT axis.

A. The efficiency of CROT overexpression was detected by qRT-PCR in SW620 and HT29 cells.

B. The proliferation of transfected cells was assessed via colony formation assay.

C. TUNEL experiment was used to analyze the apoptosis of transfected cells. Scale bar: 200 μm.

D. The expression of apoptosis-associated proteins was measured via western blot. $^{*}P < 0.01$.

Supplementary Table 1

The sequences of primers were listed.

Supplementary Table 2

The shRNA sequences were shown.