LncRNA EPIC1 Promotes Cancer Cell Proliferation and Inhibits Apoptosis in Gallbladder Cancer Interacting with IncRNA LET

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Abstract

Background: LncRNA EPIC1 is likely involved in human cancer by promoting cell cycle progression. Our study was carried out to investigate the involvement of EPIC1 in gallbladder cancer (GBC).

Methods: Expression levels of EPIC1 in two types of tissues (GBC and paracancerous) and plasma were measured by performing qPCR. GBC-SD and SGC-996 cells were transfected with LET and EPIC1 expression vectors.

Results: In the preset study we found that EPIC1 was upregulated in tumor tissues than in paracancerous tissues of GBC patients, and plasma levels of EPIC1 were significantly correlated with levels of EPIC1 in tumor tissues. LncRNA LET was downregulated in tumor tissues than in paracancerous tissues and was inversely correlated with EPIC1 in both tumor tissues and paracancerous tissues. Overexpression of EPIC1 led to downregulated LET, and LET overexpression also mediated the downregulation of EPIC1. EPIC1 led to accelerated GBC cell proliferation and inhibited apoptosis. Overexpression of LET played opposites roles. In addition, overexpression of LET also attenuated the effects of EPIC1 overexpression on cancer cell proliferation and apoptosis.

Conclusion: Therefore, therefore, lncRNA EPIC1 may promote cancer cell proliferation and inhibit apoptosis in GBC by interacting with LET.

Background

As a common malignancy originate in biliary tract, gallbladder cancer (GBC) mainly affect female and older patients [1, 2]. Incidence of GBC is not high in the United State. It has been estimated that GBC only affect about 1000 American people and causes about 500 deaths every year [3]. In China, GBC affect about 3–5 out of 100,000 people and more than 70% of GBC patients will die of this disease [4]. Occurrence of GBC is closely related to the existence of gallstones [5], while only 1/200 of gallstone patients will develop GBC [1]. At present, the pathogenesis of GBC remains poorly understood [6], which is a challenge for the development of novel therapeutic approaches.

It has been well-established that long (> 200nt) non-coding RNAs (lncRNAs) contribute significantly to the development and progression of GBC [7]. Different from mRNAs as the template for the synthesis of proteins, lncRNAs participate in cancer biology mainly by regulating the expression of certain oncogenes and tumor suppressors [8, 9]. A better understanding of the roles of lncRNAs may provide new insights to cancer prevention and treatment [10]. However, functionality of most lncRNAs remains hardly known. EPIC1 has been characterized as a oncogenic lncRNA in many types of cancers [11, 12], whereas its involvement in GBC is unclear. Our preliminary deep sequencing data showed that EPIC1 was downregulated in GBC and inversely correlated with IncRNA LET, which has been characterized as a tumor suppressor in GBC [13]. This study aimed to investigate the potential interaction between EPIC1 and LET in GBC.
Methods

Subjects

Study subjects of the present study were 60 GBC patients (gender: 26 males and 34 females; age: 58 to 79 years, 69.3±6.6 years). Those patients were selected from the 133 GBC patients admitted to Hubei Provincial Cancer Hospital between May 2015 and January 2018. Inclusion criteria: 1) all cases were newly diagnoses GBC cases; 2) therapies were not initiated. Exclusion criteria: 1) patients transferred from other hospital; 2) recurrent cases; 3) patients complicated with other severe clinical disorders. According to AJCC staging methods and clinical findings, there were 12, 13, 18 and 17 cases at stage I-IV, respectively. This study passed the review board of aforementioned hospital Ethics Committee (2015HBCH57493749). All patients were informed with experimental details and informed consent was signed.

GBC specimens and cells

Before any therapies, paracancerous and GBC tissues were collected from all the 60 GBC patients through biopsy. All tissues were subjected to pathological examinations and all tissues were correct. Fasting blood (3ml) was also extract from all GBC patients before any therapies. Blood was added into EDTA-treated tubes. Following centrifugation at 1200 xg for 15min, supernatant (plasma) was collected.

GBC-SD and SGC-996 two human GBC cell lines (Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultivated in DMEM cell culture medium (10% FBS). Cells culture conditions were 5% CO2, 37 °C and 95% humidity.

Vectors and transient transfections

EPIC1 and LET expression vectors were constructed using pcDNA3 vector (RIBOBIO, Guangzhou, China). GBC-SD and SGC-996 were harvested and counted. Lipofectamine 2000 (Invitrogen, USA) was used to achieve transient transfections of 10 nM vectors (empty pcDNA3 vector as a negative control, NC) into 10^6 cells. Control (C) cells were cells without transfections. The interval between transfections and following experiments was 24h.

Total RNA extractions

Tissues were ground in liquid nitrogen. Total RNAs in 0.01g tissue, 0.2 ml plasma and 10^6 cells were extracted using Trizol reagent (Invitrogen, USA). RNA samples were precipitated and washing using 75% Ethanol. All RNA samples were stored in liquid nitrogen before following experiments.

QPCR

Total RNA samples were digested with DNase I for 1h at 37 °C to remove genomic DNAs. The digested RNA samples were used as template to perform reverse transcriptions using AMV reverse transcriptase
(GIBCO, USA). With GAPDH as endogenous control, qPCR reaction mixtures were prepared using QuantiTect SYBR Green PCR Kits (Qiagen) to detect the expression of EPIC1 and LET. Three replicate tubes were set for each reaction and all Ct value normalizations were performed using $2^{-\Delta \Delta Cq}$ method.

**Cell proliferation analysis**

EPIC1 and LET were harvested. Cells were counted and 1ml DMEM cell culture medium (10% FBS) was mixed with $3 \times 10^4$ cells to make single cell suspensions. In an 96-well cell culture plate (0.1 ml per well), cells were cultured under conditions of 5% CO$_2$, 37 °C and 95% humidity. Three replicate wells were set for each transfection group. Following that, 10ul CCK-8 solution (Sigma-Aldrich) was added into each well at 2h before the termination of cell culture. After cell culture was ended, 10ul DMSO was added into each well and OD values were measured at 450 nm.

**Cell apoptosis analysis**

EPIC1 and LET were harvested. Cells were counted and 1ml DMEM cell culture medium (0.1% FBS) was mixed with $3 \times 10^4$ cells to make single cell suspensions. In a 6-well plate (2ml per well), cells were cultured under conditions of 5% CO$_2$, 37 °C and 95% humidity. Three replicate wells were set for each transfection group. Cell culture was performed for 48h, followed by cell digestion using 0.25% trypsin. Following staining using propidium iodide (PI) and Annexin V-FITC (Dojindo, Japan), flow cytometry was performed to separate apoptotic cells.

**Statistical analysis**

All qPCR, cell proliferation and cell apoptosis experiments were repeated 3 times. Data were expressed as mean values. Differences were explored by either paired t test (GBC vs. paracancerous) or ANOVA (one-way) combined with Tukey test (among different cell transfection groups). Correlations were analyzed by linear regression. p<0.05 was statistically significant.

**Results**

**EPIC1 was upregulated in GBC and positively correlated with its level in plasma**

Expression levels of EPIC1 in two types of tissues (GBC and paracancerous) and plasma were measured by performing qPCR. Differences in expression levels were explored by paired t test (GBC vs. paracancerous). Comparing to paracancerous tissues, expression levels of EPIC1 were significantly higher in GBC tissues (Fig. 1A, p < 0.05). Correlations between EPIC1 in two types of tissues (GBC and paracancerous) and its plasma levels were analyzed by linear regression. It was observed that plasma levels of EPIC1 were significantly correlated with levels of EPIC1 in GBC tissues (Fig. 1B) but not in paracancerous tissues (Fig. 1C).
LET was downregulated in GBC and was inversely correlated with EPIC1

Expression levels of EPIC1 in two types of tissues (GBC and paracancerous) were also measured by performing qPCR. Differences in expression levels were explored by paired t test (GBC vs. paracancerous). Comparing to paracancerous tissues, expression levels of LET were significantly lower in GBC tissues (Fig. 2A, p < 0.05). Correlations between EPIC1 and LET were analyzed by performing linear regression. It was observed that LET was inversely correlated with EPIC1 in both tumor tissues (Fig. 2B) and paracancerous tissues (Fig. 2C).

LET and EPIC1 negatively regulated each other in GBC tissues

GBC-SD and SGC-996 cells were transfected with LET and EPIC1 expression vectors. At 24 h post-transfections, expression levels of LET and EPIC1 were significantly increased comparing to C and NC (empty vector) groups (Fig. 3A, p < 0.05). In addition, overexpression of EPIC1 led to downregulated LET (Fig. 3B), and LET overexpression also mediated the downregulation of EPIC1 (Fig. 3C) in cells of both cell lines (p < 0.05).

The interaction between EPIC1 and LET participate in the regulation of GBC cell proliferation and apoptosis

Comparing to C and NC two groups, EPIC1 led to increased proliferation rate (Fig. 4A) and reduced apoptotic rate (Fig. 4B) of cells of both GBC-SD and SGC-996 cell lines (p < 0.05). Overexpression of LET played opposites roles. In addition, Overexpression of LET also attenuated the effects of EPIC1 overexpression on cancer cell proliferation and apoptosis.

Discussion

This study analyzed the interactions between EPIC1 and LET two IncRNAs in GBC. We found that EPIC1 was upregulated and LET was downregulated in GBC. EPIC1 and LET may form a negative feedback regulation loop to regulate the apoptosis and proliferation of GBC cells.

The function of EPIC1 has been studies in many different types of cancers. Most studies supported the role of EPIC1 as a oncogenic IncRNA in cancer biology [12, 13]. For instance, EPIC1 can promote cell cycle progression through the interactions with MYC [12]. In lung cancer, EPIC1 was upregulated and the overexpression of EPIC1 resulted in the enhanced cell proliferation [11]. However, in a recent study, Zhao et al. reported that EPICA promoted MEF2D ubiquitylation to suppress the invasion and viability of cancer cells [14], indicating is role as a tumor suppressor in this disease. Therefore, EPIC1 may have different functions in different types of cancers. Our study is the first to report the overexpression of EPIC1 in GBC. We also showed the enhanced GBC cell proliferation and inhibited apoptosis after EPIC1 overexpression. Our data suggest that EPIC1 is an oncogenic IncRNA in GBC.

LET plays a tumor suppressive role in different types of cancers through the regulation of multiple cell behaviors, such as proliferation, apoptosis and invasion [15, 16]. Downregulation of LET predicted poor
survival of GBC patients [13]. In our study we showed that LET may inhibit GBC by regulating cancer cell proliferation and apoptosis. Our data further confirmed the role of LET as a tumor suppressor in GBC. It is known that the expression of LET in cancer can be regulated by other lncRNAs, such as lncRNA DANCR. In the present study we found that EPIC1 and LET can form a negative feedback regulation loop to regulate GBC cell behaviors. However, the mechanism mediates the interaction between these two lncRNAs remains unclear. Our future studies will try to explore more details of the interactions between EPIC1 and LET.

**Conclusions**

In conclusion, EPIC1 is an oncogenic lncRNA in GBC and may form and negative feedback regulation loop with LET to promote the apoptosis and inhibit proliferation of GBC cells.

**Abbreviations**

gallbladder cancer (GBC)

**Declarations**

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Not applicable

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Not applicable

**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Ethical statement**

This study passed the review board of Hubei Provincial Cancer Hospital Ethics Committee (2015HBCH57493749).

**Informed consent**

All patients were informed with experimental details and informed consent was signed.
Consent for publication

Not applicable

Authors’ contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work

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Figures

Figure 1

EPIC1 was upregulated in GBC and positively correlated with its level in plasma. Expression levels of EPIC1 in two types of tissues (GBC and paracancerous) and plasma were measured by performing qPCR. Differences in expression levels between GBC and paracancerous were explored by paired t test (A). Correlations between EPIC1 GBC (A) and paracancerous (B) and its plasma levels were analyzed by linear regression. qPCR was performed 3 times and data were mean values, *, p<0.05.

Figure 2
LET was downregulated in GBC and was inversely correlated with EPIC1 Expression levels of EPIC1 in two types of tissues (GBC and paracancerous) were also measured by performing qPCR. Differences in expression levels between GBC and paracancerous were explored by paired t test (A). Correlations between EPIC1 and LET in both tumor tissues (B) and paracancerous tissues (C) were analyzed by performing linear regression. qPCR was performed 3 times and data were mean values, *, p<0.05.

Figure 3

LET and EPIC1 negatively regulated each other in GBC tissues GBC-SD and SGC-996 cells were transfected with LET and EPIC1 expression vectors. At 24h post-transfections, overexpression of LET and EPIC1 were confirmed by performing qPCR (Fig.3A, p<0.05). The effects of EPIC1 overexpression on LET (B), and the effects of LET overexpression on EPIC1 (Fig.3C) were also explored by performing qPCR. All experiments were replicated 3 times and all data were mean values, *,p<0.05.
Figure 4

The interaction between EPIC1 and LET participate in the regulation of GBC cell proliferation and apoptosis. The effects of EPIC1 and LET overexpression on GBC (GBC-SD and SGC-996) cell proliferation and apoptosis were analyzed by performing cell proliferation (A) and apoptosis (B) assays. All experiments were replicated 3 times and all data were mean values, *, p<0.05.