The long noncoding RNA DLGAP1-AS2 facilitates cholangiocarcinoma progression via miR-505 and GALNT10

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Cholangiocarcinoma (CCA) is a highly invasive malignant tumor with high mortality, most cases of CCA are already advanced when they are detected, resulting in poor prognosis. As such, there is an ongoing need for the identification of effective biomarkers for CCA. The long noncoding RNA DLGAP1-AS2 has been reported to have prognostic value in glioma and Wilms’ tumor. Here, we investigated the function of DLGAP1-AS2 in CCA. The differential expression of DLGAP1-AS2 in CCA tissues and normal tissues was first examined using data from the The Cancer Genome Atlas database and then in CCA cell lines by quantitative RT-PCR (qRT-PCR). The target gene was predicted by bioinformatics analysis, and the binding sites were confirmed using luciferase assay. DLGAP1-AS2 is up-regulated in CCA, and high DLGAP1-AS2 expression promotes cell viability and is associated with poor prognosis. Notably, DLGAP1-AS2 acts as a sponge to suppress miR-505 expression, and miR-505 reduces the expression of N-acetylgalactosaminyltransferase 10 (GALNT10) in CCA cells. Biofunctional experiments revealed that a miR-505 inhibitor almost completely removed the inhibitory effect of si-DLGAP1-AS2 on CCA cell malignant progression, whereas the malignant phenotype of cells cotransfected with si-DLGAP1-AS2 and si-GALNT10 was significantly reduced as compared with the control. In summary, the DLGAP1-AS2/miR-505/GALNT10 axis may contribute to regulating the malignant progression of CCA and may have potential as a novel target for CCA therapy.

Abbreviations
CCA, cholangiocarcinoma; CCK-8, Cell Counting Kit-8; GALNT, N-acetylgalactosaminyltransferase 10; IncRNA, long noncoding RNA; NC, negative control; qRT-PCR, quantitative RT-PCR; SD, standard deviation; TCGA, The Cancer Genome Atlas.
form of RNA from three levels: transcription level, posttranscriptional regulation and epigenetics [6–8]. Importantly, lncRNA has been confirmed to act as a regulatory factor in tumor formation, invasion, metastasis and drug resistance mechanisms by affecting the expression of oncogenes [9,10], including CCA. For example, lncRNA-EPIC1 is up-regulated in CCA and accelerates the malignant biological behaviors of CCA by targeting Myc [11]. lncRNA-RMRP contributes to accelerating the tumorigenesis and development of CCA by sponging miR-217 [12]. Hence the development of lncRNA will provide the feasible targets for the targeted therapy of CCA. lncRNA DLGAP1-AS2 is located on human chromosome 18p11, and its role in the physiological process is rarely reported. Recently, two studies have confirmed its prognostic value in glioma [13] and Wilms’ tumor [14]. Wherein, Miao et al. [13] indicated that DLGAP1-AS2 was highly expressed in gliomas and promoted the malignant phenotype of glioma cells by targeting YAP1. Nevertheless, the expression and function of DLGAP1-AS2 in CCA have not been studied.

Herein, we researched the level of DLGAP1-AS2 in CCA and assessed the impact of DLGAP1-AS2 on the malignant biological behaviors of CCA cells in vitro, as well as its potential molecular mechanism, which laid a theoretical foundation for the application of DLGAP1-AS2 as a therapeutic target of CCA.

Materials and methods

Data acquisition

We downloaded the RNA-seq data from The Cancer Genome Atlas (TCGA) database. These data were applied to analyze the differential expression of DLGAP1-AS2, miR-505 and N-acetylgalactosaminyltransferase 10 (GALNT10), which contains 36 patients with CCA and nine healthy control subjects. Moreover, the prognostic value of DLGAP1-AS2 was also assessed using data from TCGA database. Furthermore, patient information was extracted from TCGA database.

Cell culture and transfection

CCA cell lines HUCC1 and RBE were obtained from the Shanghai Cell Bank (Shanghai, China). Normal bile duct cell HEBEpic was purchased from the ScienCell Research Laboratories (Carlsbad, CA, USA). Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 100 U mL⁻¹ penicillin–streptomycin was used to culture the cells at 37 °C with 5% CO₂.

DLGAP1-AS2 negative control (si-NC, 5’-TTTCCGAACGTGTACGT-3’), si-DLGAP1-AS2#1 (5’-CUUGCGUGAGAUGAAUAA-3’), si-DLGAP1-AS2#2 (5’-GGCGGAAAUUGUGUUGU UU-3’), pcDNA3.1-DLGAP1-AS2, miR-505 inhibitor, miR-505 mimic, negative control (miR-NC) and si-GALNT10 (5’-ATGAGTACGAGGTACAT-3’) were synthesized by Dharmaco. HUCC1 and RBE cells were transiently transfected with these sequences by Lipofectamine 2000 as directed by the supplier. After 48 h, the transfection efficiency was measured using qRT-PCR. The transfected cells were used for subsequent experiments.

Total RNA extraction and qRT-PCR

RNeasy Mini Kit (Qiagen, Dusseldorf, Germany) was used to isolate the total RNA from cells. One microgram of total RNA was used as a template, and cDNA was formed using the reverse transcription reaction system following the standard of the RT-PCR kit. Then, qRT-PCR was conducted to assess the mRNA levels of DLGAP1-AS2 and GALNT10 with the SYBR Premix Ex Taq II kit (Takara, Dalian, China) following the supplier’s instructions, and the data were normalized to GAPDH. The primer sequences of DLGAP1-AS2 and GALNT10 were presented as follows: DLGAP1-AS2: forward (F), 5’TTCCTGTGTTTTACGAGATGATCC-3’ and reverse (R), 5’TGGTGAGCCCTGCGGATGTTGAA-3’; GALNT10: F, 5’-GAGCCCTTGACGTGGGAGATGTC-3’ and R, 5’-GAGGTTCCAGAACACTCTCCTGA-3’; and GAPDH: F, 5’-TAGATGACACCCTGGTCTCCTGA-3’ and R, 5’-ACCTCCACCTGT CCTTAGTG-3’.

Western blotting

The cells were lysed with radioimmunoprecipitation assay buffer (containing protease inhibitor) to extract protein. Then, the proteins were separated with 10% SDS/PAGE gels and then transferred to polyvinylidene difluoride membranes. The membranes were sealed in 5% skimmed milk powder for 1 h, which then incubated with primary antibodies at 4 °C overnight. The primary antibodies were as follows: GALNT10 and GAPDH (1 : 1000; Abcam, Cambridge, MA, USA). After that, HRP-conjugated secondary antibody was used to incubate the membranes for 2 h. Finally, Electro-Chemiluminescence substrate was used to measure the blots.

Bioinformatics prediction

LncBase (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbase2%2F2Findex) was applied to predict miRNAs regulated by DLGAP1-AS2, and the
target molecule of miR-505 was predicted by starBase (http://starbase.sysu.edu.cn/).

Luciferase assay
To confirm the results of bioinformatics prediction, we chose 293T cells with high transfection efficiency for luciferase activity detection. The DLGAP1-AS2 sequence was infixed with the pGL3 Basic vector. The luciferase reporter plasmid and miR-505 inhibitor/NC were cotransfected into cells and cultured for 48 h. Then, the luciferase activity was detected using the Promega dual-luciferase reporter gene detection kit following the supplier’s standards. Also, according to the earlier steps, the luciferase reporter assay was conducted to assess the regulatory effect between GALNT10 and miR-505 as well.

Proliferation assays
Cell Counting Kit-8 (CCK-8) and colony formation assays were carried out to assess the proliferative capacity of cells as described previously [15,16]. In CCK-8 assay, the cells were seeded into 96-well plates (4 × 10³ cells per well) and cultured for 0, 24, 48 and 72 h. Then, CCK-8 kit was used to detect the OD value at the specified time point as directed by supplier. Herein, 2 h before the detection, 10 μL CCK-8 solution was appended to each well for culture. For colony formation assay, in brief, about 400 cells were seeded into each medium and cultured at 37 °C with 5% CO₂ for 7–14 days until the visible colonies appeared. Then, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. Finally, we counted the number of colonies.

Invasion and migration assays
Transwell assay was performed to appraise the ability of cell invasion and migration as mentioned earlier, with minor modifications [17]. The upper surface of the Transwell chambers was precoated with (invasion assay) or without (migration assay) Matrigel. Cells were inoculated on the lower surface were fixed with 4% paraformaldehyde and stained with crystal violet. Five visual fields were randomly selected to photograph and count under the microscope.

Statistical analysis
SPSS 21.0 (IBM, New York, USA) statistical software was applied for the data analysis. All assays were performed three times, and the data were represented as mean ± standard deviation (SD) using the t-test (two groups) and one-way ANOVA (multiple groups) for comparison. The correlations between DLGAP1-AS2 and miR-505, as well as GALNT10 and DLGAP1-AS2/miR-505, were analyzed using Pearson’s correlation analysis. The survival curves were plotted using Kaplan–Meier survival analysis. A P-value <0.05 represented that the difference was significant.

Results
Anomalous overexpression of IncRNA DLGAP1-AS2 in CCA tissues and cell lines
To detect whether DLGAP1-AS2 is abnormally expressed in CCA, we measured the level of DLGAP1-AS2 in CCA tissues and normal tissues based on the TCGA database. Patient information was displayed in Table S1. As presented in Fig. 1A, the DLGAP1-AS2 level was markedly enhanced in CCA tissues compared with that in normal tissues. Then, qRT-PCR was conducted to confirm the DLGAP1-AS2 expression in CCA cell lines. Consistently, DLGAP1-AS2 presented the high level in CCA cell lines HUCCT1 and RBE compared with that in HEBEpics cells (Fig. 1B). Moreover, Kaplan–Meier survival analysis indicated that high DLGAP1-AS2 expression was related to poor outcomes of patients with CCA (Fig. 1C).

Considering the abnormal expression of DLGAP1-AS2 in CCA and its prognostic value, we speculate that DLGAP1-AS2 is involved in the malignant progression of CCA. First, we transfected si-DLGAP1-AS2#1 and si-DLGAP1-AS2#2 into HUCCT1 and RBE cells, respectively, to knock down DLGAP1-AS2, and then qRT-PCR was performed to detect the transfection efficiency. As presented in Fig. 1D,E, si-DLGAP1-AS2#1 and si-DLGAP1-AS2#2 both markedly decreased the DLGAP1-AS2 level, and si-DLGAP1-AS2#2 had the higher efficiency. Then, CCK-8 assay was performed to assess the cell viability. It can be seen from Fig. 1F,G that knockdown of DLGAP1-AS2 led to a marked decline in the cell viability of both HUCCT1 and RBE cells. These findings revealed that DLGAP1-AS2 may contribute to promoting the malignant progression of CCA.

IncRNA DLGAP1-AS2 targeted miR-505
The potential targets of DLGAP1-AS2 were predicted using miRDB and obtained 199 miRNAs. Then, the predicted miRNAs were intersected with the down-regulated miRNAs analyzed in TCGA and obtained three common miRNAs (Fig. 2A), namely, hsa-miR-3163,
hsa-miR-4705 and hsa-miR-505. Apparently, miR-505 was lowly expressed in CCA tissues (Fig. 2B). Furthermore, Pearson’s correlation analysis indicated that the miR-505 expression was negatively correlated with DLGAP1-AS2 (Fig. 2C). In view of the earlier results, we selected miR-505 for the follow-up experiments. After transfection of the miR-505 inhibitor, the level of miR-505 decreased significantly, which indicated that the miR-505 inhibitor actually inhibits miR-505 (Fig. 2D). Based on the bioinformatics prediction, the binding site of DLGAP1-AS2 and miR-505 was presented in Fig. 2E. Subsequently, we conducted luciferase reporter assay to validate this prediction. As displayed in Fig. 2F, the luciferase activity of the WT-

Fig. 1. IncRNA DLGAP1-AS2 was up-regulated in CCA and contributes to promoting cell viability of CCA cells. (A) Bioinformatics analysis of DLGAP1-AS2 expression in CCA tissues and normal tissues. $P < 0.0001$. (B) The levels of DLGAP1-AS2 in CCA cell lines HUCCT1 and RBE, as well as normal HEBEpics cells. (C) Kaplan-Meier survival analysis of the relevance of DLGAP1-AS2 expression and survival rate of patients with CCA. $P = 0.0019$. (D,E) The transfection efficiency of si-DLGAP1-AS2#1 and si-DLGAP1-AS2#2 in HUCCT1 and RBE cells was measured using qRT-PCR. (F,G) The impacts of si-DLGAP1-AS2#1 and si-DLGAP1-AS2#2 on cell viability of HUCCT1 and RBE cells were detected using CCK-8 assay. **$P < 0.01$. Error bar represents the mean ± SD derived from three independent experiments. Comparisons between groups were analyzed using t-tests (two-sided). OD, absorbance.
DLGAP1-AS2 reporter was markedly enhanced in the miR-505 inhibitor group, while that of the MUT-DLGAP1-AS2 reporter with mutated binding site was almost unchanged in the miR-505 inhibitor group. Moreover, we detected the effect of the miR-505 inhibitor on DLGAP1-AS2 level using qRT-PCR. Interestingly, inhibition of miR-505 led to a marked increase on DLGAP1-AS2 level (Fig. 2G). These data indicated that DLGAP1-AS2 directly bound to miR-505.

**GALNT10, a downstream molecule of miR-505, was regulated via DLGAP1-AS2**

Next, starBase was used to predict the downstream molecules of miR-505 and obtained 1488 target genes. Then, we analyzed the coexpression genes of DLGAP1-AS2. Under the condition of $P < 0.05$ and correlation coefficient $\geq 0.3$, 2646 genes with positive correlation were obtained. The predicted targets of miR-505 and the coexpression genes of DLGAP1-AS2 were intersected with the up-regulated mRNA analyzed in TCGA database and obtained four common genes (Fig. 3A), namely, ENTPD6, MECOM, GALNT10 and HEY1. The up-regulated GALNT10 (Fig. 3B) with the largest difference and the strongest correlation (Fig. 3C) was selected for the study. The binding site of GALNT10 and miR-505 was presented in Fig. 3D. Then, luciferase reporter assay revealed that the relative luciferase activity was markedly increased in the WT+miR-505 inhibitor group compared with NC or mutant (MUT) groups (Fig. 3E). Our results verified that GALNT10 is a downstream molecule of miR-505.

Because Pearson’s correlation analysis also revealed that GALNT10 expression was negatively interrelated to miR-505 expression and positively correlated with...
miR-505 targeted GALNT10. (A) Wayne diagram was used to ascertain the possible targets of miR-505. (B) Bioinformatics analysis of GALNT10 expression in CCA tissues and normal tissues. \( P < 0.0001 \). (C) The correlation between GALNT10 and DLGAP1-AS2. \( R = 0.5206, P = 0.0002 \). (D) Comparison of miR-505 with GALNT10 sequences. (E) Dual-luciferase reporter assay was performed to confirm that miR-505 directly binds to the 3' UTR of GALNT10. **\( P < 0.01 \). (F) The correlation between GALNT10 and DLGAP1-AS2. \( R = 0.5206, P = 0.0002 \). (G–I) The impacts of si-DLGAP1-AS2, miR-505 inhibitor and si-DLGAP1-AS2+miR-505 inhibitor on GALNT10 mRNA and protein levels were detected using qRT-PCR and western blotting. **\( P < 0.01 \) versus NC group, ###\( P < 0.01 \) versus si-DLGAP1-AS2 group, ^^\( P < 0.01 \) versus miR-505 inhibitor group. (J–L) The impacts of DLGAP1-AS2, miR-505 mimic and DLGAP1-AS2+miR-505 mimic on GALNT10 mRNA and protein levels were detected using qRT-PCR and western blotting. **\( P < 0.01 \) versus NC group, ###\( P < 0.01 \) versus DLGAP1-AS2 group, ^^\( P < 0.01 \) versus miR-505 mimic group. Error bar represents the mean ± SD derived from three independent experiments. Comparisons between groups were analyzed using t-tests (two-sided).
DLGAP1-AS2 promotes the malignant biological behaviors of CCA cells by increasing the GALNT10 level via restraining miR-505

In view of the differential expression of DLGAP1-AS2, miR-505 and GALNT10, as well as their regulatory relationship, we conducted the functional experiments in vitro to verify the biological significance of the DLGAP1-AS2/miR-505/GALNT10 axis. CCK-8 (Fig. 4A,B), colony formation (Fig. 4C,D) and Transwell assays (Fig. 4E,F) revealed that suppression of miR-505 can markedly accelerate cell proliferation, migration and invasion compared with the NC group, whereas the phenotype of cells cotransfected with si-DLGAP1-AS2 and miR-505 inhibitors was almost no different compared with the NC group. Also, after cells cotransfected with si-DLGAP1-AS2 and si-GALNT10, the malignant biological behaviors of both HUCCT1 and RBE cells were significantly reduced compared with that of the NC group. These results demonstrated that DLGAP1-AS2 facilitates the malignant progression of CCA cells via regulating the miR-505/GALNT10 cascade.

Discussion

To our knowledge, most early-stage CCAs are asymptomatic [1]. The lack of effective biomarkers leads to the loss of the best treatment opportunity for most patients. Herein, we expounded the possibility of the DLGAP1-AS2/miR-505/GALNT10 axis as a diagnostic marker and therapeutic target for CCA. As a result, high expression of DLGAP1-AS2 in both CCA tissues and cell lines was associated with poor prognosis. Also, knockdown of DLGAP1-AS2 decreased the viability of CCA cells. Importantly, miR-505 was negatively regulated by DLGAP1-AS2, and it inhibited GALNT10 expression. Then, functional experiments in vitro revealed that inhibition of miR-505 could reverse the facilitating effects of si-DLGAP1-AS2 on cell phenotype, while the malignant biological behaviors of cells cotransfected with si-DLGAP1-AS2 and si-GALNT10 were significantly inhibited compared with that of the NC group.

Extensive research reported that the abnormal expression of lncRNA in malignant tumors is closely related to the tumorigenesis, development and prognosis of cancer [18]. Some lncRNAs are highly expressed in tumors, which presented the characteristics of oncogenes [19–21]. As a little-reported lncRNA, DLGAP1-AS2 was up-regulated in CCA tissues and cell lines, and down-regulation of DLGAP1-AS2 significantly decreased the cell viability of CCA cell lines HUCC1 and RBE. These findings suggest that DLGAP1-AS2 has tumor-promoting properties.

To further explore the functional mechanism of DLGAP1-AS2, we searched for its target and found that DLGAP1-AS2 directly targets miR-505. In recent years, multiple studies indicated that numerous micro-RNAs are aberrantly expressed in CCA and functioned as oncogenes or tumor suppressors in the proliferation, apoptosis, migration and invasion of CCA. For example, miR-21 is highly expressed in CCA, which enhances the proliferation but restrains the apoptosis of CCA cells [22]. Contrarily, miR-186 is down-regulated in CCA, and overexpression of miR-186 can suppress the proliferation of CCA cells [23]. Recently, miR-505 has been widely studied in cancer. It is generally considered that miR-505 has tumor-suppressor properties, which has been confirmed to be down-regulated in colorectal cancer [24], gastric cancer [25], endometrial cancer [26], osteosarcoma [27] and hepatocellular carcinoma [28]. Nevertheless, the role of miR-505 in CCA has not been elucidated. In this work, we have reached a consistent conclusion. miR-505 was down-regulated in CCA tissues based on public data, and inhibition of miR-505 led to a marked increase in cell proliferation, migration and invasion. Importantly, inhibition of miR-505 can also reverse the si-DLGAP1-AS2-induced inhibitory effect on cell viability. These data revealed that the DLGAP1-AS2/miR-505 axis contributes to regulating the malignant progression of CCA.

Interestingly, we found a negative correlation between miR-505 and GALNT10 expression in CCA tissues. GALNT10, a member of the GALNT family,
has been studied in a variety of cancers [29]. GALNT10 can be applied as an independent prognostic factor for advanced ovarian serous carcinoma, which can predict poor prognosis and promote tumor progression [30]. Moreover, GALNT10 is up-regulated via HBV-mediated inhibition of miR-122 and functioned as an oncogene in the progression of hepatocellular carcinoma [31]. Our data indicated that GALNT10 was overexpressed in CCA tissues and related to the poor outcomes of patients with CCA.

Notably, a biological function experiment revealed that knockdown of GALNT10 and DLGAP1-AS2 together inhibited the proliferation, migration and invasion of tumor cells.

In conclusion, we elucidated the new function of lncRNA DLGAP1-AS2 in facilitating the malignant progression of CCA cells by modulating the miR-505/GALNT10 axis. These findings revealed the significance of the lncRNA DLGAP1-AS2/miR-505/GALNT10 axis in the malignant progression of CCA.
and supply a novel target for the CCA diagnosis and therapy.

**Conflict of interest**
The authors declare no conflict of interest.

**Data accessibility**
The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author contributions**
ZL, LP and XD designed the study and collected data. ZL and XY analyzed the data. ZL and LP wrote the manuscript. ZL and XD reviewed and edited the manuscript. All authors read and approved the final manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Patient information.