Long non-coding RNA GCASPC, a target of miR-17-3p, negatively regulates pyruvate carboxylase-dependent cell proliferation in gallbladder cancer

Ming-zhe Ma1,2,3†, Yan Zhang4†, Ming-zhe Weng1,2†, Shou-hua Wang1,2†, Ye Hu5, Zhao-yuan Hou6, Yi-yu Qin1,2, Wei Gong1,2, Yong-Jie Zhang7, Xiang Kong8*, Jian-dong Wang1,2*, Zhi-wei Quan1,2*

1Department of General Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China
2Institute of Biliary Tract Disease, Shanghai Jiao Tong University School of Medicine, Shanghai, China
3Department of Gastric Cancer and Soft Tissue Sarcoma, Fudan University Shanghai Cancer Center, Shanghai, China
4Department of Gastroenterology, Yijishan Hospital, the First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui, China
5State Key Laboratory for Oncogenes and Related Genes; Division of Gastroenterology and Hepatology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
6Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Shanghai Jiaotong University School of Medicine, Shanghai, China
7Second Department of Biliary Surgery and Department of Special Treatment, Eastern
Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China

8Department of Endocrinology, Yijishan Hospital, the First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui, China.

†These authors contributed equally to this work

*To whom correspondence should be addressed:

*Prof. Xiang Kong, Ph.D.. Department of Endocrinology, Yijishan Hospital, the First Affiliated Hospital of Wannan Medical College, 2 West Zheshan Road, Wuhu, 241001, Anhui, China. Fax: +86-0553-5739999. Email: wnmcyaolikx@sina.com.

*Prof. Zhi-wei Quan, M.D. and Jian-dong Wang, Ph.D.. Department of General Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, China. Fax: +86-21-25078999. Email: zhiwquan@163.com and shence201511@sina.com.

Running Title: GCASPC suppresses GBC progression

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**Abbreviations:** lncRNA, long non-coding RNA; GBC, gallbladder cancer; PC, pyruvate carboxylase; *HOTAIR*, HOX transcript antisense RNA; *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1;

**Abstract**

Long non-coding RNAs (lncRNAs) are being implicated in the development of many cancers. Here we report the discovery of a critical role for the lncRNA GCASPC in determining the progression of gallbladder cancer (GBC). Differentially expressed lncRNAs and mRNAs between GBC specimens and paired adjacent non-tumor tissues from 5 patients were identified by and validated by an expression microarray analysis. qRT-PCR was used to measure GCASPC levels in tissues from 42 GBC patients, and levels of GCASPC were confirmed further in a separate cohort of 89 GBC patients. GCASPC was overexpressed or silenced in several GBC cell lines where molecular and biological analyses were performed. GCASPC levels were significantly lower in GBC than adjacent non-tumor tissues, and were associated with tumor size, AJCC tumor stage and patient outcomes. GCASPC overexpression suppressed cell proliferation in vitro and in vivo whereas GCASPC silencing had opposite effects. By RNA pulldown and mass spectrometry we identified pyruvate carboxylase as an RNA-binding protein that associated with GCASPC. Since GCASPC is a target of miR-17-3p, we confirmed that both miR-17-3p and GCASPC
downregulated pyruvate carboxylase level and activity by limiting protein stability. Taken together, our results defined a novel mechanism of lncRNA-regulated cell proliferation in gallbladder cancer, illuminating a new basis for understanding its pathogenicity.

**Key words**: gallbladder cancer; tumor progression; pyruvate carboxylase; long non-coding RNA

**Introduction**

Gallbladder cancer (GBC) is the most common biliary tract cancer and the fifth most common gastrointestinal malignancy worldwide (1). The prognosis of GBC remains extremely poor despite recent advances in GBC treatment, with a median survival time of 9.2 months for suspected carcinomas and 26.5 months for incidental GBC (2,3). Although great efforts have been put into clarifying the pathophysiological mechanisms contributing to the progression of GBC, much of it remains unknown (4,5). Thus, it is vital to reveal the molecular mechanisms of gallbladder carcinogenesis to facilitate development of novel cancer biomarkers and appropriate therapeutic strategies.

Long non-coding RNAs (lncRNAs), a subgroup of non-coding RNAs (ncRNAs), are longer than 200 nucleotides in length and with little protein-coding potential (6,7). lncRNAs are abundantly expressed in mammalian cells and a number of them have been identified as critical regulators in a diverse array of cellular processes via...
controlling multiple levels of the gene expression, including carcinogenesis (8-11). A growing volume of literature has demonstrated that lncRNAs expression profiling may facilitate the diagnosis of human cancers (12-14). They have the potential to serve as prognostic indicators and therapeutic targets. Although over 95000 human lncRNAs have been annotated (15), only a few of them have been functionally characterized. In our previous studies, we have identified several dysregulated lncRNAs in GBC (16-18). Yet, there have been no systematic profiling studies of lncRNAs in gallbladder cancer up until now.

Although emerging evidence has shown the paramount role of lncRNAs in tumor development, only a small portion of them, such as HOX transcript antisense RNA (HOTAIR) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) have been well characterized in various carcinomas (6). Unlike the well-established molecular mechanism of microRNAs (miRs) action (19), which is based on seed sequence base-pairing, the action mode of lncRNAs remains to be explored. Studies revealed that lncRNAs may interact with DNA, RNA or protein and regulate a large number of genes with different mechanisms, thereby impacting a variety of cellular pathways (9-15). Thus, the molecular mechanisms of lncRNAs action can be diversified and require intensive investigations.

In the present study, through transcriptome microarray analysis, we found a number of lncRNAs dysregulated in GBC compared with paired nontumoral tissues. Among
the downregulated lncRNAs, we further characterized the clinicopathologic relevance of a novel lncRNA GCASPC (gallbladder cancer associated suppressor of pyruvate carboxylase lncRNA) in GBC progression. GCASPC interacted with pyruvate carboxylase protein in GBC cells, and the anti-proliferative functions of GCASPC can be neutralized by pyruvate carboxylase. We provided in vitro and in vivo data to demonstrate that GCASPC, which is a target of miR-17-3p, suppressed cell proliferation in GBC by destabilization of pyruvate carboxylase protein.

Materials and Methods

Microarray and computational analysis

Briefly, samples (five GBC tissues and five corresponding nontumor tissues; Supplemental Table 1) were used to synthesize double-stranded complementary DNA (cDNA), and double-stranded cDNA was labeled and hybridized to Human Gene 2.0 arrays (Affymetrix, CA, USA) according to manufacturer’s protocol, and Affymetrix Expression Console Software (version 1.3.1) was used for microarray analysis. Raw data (CEL files) were normalized at the transcript level using the robust multiaverage method (RMA workflow). Median summarization of transcript expression was calculated. The random variance model (RVM) t-test was used to identify differentially expressed genes between the GBC and nontumoral groups. A p value was calculated using the paired t-test. The threshold set for dysregulated genes was a fold change >2.0 and a p value <0.05. Hierarchical clustering (Cluster3.0) and TreeView analysis (Stanford University, USA) were performed based on the results of
differentially expressed genes. Data are available via Gene Expression Omnibus (GEO) GSE62335.

**Patients and Clinical Samples**

The human specimens in this study were sanctioned by the local ethics committee at the Shanghai Jiao-Tong University School of Medicine, Xinhua Hospital (Shanghai, China). Two independent cohorts involving 131 GBC patients were enrolled in this study. Forty-two fresh GBC tissue pairs were collected from patients at Xinhua Hospital (Shanghai Jiaotong University School of Medicine, Shanghai, China) from April 2008 to May 2013. Another 89 fresh GBC tissue pairs were collected from Eastern Hospital of Hepatobiliary (Second Military Medical college, Shanghai, China) from August 2007 to September 2014 and used for further validation. Tissue samples were collected in the operating room and processed immediately within 15 minutes. Patients’ clinical information is listed in Supplementary Table 2 and Supplementary Table 3. The data do not contain any information that could identify patients. None of the patients received preoperative treatment, including chemotherapy or radiotherapy. The nontumorous samples were taken at a distance of at least 5 cm from the tumor, and all tissues were examined histologically.

**Cell culture**

Four human GBC cell lines (GBC-SD, SGC-996, NOZ and OCUG-1) were used in this study. GBC-SD, SGC-996, NOZ, OCUG-1 and the non-tumorigenic human
intrahepatic biliary epithelial cell line H69 were purchased from the Health Science Research Resources Bank (Osaka, Japan) on July 2013 where they were characterized by Mycoplasma detection, DNA-fingerprinting, isozyme detection, and cell vitality detection. The last cell characterization with the above methods was performed on March, 2015. These cell lines were immediately expanded and frozen such that they could be restarted every 3 to 4 months from a frozen vial of the same batch of cells.

Cells were cultured at 37°C in an atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Thermo Scientific). The passage numbers for GBC-SD, SGC-996, NOZ, OCUG-1 and H69 were 16, 11, 9, 23 and 10, respectively. All cell lines have been passaged for fewer than 6 months in our laboratory after resuscitation.

5’and 3’rapid amplification of cDNA ends (RACE) analysis, Subcellular fractionation analysis and assessment of protein-coding potential

RACE analysis and subcellular fractionation analysis were performed as described previously (18). We determined the protein-coding potential of transcript using an in vitro translation assay and a combination of protein-coding potential assessment software.

Plasmid construction, lentiviral construction, and cell transfections

Detailed descriptions of plasmid construction, lentiviral vector construction, and cell transfections can be found in the Supplementary Materials and Methods section.
RNA preparation, qRT-PCR and Western blot analysis

RNA preparation, qRT-PCR and Western blot analysis were performed as described previously [18].

Measurement of Cell proliferation, cell cycle

Cell proliferation was determined with Cell Counting Kit-8 (CCK-8). Cell cycle was determined with flow cytometric analysis.

In vivo tumor growth assay and immunohistochemical analysis

Nude mice (age 4～5 weeks) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences, Shanghai and housed in a pathogen-free facility in the Experimental Animal Centre of Xinhua hospital. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996). Stably overexpressing or silencing GBC cells diluted to a concentration of 1×10⁷ cells/mL in physiological saline. Mice were subcutaneously injected with 0.1ml of the suspension into either side of flank area. Tumor volumes were measured (0.5 × length × width²) in mice every 5 days. After 30 days, mice were sacrificed, and tumors were weighed, exercised and subjected to immunohistochemical analysis of Ki67.
RNA Pull-Down Assay, Mass Spectrometry and RNA Immunoprecipitation (RIP)

The experiments were performed as described by Li et al (12).

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 software (Chicago, IL, USA). All data are presented as the mean ± standard deviation. Unless otherwise noted, the differences between two groups were analyzed using Student's t-test. The Kaplan-Meier method was used to calculate survival, and significance was determined by log-rank test. Multivariate logistic regression was performed to identify the independent factors related to GBC prognosis. The relationship between GCASPC expression levels and clinical parameters was assessed with the nonparametric Mann-Whitney-Wilcoxon test. Risk score analysis was performed to investigate the effectiveness of the GCASPC for prediction. Correlations between GCASPC and miR-17-3p were analyzed by Spearman rank correlation. P-values were two-sided and a value of <0.05 was considered to be statistically significant. One asterisk and two asterisks indicate $p < 0.05$ and $p < 0.01$, respectively.

Results

LncRNAs Expression Profile in GBC

To identify transcripts that potentially drive gallbladder tumorigenesis, LncRNAs and mRNAs expression profiles were determined by microarray analysis. A hierarchical
clustering analysis showed systematic variations in transcript expression levels between GBC tissues and paired adjacent nontumor tissues from 5 GBC patients (Figure 1A). To validate our microarray findings, we randomly selected differentially expressed transcripts (10 lncRNAs and 8 mRNAs) and analyzed their expression, using quantitative real-time polymerase chain reaction (qRT-PCR), in 15 pairs of randomly-selected GBC and corresponding non-tumor tissues from cohort 1 (Supplemental Figure 1,2). Thus, qRT-PCR analysis confirmed our microarray findings, indicating that a set of lncRNAs are frequently aberrantly expressed in GBC tissues.

**Cellular characterization of GCASPC**

In the present study, we focused on the lncRNAs that are significantly downregulated in GBC tissues. We identified a modestly conserved GCASPC (lnc-SOD2-1:1, LNCipedia annotation; NONHSAT115853, NONCODE v4; RP1-56L9.7-001, GENCODE v13) on human chromosome 6; 160060339-160061133 as one of the top ranked candidates with a significant \( p \) value (\( p=0.003, p<0.05 \)). We noted that GCASPC was located within the intron of insulin-like growth factor 2 receptor (\( IGF2R \), Supplemental Figure 3A). To explore the potential relationship of the GCASPC and \( IGF2R \) transcripts, we first examined the expression levels in 27 GBC tissues (cohort 1). The results showed that no correlation (\( r^2=0.031; p=0.377 \)) existed between the transcript levels of GCASPC and \( IGF2R \) (Supplemental Figure 3B). Furthermore, GCASPC was statistically unchanged in GBC-SD and SGC-996 cells.
transfected with two different short interfering RNAs (siRNAs, designated as si-1 and si-2) against *IGF2R*, despite significant reduction in *IGF2R* messenger RNA expression (Supplemental Figure 3C). *IGF2R* was not significantly changed in GBC-SD cells with two different shRNAs (shRNAs, designated as sh-1 and sh-2) against *GCASPC* (Supplemental Figure 3D). *GCASPC* is polyadenylated (Supplemental Figure 3E). *GCASPC* was composed of two exons and spanned nearly 740 base pairs (bp), identifying it as a modestly conserved locus (Supplemental Figure 3F). The sequence of full-length *GCASPC* is presented in Supplemental Figure 4. We verified that *GCASPC* was indeed a non-coding RNA with an in vitro translation assay (Supplemental Figure 5A) and online protein-coding potential assessment softwares (Supplemental Figure 5B,C). Subcellular fractionation analysis revealed *GCASPC* is mainly located in the cytoplasm of GBC cells (Supplemental Figure 5D). The RNAfold image is presented in Supplemental Figure 5E,F.

Expression levels of *GCASPC* was significantly downregulated in GBC cell lines compared to non-tumorigenic human intrahepatic biliary epithelial cell line H69, and *GCASPC* copy number in GBC cells varied from 40 to 130 copies per cell (Supplemental Figure 6A). The expression of *GCASPC* is comparable to *H19, HOTAIR, CCAT1* and *MALAT1* (Supplemental Figure 6B). We did not find any changes in *GCASPC* expression levels in GBC cells treated with DNA methylation inhibitor 5-azacytidine (Supplemental Figure 6C). We determined that *GCASPC* was upregulated by the histone deacetylase inhibitor trichostatin A (TSA) in SGC-996 cells (Supplemental Figure 6D). These results indicate that *GCASPC* expression in
GBC is likely to be regulated by histone acetylation.

*LnRNA-GCASPC is downregulated in GBC tissues and correlated with GBC progression*

To further investigate the role of *GCASPC* in GBC, we examined 42 paired GBC/nontumor tissue specimens (Supporting Table 2, cohort 1). The transcript levels of *GCASPC* was significantly lower in GBC tissues, after normalizing to U6 expression (*p*<0.001, Figure 1B). Furthermore, receiver operating characteristic (ROC) curves were determined to evaluate the sensitivity and specificity of *GCASPC* expression in predicting gallbladder cancer tissues from normal tissues. Notably, *GCASPC* displayed considerable predictive significance, with an area under curve (AUC) of 0.697 (95% CI (confidence interval) = 0.584–0.810, *p*=0.002; Figure 1C).

According to the median ratio of relative *GCASPC* expression in tumor tissues, the GBC patients were classified into two groups: High-*GCASPC* group: *GCASPC* expression ratio $\geq$ median ratio; and Low-*GCASPC* group: *GCASPC* expression ratio $\leq$ median ratio. To determine whether *GCASPC* expression level in GBC was associated with specific clinicopathological characteristics, we measured *GCASPC* expression levels in tumor tissues from another 89 GBC patients independent from 42 GBC patients of cohort 1 (Supplemental Table 3, cohort 2) by qRT-PCR. As demonstrated in Table 1, a lower *GCASPC* expression level was significantly more frequent in tissues with increased tumor size (*p*=0.039, *p*<0.05) and advanced AJCC
tumor stage \((p=0.011, p<0.05)\) in cohort 2. These associations were confirmed by analysis of samples from cohort 1 (supplemental Table 4). Furthermore, Kaplan-Meier and log-rank test analyses suggested a correlation between low tumoral \(GCASPC\) expression and reduced overall survival (OS) and disease-free survival (DFS) rates \((p < 0.001 \text{ for both OS and DFS, Figure } 1E,F)\). In addition, multivariate analysis showed that \(GCASPC\) expression \((95\% \text{ CI: } 1.421–5.034; \ p= 0.006)\) and local invasion status \((95\% \text{ CI: } 1.342–5.579; \ p= 0.005)\) were independent factors that affected the OS of GBC patients after radical GBC resection (supplemental Table 5).

\textbf{LnmRNA-GCASPC suppresses GBC cell proliferation}

To evaluate the biological effects of \(GCASPC\) on development of GBC, we performed gain- and loss-of-function studies in GBC cells. After detecting the expression levels of \(GCASPC\) in a variety of GBC cell lines (Supplemental Figure 6A), we constructed cell lines with stable \(GCASPC\) overexpression and downregulation (Supplemental Figure 7A-D). Cell-counting kit-8 assays indicated that exogenous expression of \(GCASPC\) decreased the proliferative capacity of SGC-996 (Figure 2A) and NOZ cells (Supplemental Figure 8A), compared to that of parallel stable cell lines containing the empty vector. Consistent with decreased cell proliferation, \(GCASPC\)-overexpressing SGC-996 (Figure 2A) and NOZ (Supplemental Figure 8A) cells exhibited lower levels of proliferating cell nuclear antigen (PCNA) expression. Conversely, cell proliferation and PCNA expression were increased in GBC-SD (Figure 2B) and OCUG-1 (Supplemental Figure 8B) cells when \(GCASPC\) expression was knocked...
down. These data suggest that GCASPC play a physiological role in regulating GBC cell proliferation. Next, we examined differences in cell cycle distributions following GCASPC overexpression or silencing by fluorescence-activated cell sorting (FACS) analysis of propidium-iodide-stained cells. GCASPC overexpression resulted in significant G1/S arrest in SGC-996 and NOZ cells, whereas cell cycle progression beyond the G1/S transition was observed in GCASPC-knockdown GBC-SD and OCUG-1 cells (Figure 2C,D; Supplemental Figure 8C,D).

The growth-suppressive effect of GCASPC was confirmed by in vivo tumor growth assays. Our results showed that the growth of tumors from GCASPC-overexpressed xenografts was significantly inhibited as demonstrated by decreased mean volumes and weights as well as slower tumor growth rates, and the growth of tumors from GCASPC-downregulated xenografts was significantly promoted, compared with that of tumors formed from control xenografts. Moreover, immunohistochemical staining of tumor tissues indicated a decrease in Ki67 in GCASPC-upregulated xenografts versus vector-transduced xenografts. In contrast, GCASPC-knockdown xenografts showed stronger staining for Ki67. (Figure 2E,F; Supplemental Figure 8E,F). In summary, these data suggest that GCASPC suppressed gallbladder cancer cell proliferation.

GCASPC associates with pyruvate carboxylase (PC) and downregulates its protein level and activity by destabilizing PC protein in GBC cells
We sought to explore the molecular mechanisms by which GCASPC exerts its effects on GBC cell proliferation. As lncRNAs have been reported to exert cis-regulatory effects on nearby genes (20), we examined whether manipulation of GCASPC expression levels would affect the mRNA levels of its in cis genes. As demonstrated in Supplemental Figure 9A, no statistical changes in the transcript levels of neighbouring genes were observed in SGC-996 cells with GCASPC overexpression, as represented by SOD2, MAS1, loc729603, AIRN and SLC22A1. It suggests GCASPC may act in trans. Recent studies have suggested that lncRNAs participate in molecular regulation pathways through interacting with proteins (9,12). Thus, we hypothesized that GCASPC might function through a similar mechanism. To test this hypothesis, we performed RNA-pulldown assays to identify proteins associated with GCASPC RNA in NOZ cells as previously described (9). RNA-associated proteins were analyzed by SDS/PAGE and silver staining (Figure 3A). Three distinct bands specific to GCASPC were excised and subjected to mass spectrometry (Supplemental Table 6). PC was detected by western blotting from three independent RNA pull-down assays in cell extracts from SGC-996 and NOZ cells (Figure 3B). The specificity of this interaction was further verified with RIP (Figure 3C). Notably, deletion-mapping analyses identified that 3’-end segment (472-741 nt) of GCASPC is required for the association with PC (Figure 3D). RNA folding analyses (21) of this 3’ region indicated a stable stem-loop structure (Supplemental Figure 9B), which might provide the necessary spatial conformation for the interaction. In addition, we found that PC was significantly upregulated in GBC tissues compared to adjacent nontumor
tissues (Supplemental Figure 9C). As mentioned above (Supplemental Figure 5D), GCASPC is mainly located in the cytoplasm of GBC cells. We further detected the subcellular fractionation of GCASPC because PC is a mitochondria protein. We isolated pure mitochondria fractionation via standard cellular fractionation methods and found that GCASPC was mainly located in the mitochondria fraction (Figure 3E). Then we analyzed the interaction between GCASPC and PC in the cytoplasmic (without mitochondria) and mitochondria fraction. The RIP analysis demonstrated that the interaction between GCASPC and PC specifically takes place in the mitochondria fraction (Figure 3F).

Next, we sought to characterize the effects of GCASPC on PC. We detected a significant upregulation of the PC protein and PC activity upon GCASPC knockdown in both GBC-SD (Figure 4A) and OCUG-1 cells (Supplemental Figure 10A), and a downregulation of the PC protein and PC activity in GCASPC-overexpressing SGC-996 (Figure 4A) and NOZ cells (Supplemental Figure 10B), but we did not observe a significant change in PC mRNA levels (Supplemental Figure 10C,D). As 3’-end segment (472-741 nt) of GCASPC is required for the association with PC, we overexpressed the truncated version (472-741 nt) and analyzed its impact on cell proliferation, PC protein expression and activity. The data demonstrated that the truncated version could significantly suppress cell proliferation, PC expression and activity (Supplemental Figure 10E,F).
Based on this finding, we hypothesize that GCASPC binds to PC and affects its biological activity at the translational or post-translational level. To identify these hypotheses, we firstly observed the expression of PC proteins in GBC cells incubated with the protein synthesis inhibitor cycloheximide (CHX). As shown in Figure 4B, CHX decreased the expression of PC proteins by inhibiting protein synthesis. However, knockdown GCASPC still induced the upregulation of PC protein levels under the treatment of CHX (Figure 4B). These results suggest that GCASPC might promote the PC protein degradation. We used the proteasome inhibitor MG-132 to further clarify the possible mechanism. Firstly, ectopic expression of GCASPC downregulated the protein levels of PC (Figure 4A), suggesting that GCASPC destabilized PC protein. As illustrated in Figure 4C, MG-132 upregulated the protein levels of PC, suggesting that the inhibition of ubiquitination-proteasome pathway might ameliorate the degradation of PC. The last but not the least, MG-132 abolished the reduction of PC protein levels in GCASPC-overexpressing SGC-996 (Figure 4C) and NOZ cells (Supplemental Figure 11A). We further examined whether GCASPC affects PC protein stability by performing an ubiquitination assay and found that the PC ubiquitination level was significantly higher in cells that overexpressed GCASPC relative to control cells (Supplemental Figure 11B). These data indicate that GCASPC downregulates PC protein abundance via the ubiquitination-proteasome pathway. Functionally, PC inhibition suppressed the proliferation of GBC cells (Figure 4D; Supplemental Figure 11C) and PC overexpression promoted the proliferation of GBC-SD cells and abrogated the effect of GCASPC overexpression on suppressing
cell proliferation (Figure 4E), indicating that the function of \textit{GCASPC} depends on PC. Collectively, these data suggest that \textit{GCASPC} suppressed tumorigenesis by negatively regulates PC-dependent cell proliferation.

\textit{GCASPC} is direct target of miR-17-3p

A competitive RNA (ceRNA) hypothesis has been proposed and recent studies have suggested that interaction between lncRNAs and microRNAs (miRNAs) (10,11,16), imposing an additional level of posttranscriptional regulation. We performed a search for miRNAs that have complementary base pairing with \textit{GCASPC}, using online software program miRDB (http://mirdb.org) (22). The search results demonstrated that 30 miRNAs formed complementary base pairing with \textit{GCASPC} (Supplementary Table 5). As miR-17-3p achieved the highest score according to miRDB and formed no complementary base pairing with PC mRNA according to Targets can, we selected it for further studies. What’s more, other miRNAs on the list shared no common interaction site with miR-17-3p (Supplementary Table 7). According to the prediction results, there was one putative miR-17-3p binding site in exon 2 of \textit{GCASPC} (Figure 5A). To confirm the direct binding between \textit{GCASPC} and miR-17-3p, luciferase reporter constructs were generated. We observed that miR-17-3p mimics reduced the luciferase activities of wild-type (WT) \textit{GCASPC} reporter vector, but not a mutant \textit{GCASPC}, indicating that miR-17-3p binds to \textit{GCASPC} in a sequence-specific manner. We further clarified the regulatory relationship between \textit{GCASPC} and miR-17-3p. Overexpression of miR-17-3p significantly suppressed the expression of \textit{GCASPC} in
GBC-SD and OCUG-1 cells (Figure 5B). In contrast, inhibition of miR-17-3p enhanced the expression of GCASPC in SGC-996 and NOZ cells (Supplemental Figure 12A). However, there was no obvious difference in miR-17-3p level after overexpression or knockdown of GCASPC (Figure 5B; Supplemental Figure 12B). It suggests that GCASPC is targeted by miR-17-3p. To distinguish between a transcriptional and a posttranscriptional mechanism, we treated GBC-SD cells with alpha-amanitin, which blocked RNA Polymerase II transcription. This experiment revealed that overexpression of miR-17-3p decreased the GCASPC half-life (Figure 5C). The microRNAs are known to bind their targets and cause translational repression and/or RNA degradation in an Ago2-dependent manner. We performed RNA pull-down experiments by using GCASPC probe and then examined Ago2 and miR-17-3p simultaneously as described previously (18) to determine whether GCASPC and miR-17-3p are in the same RISC complex. The in vitro RNA pull-down experiment was performed to confirm the direct physical association between GCASPC and Ago2. As a result, we detected Ago2 (Supplemental Figure 12C). Furthermore, we detected miR-17-3p in the same pellet, supporting that miR-17-3p is bona fide GCASPC-targeting miRNA (Supplemental Figure 12D). Furthermore, miR-17-3p knockdown suppressed the proliferation and expression levels of PC in GCASPC-knockdown GBC-SD cells (Figure 5D,E). A statistically significant inverse correlation was observed between GCASPC and miR-17-3p transcript levels in 42 GBC specimens (r=-0.498, p=0.002, Figure 5F). In general, these data suggest that miR-17-3p directly binds to GCASPC and negatively regulates GCASPC-mediated...
tumor-suppressive activity.

**Discussion**

The molecular classification of GBC have identified a number of protein-coding genes as valuable biomarkers and prognostic indicators (4,5,23). However, a poor overlap exists between these biomarkers of GBC. Thus, it might be a better resolution to establish more-accurate prognostic gene signatures by using a combination of different types of transcripts (24). A growing volume of literature has demonstrated that noncoding RNAs, predominantly miRNAs, could serve as potential biomarkers of GBC (3,25). Given the fact that IncRNAs are more abundantly expressed in mammalian cells, it is plausible to speculate that IncRNAs, once regarded as ‘transcriptional noise’, may be potential prognostic indicators in GBC. Although thousands of IncRNAs have been annotated (15), functional interpretation has just started.

In the present study, we revealed signatures of a small number of IncRNAs that are aberrantly expressed in human GBC, compared to nontumor tissues. We identified a new IncRNA transcript (**GCASPC**), which was significantly downregulated in GBC tissues from two cohorts of patients. We determined that the low expression level of **GCASPC** was significantly associated with numerous clinicopathological characteristics, including tumor size, AJCC stage, frequent recurrence and cancer-related death. A multivariate analysis revealed that **GCASPC** expression level
was an independent risk factor for OS after surgery. These data suggest that *GCASPC* can be a potential prognostic indicator for GBC.

By applying loss-of and gain-of-function approaches, we identified that *GCASPC* play a role in cell proliferation and cell cycle progression. Although it has been suggested that some lncRNAs act *in cis* (neighboring genes) through transcriptional interference, the majority of lncRNAs primarily function *in trans* by targeting multiple chromatin regulatory proteins to distant genes (26). Here, we identified that *GCASPC* had no effect on the expression level of neighboring genes, suggesting that *GCASPC* functions *in trans*. The *GCASPC* transcript was found to associate with pyruvate carboxylase (PC) to promote PC protein degradation. However, the underlying mechanisms of *GCASPC* regulates the ubiquitination and/or ubiquitination associated enzymes require further investigation. PC, an enzyme that converts pyruvate to oxaloacetate, has recently been demonstrated to play an important role in cancer cell metabolism and proliferation (27, 28). We also found that the *GCASPC* abrogated PC-mediated GBC cell proliferation, indicating that *GCASPC* function in a PC-dependent manner. Enhanced glycolysis under aerobic conditions (the Warburg effect) has been a hallmark of cancer for many decades (29). However, accelerated glycolysis alone is insufficient to meet the total metabolic demands of proliferating cancer cells. The Krebs cycle is also a source of energy via the oxidation of pyruvate, fatty acids and amino acids such as glutamine (27). Continued functioning of the Krebs cycle requires the replenishment of intermediates that are diverted for anabolic
uses or glutathione synthesis, which was accomplished via 2 major pathways: carboxylation of pyruvate to oxaloacetate via ATP-dependent pyruvate carboxylase (PC) (27) and glutaminolysis (30). We presume that the inhibition of gallbladder cancer cell proliferation by PC suppression with GCASPC upregulation is accompanied by a decrease in anaplerotic input into the Krebs cycle, which has been verified in NSCLC cells with PC suppression (27). However, whether it is true in this case requires further investigation.

A growing number of reports suggests the existence of a widespread interaction network involving ceRNAs, where ncRNAs could regulate modulatory RNA by binding and titrating them off their binding sites on protein coding messengers (31,32). Inspired by the discoveries of the interaction between lncRNAs and miRs (33,34), we sought to identify the role of miRNAs in the regulation of lncRNAs. Luciferase assays indicated that miR-17-3p directly binds to GCASPC. MiR-17-3p overexpression silenced GCASPC in GBC cells. Furthermore, GCASPC transcript level was inversely correlated with miR-17-3p mRNA level in GBC tissues. However, the alignment between the GCASPC and miR-17-3p is not very specific, as 30 miRNAs were predicted to form complementary base pairing with GCASPC. What’s more, miR-17-3p may also act independently of miR-17-3p, as it shares homology with a number of protein-coding genes such as TIMP3 (35) and MDM2 (36). In addition to miRNAs, lncRNAs could also be regulated by typical transcriptional factor [37, 38], mRNA binding protein [39], promoter methylation [40] and histone acetylation levels
Our data revealed that *GCASPC* was upregulated by the histone deacetylase inhibitor trichostatin A (TSA), suggesting that *GCASPC* expression in GBC is likely to be regulated by histone acetylation. The precise molecular mechanism of the downregulation of *GCASPC* in GBC calls for further research.

In summary, we showed the detailed mechanistic insight of miR-17-3p-*GCASPC*-pyruvate carboxylase axis in gallbladder cancer. This finding suggests that *GCASPC* may be the important target for tumor therapy.

**Reference**


Table 1. Correlation between GCASPC expression and GBC clinicopathological characteristics in 89 patients (Cohort 2)

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<th>Characteristics</th>
<th>GCASPC expression levels</th>
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Differences among variable were assessed by chi-square test. *, the values had statistical significant differences.

Figure Legends

**Figure 1. GCASPC downregulation in GBC tissues.** (A) Hierarchical clustering analysis of the top 100 lncRNAs that were differentially expressed (>2-fold; \( p < 0.05 \)) between GBC samples (T, tumor) and paired non-tumor samples (NT, non-tumor). (B) GCASPC expression was analyzed by qRT-PCR in GBC samples and adjacent non-tumor liver tissues (cohort 1, \( n = 42 \)). GCASPC expression level was normalized to that of U6. Horizontal lines in the box plots represent the medians, the boxes represent the interquartile range, and the whiskers represent the 2.5th and 97.5th percentiles. The significant differences between samples were analyzed using the Wilcoxon signed-rank test. **, \( p < 0.01 \). (C) ROC
curve for prediction of gallbladder cancer using RT-qPCR-based GCASPC expression level. The AUC was 0.697, with 95% CI and p value indicated. Kaplan-Meier survival analysis of overall survival (OS) (E) and disease-free survival (DFS) (F) in GBC patients (p<0.001 for both OS and DFS) based on GCASPC expression.

**Figure 2. GCASPC inhibits GBC cell proliferation and tumor growth.** (A) The cell growth rates were determined with CCK-8 proliferation assays. GCASPC overexpression in SGC-996 cells significantly inhibited cell proliferation. (B) GCASPC depletion enhanced the proliferation of GBC-SD cells. Changes in the proliferation marker, PCNA, were shown by western blotting analysis and normalized to β-Tublin (n=3). (C,D) FACS determined the relative cell numbers in each cell-cycle phase after propidium iodide staining of GCASPC-overexpressed SGC-996 cells (C) or –downregulated GBC-SD cells (D). Numbers inside bars represent percentages of cells in each phase. Data are the mean ± S.D. Effects of GCASPC overexpression (E) or GCASPC knockdown (F) on tumor growth in vivo. Up Left: Representative images of tumors formed in nude mice injected subcutaneously with SGC-996 cells overexpressing GCASPC (E) or GCASPC- silencing GBC-SD cells (F). Tumor weights and tumor growth curves. *, p < 0.05; **, p < 0.01. Right: Representative images of IHC staining of Ki67. (original magnification ×200, bar=50 µM)

**Figure 3. GCASPC binds to pyruvate carboxylase protein.** (A) Silver stained
SDS-PAGE gel of proteins immunoprecipitated from NOZ cell extract by \textit{GCASPC} and its antisense RNA. The arrow indicates the region of the gel excised for mass spectrum determination by the liquid chromatography dual mass spectrometry method. (B) Biotinylated \textit{GCASPC} or antisense RNA was incubated with cell extracts of NOZ and SGC-996 cells, targeted with streptavidin beads, and washed, and the associated proteins were resolved on a gel. Western blot analysis detected the specific association of PC and \textit{GCASPC} (n=3). (C) RIP experiments were performed using the PC antibody for immunoprecipitation (IP) and a primer to detect \textit{GCASPC}. RIP enrichment was determined relative to the input controls (n=3). (D) Biotinylated RNAs corresponding to different fragments of \textit{GCASPC} or its antisense sequence (dotted line) were incubated with NOZ cell lysates and associated proteins were resolved electrophoretically. Western blot analysis of the specific association of PC and \textit{GCASPC} (n = 3). (E) RNA was extracted from the total or only mitochondria of GBC-SD cells. One µg of RNA was used for the qRT-PCR analysis of \textit{GCASPC}, U2 snRNA (nuclear retained), and COX IV (mitochondria retained). (F) RIP experiments with different fractions of cells (non-mitochondria, mitochondria fraction and total cell) were performed using PC for IP and a primer to detect \textit{GCASPC}.

\textbf{Figure 4.} \textit{GCASPC} decreases the protein level and activity of pyruvate carboxylase by inhibiting its protein stability. (A) The protein levels of PC were detected in \textit{GCASPC}-knockdown GBC-SD cells and \textit{GCASPC}-up-regulated SGC-996 cells by Western Blot analysis. PC activity were measured in total cell lysate as
described in methods. (B) GCASPC stable knockdown GBC-SD cells and control
cells were incubated with the protein synthesis inhibitor cycloheximide (CHX, 0.5
μg/μl) for 24 hours. (C) GCASPC stable overexpressing SGC-996 cells and control
cells were incubated with MG132 (5 μM) for 24 hours. The levels of PC proteins
were detected by western blots (n=3). (D) PC specific siRNA 1,2 effectively
suppressed the protein level of PC and the expression of proliferation marker, PCNA
in SGC-996 cells. (E) CCK-8 assays showed that cell proliferation was promoted in
GCASPC-overexpressing GBC-SD cells after the cells were transfected with PC
overexpression vector.

Figure 5. GCASPC is a target of miR-17-3p. (A) The miR-17-3p target site in the
sequence of GCASPC, as predicted by MirTarget2 software (upper panel). Dual
luciferase assays showed a decrease in reporter activity following cotransfection of
pmirGLO-wt-GCASPC and miR-17-3p mimics in GBC-SD cells (p = 0.008), whereas
the cotransfection of pmirGLO-mut-GCASPC and miR-17-3p had no effect on
reporter activity (lower panel). (B) Left: Decreased GCASPC expression in GBC-SD
and OCUG-1 cells after the transfection of miR-17-3p mimics. Right: miR-17-3p
expression levels in GBC-SD and OCUG-1 cells after GCASPC knockdown. (C)
GCASPC stability analysis in GBC-SD cells after alpha-amanitin treatment. Cells
were transfected with miR-17-3p mimics and 48 hours later, a time course for RNA
stability was started by adding the RNA-Polymerase II inhibitor. Cells were harvested
at the indicated time points. Expression levels were normalized to “0 h”. (D)
miR-17-3p inhibition suppressed the protein level of PC as demonstrated by western blotting analysis and normalized to β-Tublin. (E) miR-17-3p inhibition abolished the growth-ability of GCASPC knockdown, as confirmed with CCK8 assays. (E) Western blot analysis showing that miR-17-3p inhibition decreased the expression levels of PC and PCNA in GCASPC-knockdown cells. (F) Scatter diagram exhibited a negative correlation of GCASPC and miR-17-3p in 42 pairs of GBC tissues by qRT-PCR. **, p < 0.01.
Table 1. Correlation between GCASPC expression and GBC clinicopathological characteristics in 89 patients (Cohort 2)

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Differences among variable were assessed by chi-square test.*, the values had statistical significant differences.
Figure 3

A

B

C

D

E

F

Figure 3

A

B

C

D

E

F

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Figure 5

A

GCASPC-wt

miR-17-3p

GCASPC-mut

5'-GAGTGCAGAATTGCA3'

3'-UUCACGGAAG--UGACGUCAC-5'

Luciferase activity

GBC-SD

OCUG-1

B

scramble

miR-17-3p

GCASPC shRNA-1

GCASPC shRNA-2

GBC-SD

OCUG-1

C

scramble

miR-17-3p mimics

% Remaining RNA of GCASPC

alpha-amanitin (hours)

D

PC

β-Tublin

1.00

0.52

E

scramble

shRNA-GCASPC

miR-17-3p inhibitor

shRNA-GCASPC+miR-17-3p inhibitor

CCK8-OD450

0.0 1 2 3 4 5 6 7

F

r=-0.498, p=0.001

GCASPC expression (ΔCt)

miR-17-3p expression (ΔCt)

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Ming Z Ma, Yan Zhang, MINGZHE WENG, et al.

Cancer Res  Published OnlineFirst July 22, 2016.

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