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Long Noncoding RNA *GCASPC*, a Target of miR-17-3p, Negatively Regulates Pyruvate Carboxylase–Dependent Cell Proliferation in Gallbladder Cancer

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Abstract

Long noncoding RNAs (lncRNA) 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. Differentially expressed lncRNAs and mRNAs between gallbladder cancer specimens and paired adjacent nontumor tissues from five patients were identified and validated by an expression microarray analysis. Quantitative real-time PCR was used to measure GCASPC levels in tissues from 42 gallbladder cancer patients, and levels of GCASPC were confirmed further in a separate cohort of 89 gallbladder cancer patients. GCASPC was overexpressed or silenced in several gallbladder cancer cell lines where molecular and biological analyses were performed. GCASPC levels were significantly lower in gallbladder cancer than adjacent nontumor tissues and were associated with tumor size, American Joint Committee on Cancer tumor stage, and patient outcomes. GCASPC overexpression suppressed cell proliferation *in vitro* and *in vivo*, whereas GCASPC silencing had opposite effects. By RNA pull-down and mass spectrometry, we identified pyruvate carboxylase as an RNA-binding protein that associated with GCASPC. Because 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. *Cancer Res; 76(18); 5361–71. ©2016 AACR.*

Introduction

Gallbladder cancer is the most common biliary tract cancer and the fifth most common gastrointestinal malignancy worldwide (1). The prognosis of gallbladder cancer remains extremely poor despite recent advances in gallbladder cancer treatment, with a median survival time of 9.2 months for suspected carcinomas and 26.5 months for incidental gallbladder cancer (2, 3). Although great efforts have been put into clarifying the pathophysiologic mechanisms contributing to the progression of gallbladder cancer, 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 noncoding RNAs (lncRNA), a subgroup of noncdoing RNAs (ncRNA), 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 95,000 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

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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gallbladder cancer (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 miRNAs 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 affecting 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 gallbladder cancer compared with paired nontumoral tissues. Among the downregulated lncRNAs, we further characterized the clinicopathologic relevance of a novel gallbladder cancer–associated suppressor of pyruvate carboxylase lncRNA (lncRNA *GCASPC*) in gallbladder cancer progression. *GCASPC* interacted with pyruvate carboxylase (PC) protein in gallbladder cancer cells, and the antiproliferative functions of *GCASPC* can be neutralized by PC. We provided *in vitro* and *in vivo* data to demonstrate that *GCASPC*, which is a target of miR-17-3p, suppressed cell proliferation in gallbladder cancer by destabilization of PC protein.

Materials and Methods

Microarray and computational analysis

Briefly, samples (five gallbladder cancer tissues and five corresponding nontumor tissues; Supplementary Table S1) were used to synthesize double-stranded complementary DNA (cDNA), and double-stranded cDNA was labeled and hybridized to Human Gene 2.0 arrays (Affymetrix) according to the 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 gallbladder cancer 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, Stanford, CA) 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 gallbladder cancer patients were enrolled in this study. Forty-two fresh gallbladder cancer tissue pairs were collected from patients at Xinhua Hospital (Shanghai Jiao Tong University School of Medicine) from April 2008 to May 2013. Another 89 fresh gallbladder cancer 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 Tables S2 and S3. 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 gallbladder cancer cell lines (GBC-SD, SGC-996, NOZ, and OCUG-1) were used in this study. GBC-SD, SGC-996, NOZ, OCUG-1, and the nontumorigenic human intrahepatic biliary epithelial cell line H69 were purchased from the Health Science Research Resources Bank 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% CO_2 in DMEM (Gibco BRL) supplemented with 10% FBS, 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 analysis, subcellular fractionation analysis, and assessment of protein-coding potential

Rapid amplification of cDNA ends (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.

RNA preparation, quantitative real-time PCR, and Western blot analysis

RNA preparation, quantitative real-time PCR (qRT-PCR), and Western blot analysis were performed as described previously (18).

Measurement of cell proliferation and cell cycle

Cell proliferation was determined with the 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 NIH (NIH publication number 85-23, revised 1996). Stably overexpressing or silencing gallbladder cancer cells diluted to a concentration of 1×10^7 cells/mL in physiologic saline. Mice were subcutaneously injected with 0.1 mL of the suspension into either side of flank area. Tumor volumes were measured ($0.5 \times \text{length} \times \text{width}^2$) 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

The experiments were performed as described by Li and colleagues (12).

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 software. All data are presented as the mean ± SD. Unless otherwise noted, the differences between two groups were analyzed using the Student *t* test. The Kaplan–Meier method was used to calculate survival, and significance was determined by the log-rank test. Multivariate logistic regression was performed to identify the independent factors related to gallbladder cancer 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 gallbladder cancer

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 gallbladder cancer tissues and paired adjacent nontumor tissues from 5 gallbladder cancer patients (Fig. 1A). To validate our microarray findings, we randomly selected differentially expressed transcripts (10 lncRNAs and 8 mRNAs) and analyzed their expression, using qRT-PCR, in 15 pairs of randomly selected gallbladder cancer and corresponding nontumor tissues from cohort 1 (Supplementary Figs. S1 and S2). Thus, qRT-PCR analysis confirmed our microarray findings, indicating that a set of lncRNAs are frequently aberrantly expressed in gallbladder cancer tissues.

Cellular characterization of *GCASPC*

In the present study, we focused on the lncRNAs that are significantly downregulated in gallbladder cancer 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*; Supplementary Fig. S3A). To explore the potential relationship of the *GCASPC* and *IGF2R* transcripts, we first examined the expression levels in 27 gallbladder cancer 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* (Supplementary Fig. S3B). Furthermore, *GCASPC* was statistically unchanged in GBC-SD and SGC-996 cells transfected with two different siRNAs (designated as si-1 and si-2) against *IGF2R*, despite significant reduction in *IGF2R* messenger RNA expression (Supplementary Fig. S3C). *IGF2R* was not significantly changed in GBC-SD cells with two different shRNAs (shRNAs, designated as sh-1 and sh-2) against *GCASPC* (Supplementary Fig. S3D). *GCASPC* is polyadenylated (Supplementary Fig. S3E). *GCASPC* was composed of two exons and spanned nearly 740 base pairs (bp), identifying it as a modestly conserved locus (Supplementary Fig. S3F). The sequence of full-length *GCASPC* is presented in Supplementary Fig. S4. We verified that *GCASPC* was indeed a noncoding RNA with an *in vitro* translation assay (Supplementary Fig. S5A) and online protein-coding potential assessment softwares (Supplementary Fig. S5B and S5C). Subcellular fractionation analysis revealed *GCASPC* is mainly located in the cytoplasm of gallbladder cancer cells (Supplementary Fig. S5D). The RNAfold image is presented in Supplementary Fig. S5E and S5F. Expression levels of *GCASPC* were significantly downregulated in gallbladder cancer cell lines compared with nontumorigenic human intrahepatic biliary epithelial cell line H69, and *GCASPC* copy number in gallbladder cancer cells varied from 40 to 130 copies per cell (Supplementary Fig. S6A). The expression of *GCASPC* is comparable with *H19*, *HOTAIR*, *CCAT1*, and *MALAT1* (Supplementary Fig. S6B). We did not find any changes in *GCASPC* expression levels in gallbladder cancer cells treated with DNA methylation inhibitor 5-azacytidine (Supplementary Fig. S6C). We determined that *GCASPC* was upregulated by the histone deacetylase inhibitor trichostatin A (TSA) in SGC-996 cells (Supplementary Fig. S6D). These results indicate that *GCASPC* expression in gallbladder cancer is likely to be regulated by histone acetylation.

LnRNA GCASPC is downregulated in gallbladder cancer tissues and correlated with gallbladder cancer progression

To further investigate the role of *GCASPC* in gallbladder cancer, we examined 42 paired gallbladder cancer/nontumor tissue specimens (Supporting Table 2, cohort 1). The transcript levels of *GCASPC* were significantly lower in gallbladder cancer tissues, after normalizing to U6 expression ($P < 0.001$; Fig. 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 the curve (AUC) of 0.697 [95% confidence interval (CI), 0.584–0.810; $P = 0.002$; Fig. 1C].

According to the median ratio of relative *GCASPC* expression in tumor tissues, the gallbladder cancer patients were classified into two groups: High-*GCASPC* group: *GCASPC* expression ratio ≥ median ratio; and Low-*GCASPC* group: *GCASPC* expression ratio ≤ median ratio. To determine whether *GCASPC* expression level in gallbladder cancer was associated with specific clinicopathologic characteristics, we measured *GCASPC* expression levels in tumor tissues from another 89 gallbladder cancer patients independent from 42 gallbladder cancer patients of cohort 1 (Supplementary Table S3, 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 American Joint Committee on Cancer (AJCC)

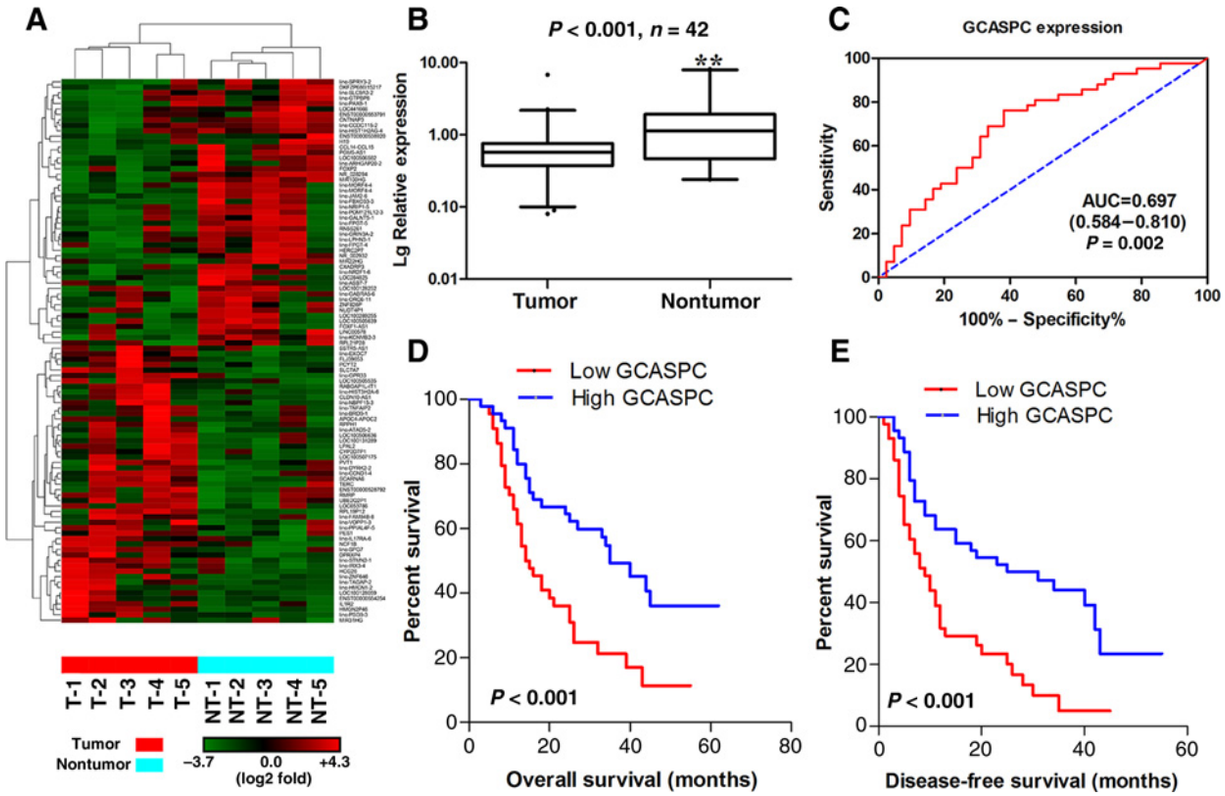

Figure 1.

GCASPC downregulation in gallbladder cancer tissues. **A,** hierarchical clustering analysis of the top 100 lncRNAs that were differentially expressed (>2-fold; $P < 0.05$) between gallbladder cancer (T, tumor) samples and paired nontumor (NT) samples. **B,** *GCASPC* expression was analyzed by qRT-PCR in gallbladder cancer samples and adjacent nontumor 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 OS (**D**) and DFS (**E**) in gallbladder cancer patients ($P < 0.001$ for both OS and DFS) based on GCASPC expression.

tumor stage ($P = 0.011$; $P < 0.05$) in cohort 2. These associations were confirmed by analysis of samples from cohort 1 (Supplementary Table S4). 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 ($P < 0.001$ for both OS and DFS; Fig. 1E and F). In addition, multivariate analysis showed that *GCASPC* expression (95% CI, 1.421–5.034; $P = 0.006$) and local invasion status (95% CI, 1.342–5.579; $P = 0.005$) were independent factors that affected the OS of gallbladder cancer patients after radical gallbladder cancer resection (Supplementary Table S5).

LnRNA GCASPC suppresses gallbladder cancer cell proliferation

To evaluate the biological effects of *GCASPC* on development of gallbladder cancer, we performed gain- and loss-of-function studies in gallbladder cancer cells. After detecting the expression levels of *GCASPC* in a variety of gallbladder cancer cell lines (Supplementary Fig. S6A), we constructed cell lines with stable *GCASPC* overexpression and downregulation (Supplementary Fig. S7A–S7D). Cell-counting kit-8 assays indicated that exogenous expression of *GCASPC* decreased the proliferative capacity of SGC-996 (Fig. 2A) and NOZ cells (Supplementary Fig. S8A), compared with that of parallel stable cell lines containing the empty vector. Consistent with decreased cell proliferation, *GCASPC*-overexpressing SGC-996 (Fig. 2A) and NOZ (Supplementary Fig. S8A) cells exhibited lower levels of proliferating cell nuclear antigen (PCNA) expression. Conversely, cell proliferation and PCNA expression were increased in GBC-SD (Fig. 2B) and OCUG-1 (Supplementary Fig. S8B) cells when *GCASPC* expression was knocked down. These data suggest that *GCASPC* plays a physiologic role in regulating gallbladder cancer cell proliferation. Next, we examined differences in cell-cycle distributions following *GCASPC* overexpression or silencing by FACS analysis of propidium-iodide–stained cells. *GCASPC* overexpression resulted in significant G_1–S arrest in SGC-996 and NOZ cells, whereas cell-cycle progression beyond the G_1–S transition was observed in *GCASPC*-knockdown GBC-SD and OCUG-1 cells (Fig. 2C and D; Supplementary Fig. S8C and S8D).

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

Table 1. Correlation between GCASPC expression and gallbladder cancer clinicopathologic characteristics in 89 patients (Cohort 2)

Characteristics	GCASPC expression levels		*P* value
	Low expression	High expression	
Gender			
Male	28	31	0.600
Female	16	14	
Age (years)			
≤59	22	26	0.462
>59	22	19	
Tumor size (cm)			
≤3	10	20	0.030[a]
>3	34	25	
Local invasion			
Yes	17	14	0.456
No	27	31	
Lymph node metastasis			
Yes	15	6	0.021[a]
No	29	39	
Distant metastasis			
Yes	11	13	0.679
No	33	32	
TNM stage			
I–II	13	27	0.004[a]
III–IV	31	18	
CA19-9 (U/ml)			
≤117	18	19	0.900
>117	26	26	

NOTE: Differences among variable were assessed by the χ^2 test.
Abbreviation: TNM, tumor node metastasis.
[a]The values had statistical significant differences.

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 (Fig. 2E and F; Supplementary Fig. S8E and S8F). In summary, these data suggest that *GCASPC* suppressed gallbladder cancer cell proliferation.

GCASPC associates with PC and downregulates its protein level and activity by destabilizing PC protein in gallbladder cancer cells

We sought to explore the molecular mechanisms by which *GCASPC* exerts its effects on gallbladder cancer 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 Supplementary Fig. S9A, no statistical changes in the transcript levels of neighboring genes were observed in SGC-996 cells with *GCASPC* overexpression, as represented by *SOD2*, *MAS1*, *loc729603*, *AIRN*, and *SLC22A1*. It suggests that 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 pull-down 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 (Fig. 3A). Three distinct bands specific to *GCASPC* were excised and subjected to mass spectrometry (Supplementary Table S6). PC was detected by Western blotting from three independent RNA pull-down assays in cell extracts from SGC-996 and NOZ cells (Fig. 3B). The specificity of this interaction was further verified with RNA immunoprecipitation (RIP; Fig. 3C). Notably, deletion-mapping analyses identified that 3′-end segment (472–741 nt) of *GCASPC* is required for the association with PC (Fig. 3D). RNA folding analyses (21) of this 3prime; region indicated a stable stem-loop structure (Supplementary Fig. S9B), which might provide the necessary spatial conformation for the interaction. In addition, we found that PC was significantly upregulated in gallbladder cancer tissues compared with adjacent nontumor tissues (Supplementary Fig. S9C). As mentioned above (Supplementary Fig. S5D), *GCASPC* is mainly located in the cytoplasm of gallbladder cancer 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 (Fig. 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 (Fig. 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 (Fig. 4A) and OCUG-1 cells (Supplementary Fig. S10A), and a downregulation of the PC protein and PC activity in *GCASPC*-overexpressing SGC-996 (Fig. 4A) and NOZ cells (Supplementary Fig. S10B), but we did not observe a significant change in PC mRNA levels (Supplementary Fig. S10C and S10D). 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 (Supplementary Fig. S10E and S10F).

Based on this finding, we hypothesize that *GCASPC* binds to PC and affects its biological activity at the translational or posttranslational level. To identify these hypotheses, we firstly observed the expression of PC proteins in gallbladder cancer cells incubated with the protein synthesis inhibitor cycloheximide (CHX). As shown in Fig. 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 (Fig. 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 (Fig. 4A), suggesting that *GCASPC* destabilized PC protein. As illustrated in Fig. 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 (Fig. 4C) and NOZ cells (Supplementary Fig. S11A). 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 (Supplementary Fig. S11B). These data indicate that *GCASPC* downregulates PC protein abundance via the ubiquitination-proteasome pathway. Functionally, PC inhibition suppressed the proliferation of gallbladder cancer cells

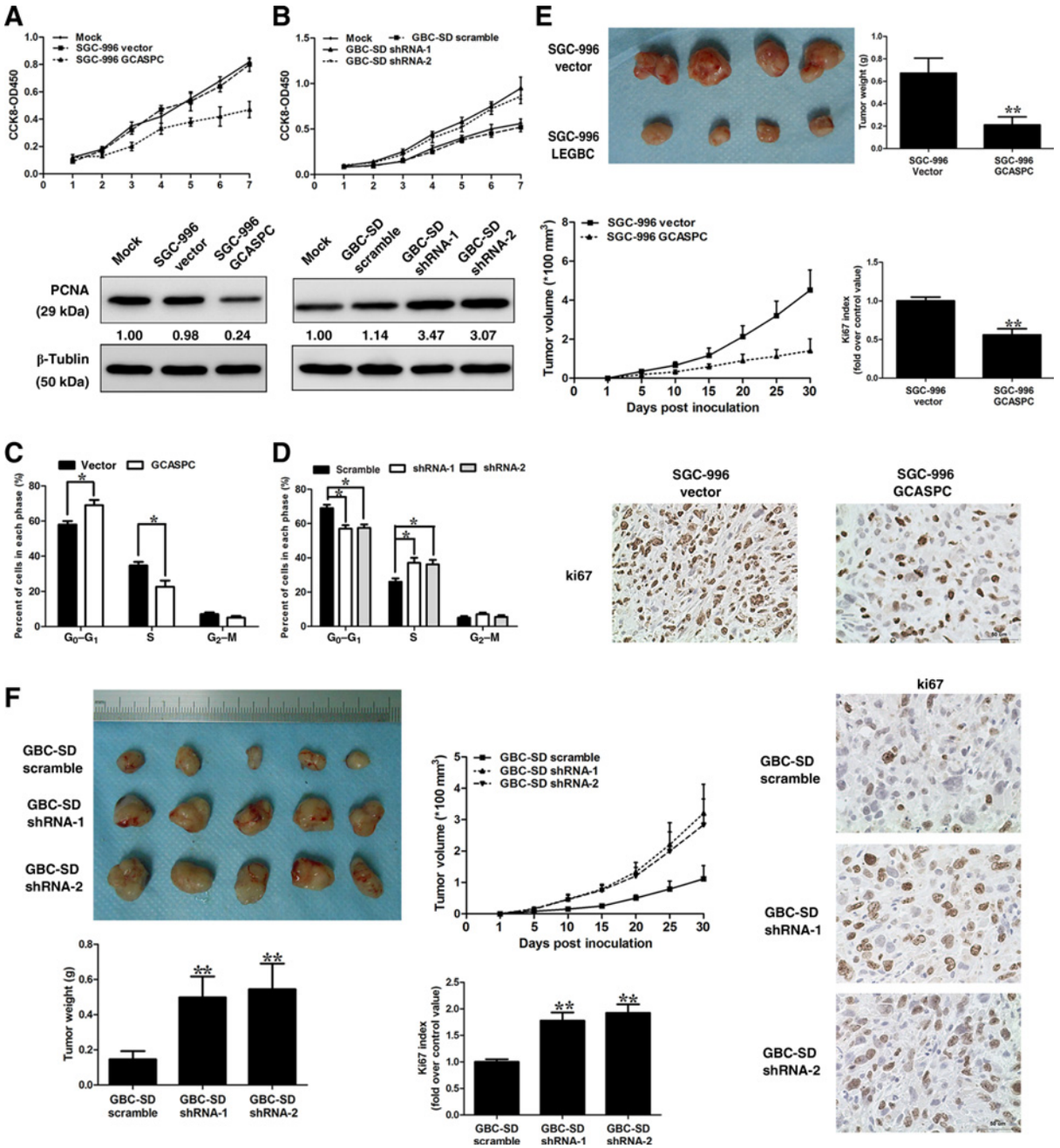

Figure 2.

GCASPC inhibits gallbladder cancer (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-tubulin ($n = 3$). **C** and **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 ± SD. Effects of *GCASPC* overexpression (**E**) or *GCASPC* knockdown (**F**) on tumor growth *in vivo*. Top 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).

(Fig. 4D; Supplementary Fig. S11C), and PC overexpression promoted the proliferation of GBC-SD cells and abrogated the effect of *GCASPC* overexpression on suppressing cell proliferation (Fig. 4E), indicating that the function of *GCASPC* depends on PC. Collectively, these data suggest that *GCASPC* suppressed tumorigenesis by negatively regulating PC-dependent cell proliferation.

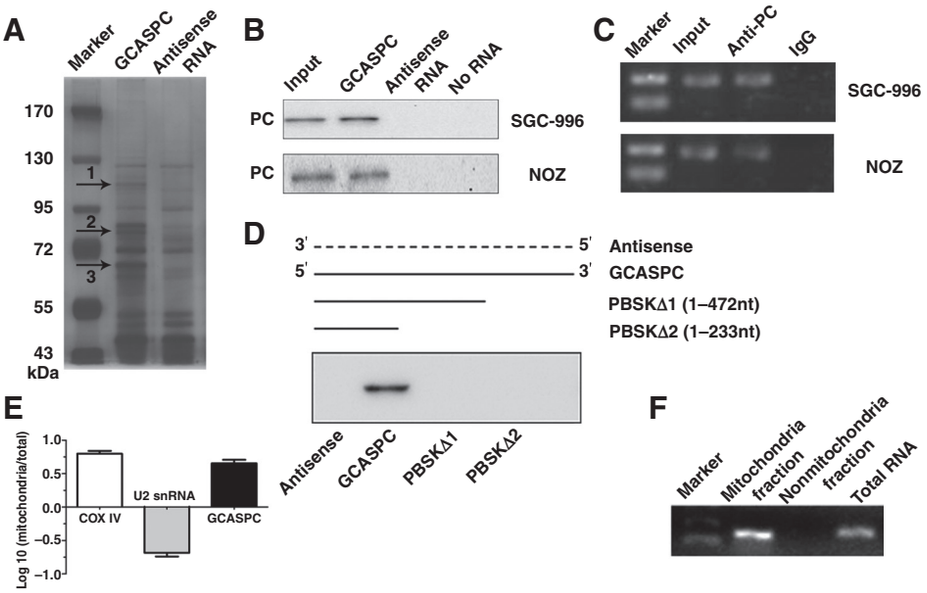

Figure 3.

GCASPC binds to PC protein. **A,** silver-stained SDS-PAGE gel of proteins immunoprecipitated from NOZ cell extract by *GCASPC* and its antisense RNA. Arrow, the region of the gel excised for mass spectrum determination by the liquid chromatography dual mass spectrometry method. **B,** biotinylated *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 *GCASPC* ($n = 3$). **C,** RIP experiments were performed using the PC antibody for immunoprecipitation and a primer to detect *GCASPC*. RIP enrichment was determined relative to the input controls ($n = 3$). **D,** biotinylated RNAs corresponding to different fragments of *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 *GCASPC* ($n = 3$). **E,** RNA was extracted from the total or only mitochondria of GBC-SD cells. One microgram of RNA was used for the qRT-PCR analysis of *GCASPC*, U2 snRNA (nuclear retained), and COX IV (mitochondria retained). **F,** RIP experiments with different fractions of cells (nonmitochondria, mitochondria fraction, and total cell) were performed using PC for immunoprecipitation and a primer to detect *GCASPC*.

GCASPC is direct target of miR-17-3p

A competitive RNA (ceRNA) hypothesis has been proposed and recent studies have suggested the existence of the interaction between lncRNAs and miRNAs (10, 11, 16), imposing an additional level of posttranscriptional regulation. We performed a search for miRNAs that have complementary base pairing with *GCASPC*, using online software program miRDB (http://mirdb.org; ref. 22). The search results demonstrated that 30 miRNAs formed complementary base pairing with *GCASPC* (Supplementary Table S5). As miR-17-3p achieved the highest score according to miRDB and formed no complementary base pairing with PC mRNA according to Targetscan, we selected it for further studies. What is more, other miRNAs on the list shared no common interaction site with miR-17-3p (Supplementary Table S7). According to the prediction results, there was one putative miR-17-3p binding site in exon 2 of *GCASPC* (Fig. 5A). To confirm the direct binding between *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) *GCASPC* reporter vector, but not a mutant *GCASPC*, indicating that miR-17-3p binds to *GCASPC* in a sequence-specific manner. We further clarified the regulatory relationship between *GCASPC* and miR-17-3p. Overexpression of miR-17-3p significantly suppressed the expression of *GCASPC* in GBC-SD and OCUG-1 cells (Fig. 5B). In contrast, inhibition of miR-17-3p enhanced the expression of *GCASPC* in SGC-996 and NOZ cells (Supplementary Fig. S12A). However, there was no obvious difference in miR-17-3p level after overexpression or knockdown of *GCASPC* (Fig. 5B; Supplementary Fig. S12B). 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 (Fig. 5C). The miRNAs 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 (Supplementary Fig. S12C). Furthermore, we detected miR-17-3p in the same pellet, supporting that miR-17-3p is bona fide *GCASPC*-targeting miRNA (Supplementary Fig. S12D). Furthermore, miR-17-3p knockdown suppressed the proliferation and expression levels of PC in *GCASPC*-knockdown GBC-SD cells (Fig. 5D and E). A statistically significant inverse correlation was observed between *GCASPC* and miR-17-3p transcript levels in 42 gallbladder cancer specimens ($r = -0.498$, $P = 0.002$, Fig. 5F). In general, these data suggest that miR-17-3p directly binds to *GCASPC* and negatively regulates *GCASPC*-mediated tumor-suppressive activity.

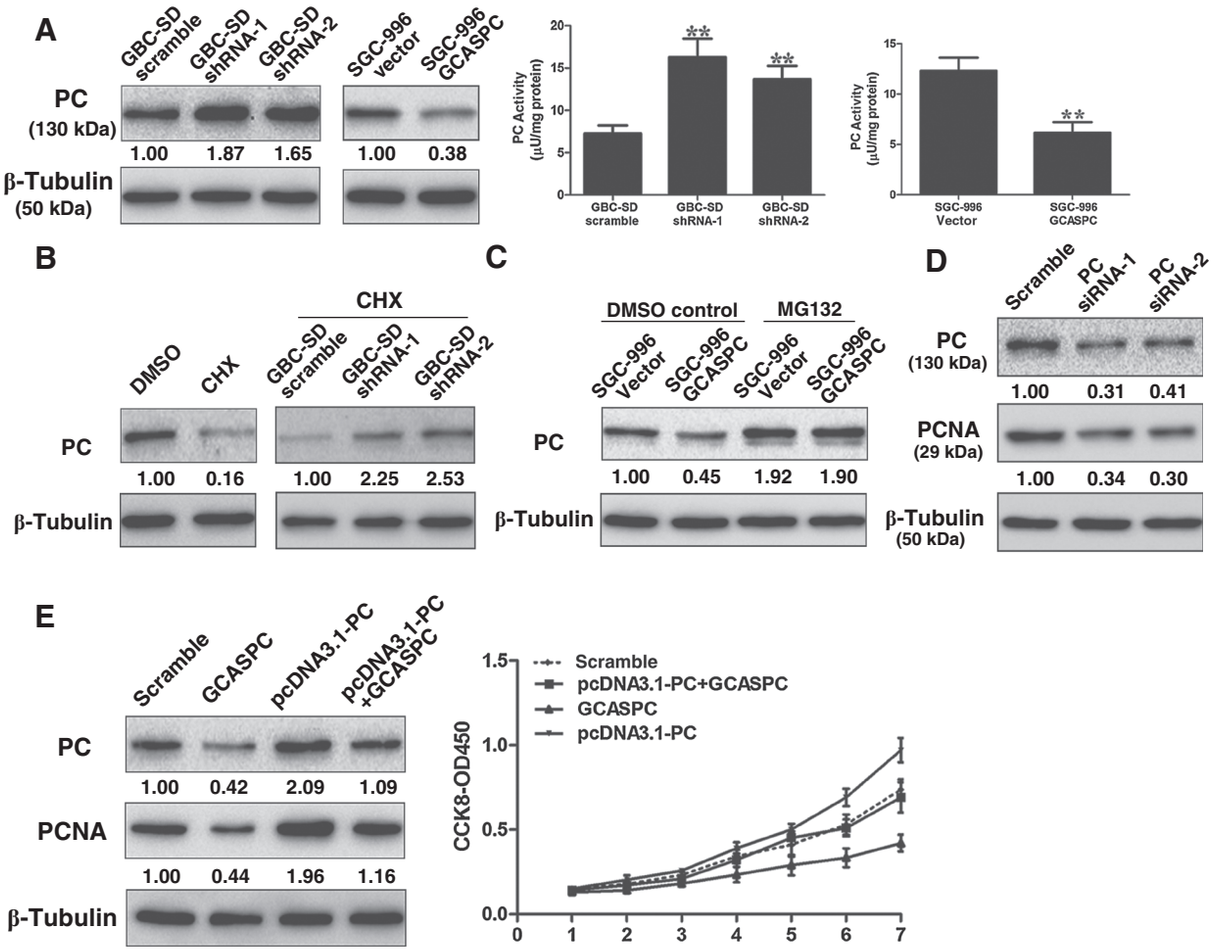

Figure 4.

GCASPC decreases the protein level and activity of PC by inhibiting its protein stability. **A,** the protein levels of PC were detected in *GCASPC*-knockdown GBC-SD cells and *GCASPC*-upregulated SGC-996 cells by Western blot analysis. PC activity was measured in total cell lysate as described in Materials and Methods. **B,** *GCASPC* stable knockdown GBC-SD cells and control cells were incubated with the protein synthesis inhibitor CHX (0.5 μg/μL) for 24 hours. **C,** *GCASPC* stable overexpressing SGC-996 cells and control cells were incubated with MG132 (5 μmol/L) 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.

Discussion

The molecular classification of gallbladder cancer has 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 gallbladder cancer. 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 gallbladder cancer (3, 25). Given the fact that lncRNAs are more abundantly expressed in mammalian cells, it is plausible to speculate that lncRNAs, once regarded as "transcriptional noise," may be potential prognostic indicators in gallbladder cancer. Although thousands of lncRNAs have been annotated (15), functional interpretation has just started.

In the present study, we revealed signatures of a small number of lncRNAs that are aberrantly expressed in human gallbladder cancer, compared with nontumor tissues. We identified a new lncRNA transcript (*GCASPC*), which was significantly downregulated in gallbladder cancer tissues from two cohorts of patients. We determined that the low expression level of *GCASPC* was significantly associated with numerous clinicopathologic 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 gallbladder cancer.

By applying loss- and gain-of-function approaches, we identified that *GCASPC* plays 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,

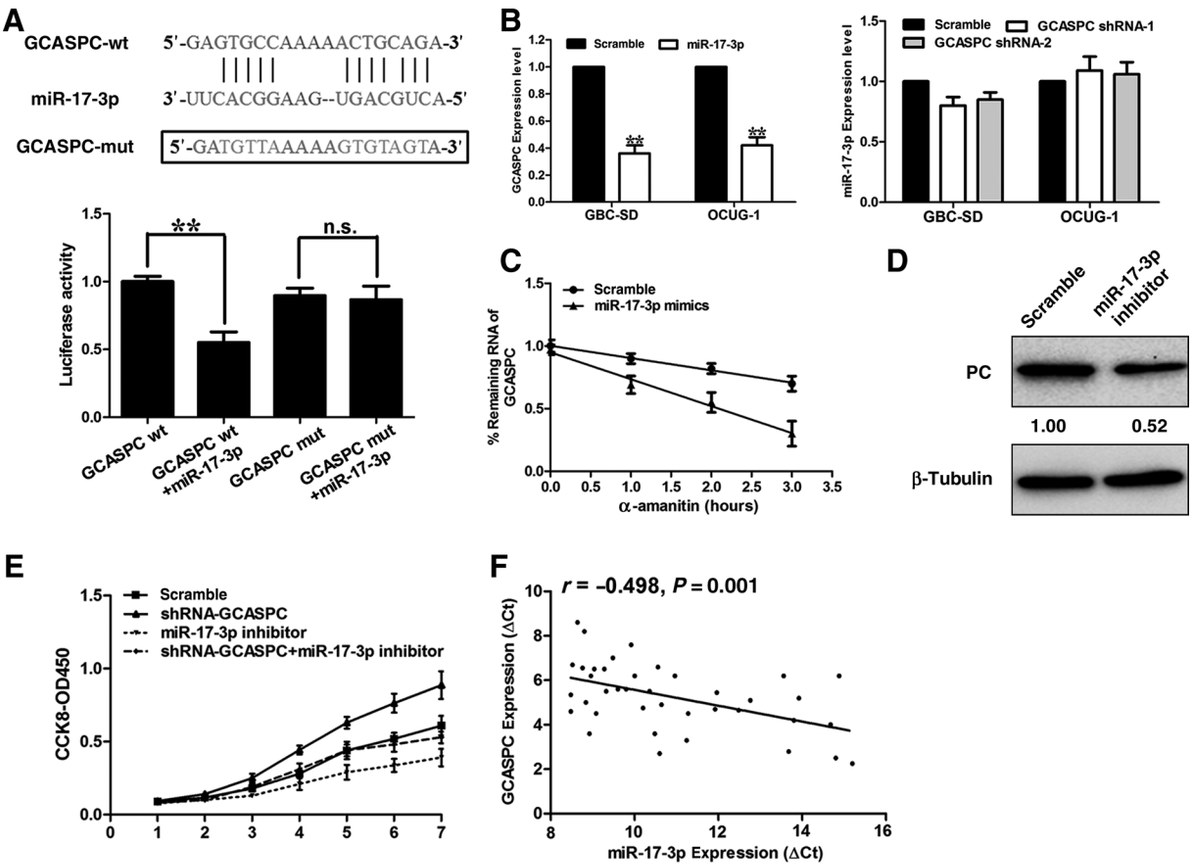

Figure 5.

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 (top). 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 (bottom). **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-tubulin. **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 gallbladder cancer tissues by qRT-PCR. **, $P < 0.01$.

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 PC to promote PC protein degradation. However, the underlying mechanisms of how *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 gallbladder cancer 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 two major pathways: carboxylation of pyruvate to oxaloacetate via ATP-dependent 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 non-small cell lung cancer cells with PC suppression (27). However, whether it is true in this case requires further investigation.

A growing number of reports suggest 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 miRNAs (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 gallbladder cancer cells. Furthermore, *GCASPC* transcript level was inversely correlated with miR-17-3p mRNA level in gallbladder cancer 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 is 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 (41). Our data revealed that *GCASPC* was upregulated by the histone deacetylase inhibitor TSA, suggesting that *GCASPC* expression in gallbladder cancer is likely to be regulated by histone acetylation. The precise molecular mechanism of the downregulation of *GCASPC* in gallbladder cancer calls for further research.

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.-z. Ma, Y. Zhang, Z.-y. Hou, W. Gong, X. Kong, J.-d. Wang, Z.-w. Quan

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.-z. Ma, Y. Zhang, M.-z. Weng, S.-h. Wang, Z.-y. Hou, Y.-y. Qin, W. Gong, Y.-J Zhang, J.-d. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-z. Ma, Y. Zhang, M.-z. Weng, Y.-y. Qin, J.-d. Wang

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Study supervision: S.-h. Wang, Z.-y. Hou, Z.-w. Quan

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