Basic Study

Linc00675 is a novel marker of short survival and recurrence in patients with pancreatic ductal adenocarcinoma

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Author contributions: Li DD and Fu ZQ contributed equally to this study; Li DD and Fu ZQ performed the majority of experiments; Lin Q conducted part of the experiments, and contributed to the revision of this paper; Zhou Y and Zhou QB conceived the experiments, analyzed and interpreted the data, and wrote the manuscript; Li ZH and Tan LP participated in the experiments and helped to analyze the data; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Sun Yat-sen Memorial Hospital.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors disclose no conflicts.

Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at liuyimin_sysu@163.com. Consent was not obtained but the presented data are anonymized and the risk of identification is low.

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Received: January 28, 2015
Peer-review started: January 29, 2015
First decision: March 10, 2015
Revised: April 7, 2015
Accepted: June 16, 2015
Article in press: June 16, 2015
Published online: August 21, 2015

Abstract

AIM: To detect linc00675 expression in pancreatic ductal adenocarcinoma (PDAC), to analyze the relationship between the expression level of linc00675 and the clinical pathological characteristics, to explore the biological functions of linc00675, and to determine whether linc00675 has independent prognostic value in PDAC.

METHODS: We studied linc00675 expression among eight histologically confirmed PDAC tissue samples and four chronic pancreatitis tissue samples through microarray screening. RT-qPCR was conducted to further investigate linc00675 expression in PDAC cell lines as well as archived tissues from a large cohort of PDAC patients. The correlations between the level of linc00675 and clinicopathological characteristics and survival in patients with pancreatic cancer were evaluated using Correlation analysis. Univariate and...
multivariate analyses were conducted to predict whether linc00675 expression is an independent prognostic and recurrence factor in patients with pancreatic cancer. After downregulating the expression of linc00675 through siRNA, MTT assay, flow cytometry, transwell assay and Western blot were used to explore the biological function of linc00675 in proliferation, invasion, and cell cycle progression of pancreatic cancer cells. The relative molecular expression levels of epithelial-mesenchymal transition were determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot.

RESULTS: The expression of Linc00675 in PDAC tissue samples was shown to be 672 times that in chronic pancreatitis tissue samples by microarray screening ($P = 3.69 \times 10^{-5}$). This finding was confirmed in tumor tissues from 90 patients with PDAC compared with adjacent normal tissue samples by quantitative RT-PCR. We found that linc00675 overexpression positively correlated with lymph node metastasis ($P = 0.005$), perineural invasion ($P = 0.006$), and poor survival ($P < 0.001$). Univariate and multivariate analyses showed that linc00675 expression served as an independent predictor of overall survival ($P = 0.009$). Additionally, receiver operating characteristic curve analysis showed that high linc00675 might serve as a predictor of tumor progression within 6 mo to a year after surgery. In vitro functional analysis demonstrated that knockdown of linc00675 attenuated pancreatic cancer cell proliferation and invasion as well as induced S phase arrest. Suppression of linc00675 in pancreatic cancer cells resulted can reverse the progress of epithelial-mesenchymal transition.

CONCLUSION: Linc00675 may function as an oncogene during PDAC development, and its expression is an independent predictor of unfavorable prognosis in patients with PDAC.

Key words: Linc00675; Long noncoding RNAs; Prognosis biomarker; Pancreatic cancer; Epithelial-mesenchymal transition

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Core tip: This is the first study to report that linc00675 is more highly expressed in pancreatic ductal adenocarcinoma (PDAC) tissues than in adjacent normal tissues. Overexpression of linc00675 in PDAC tissues positively correlated with short survival and tumor progression. The prominent finding in this study is that linc00675 is an independent prognostic marker for predicting the survival of PDAC patients after surgery.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) that originates in the glandular epithelium accounts for approximately 90% of all pancreatic tumors and exhibits a high grade of malignancy[13]. PDAC patients have an extremely poor prognosis, with a 5-year survival rate of approximately 6%. Even in patients who undergo surgical resection, the disease commonly recurs and the 5-year survival rate remains low at 15%-25%[12]. This dismal prognosis is due to the aggressive nature of this disease, and its resistance to traditional therapeutic strategies. Therefore, the development of an effective treatment for PDAC requires further research to reveal the molecular mechanisms underlying its aggressive pathogenesis.

Long noncoding RNAs (lncRNAs) are RNA molecules over 200 nucleotides in length with little protein-coding potential. Long intergenic noncoding RNAs (lincRNAs) have transcription loci that fall between two protein-coding genes and function to regulate gene expression at various levels, including transcription, epigenetic regulation and post-transcriptional processing[13-17]. Accumulating evidence suggests that deregulation of lincRNAs may contribute to many types of human diseases, including cancer[18,9]. Moreover, they play critical roles in cancer initiation, progression and metastasis[10-12]. LncRNA expression signatures have been associated with patient survival and may be useful in the patient management and the design of anticancer treatments[13]. Several lincRNAs have been implicated in tumorigenesis. However, the biological functions and prognostic value of lincRNAs in pancreatic cancer remain largely unexplored. Thus, there is an urgent need to identify the etiology and biological function of lincRNAs that may serve as markers of diagnosis and prognosis in PDAC to improve survival in this disease.

In the present study, based on microarray analysis, we focused on a long intergenic noncoding RNA named linc00675 that showed 672-fold upregulation in PDAC compared with normal pancreatic tissues. Building on this finding, we determined the significance of linc00675 in PDAC by investigating the relationship between aberrantly expressed linc00675 and patients’ clinicopathological features, as well as performing further in vitro study of PDAC cell lines. We found that upregulation of linc00675 was associated with short survival. In addition to affecting the cell cycle, overexpression of linc00675 could therefore promote cancer cell proliferation, migration and invasion. Thus, our study revealed that linc00675 is a promising prognostic biomarker in pancreatic cancer, and could be useful in pancreatic cancer risk assessment and
Table 1  Correlation between linc00675 expression and clinical characteristics

| Factor          | Linco0675 expression | P value |
|-----------------|----------------------|---------|
|                 | High (n = 45)        | Low (n = 45) |
| Age (yr)        |                      |         |
| < 60            | 22                   | 23      | 0.833 |
| ≥ 60            | 23                   | 22      |       |
| Sex             |                      |         |
| Male            | 30                   | 27      | 0.512 |
| Female          | 15                   | 18      |       |
| Differentiation |                      |         |
| Well            | 16                   | 17      | 0.304 |
| Moderate        | 15                   | 20      |       |
| Poor            | 14                   | 8       |       |
| UICC stage      |                      |         |
| p I             | 9                    | 15      | 0.153 |
| p II            | 36                   | 30      |       |
| T stage         |                      |         |
| T1              | 6                    | 9       | 0.697 |
| T2              | 16                   | 15      |       |
| T3              | 23                   | 21      |       |
| N stage         |                      |         |
| N0              | 12                   | 25      | 0.005 |
| N1              | 33                   | 20      |       |
| Perineural invasion |              |         |
| Negative        | 17                   | 30      | 0.006 |
| Positive        | 28                   | 15      |       |

1Pearson χ² test.

future therapeutic targeting.

**MATERIALS AND METHODS**

**Patients and tissue samples**

Samples of fresh frozen cancer tissues, together with normal adjacent tissues, were obtained during surgical resection from Sun Yat-sen Memorial Hospital of Sun Yat-sen University. Informed consent was obtained from the patients before sample collection, and approved by the hospital’s Ethics Review Committee. All samples were confirmed by pathological examination.

**Cell culture**

The human pancreatic cancer cell lines PANC1, Capan2, BXPC-3, Mia PaCa2, SW1990, and immortalized human pancreatic ductal epithelial cells (HPDE6) were purchased from the American Type Culture Collection and grown in complete growth medium with 10% FBS and 1% penicillin/streptomycin as recommended by the manufacturer. All the cells were cultured in a humidified 5% CO₂ incubator at 37 °C.

**RNA isolation, microarrays, and quantitative reverse transcription-PCR**

Total mRNA was extracted, purified using the mRNA-ONLY™ Eukaryotic mRNA Isolation Kit (Epicentre, Madison, CA). Total RNA was fragmented and then labeled (One-Color, Cy3, Agilent). After purification, the labeled RNA was hybridized to probes on the Hybridization Chamber gasket slides (Agilent).

After being washed, the slides were scanned using an Agilent Microarray Scanner. The raw data were extracted with the Feature Extraction software (Agilent Technology). This software utilizes the robust multiarray average algorithm to adjust the background signals. Normalized data were obtained using the quantile method of intra-microarray normalization and median method of baseline transformation between the microarrays. Differentially expressed genes with a raw expression level of over 400 in more than 4 of the 12 samples used for profiling were extracted. Then they were ordered by P value. The 10 most significantly de-regulated genes (those with the smallest P values) were selected for validation. We also computed the maximum false discovery rate based on a single gene-probe P value threshold of 0.05. We considered as significant signatures with a false discovery rate ≤ 0.1. The microarray platform and data were submitted to the Gene Expression Omnibus public database at the National Center for Biotechnology Information (accession number: GSE61166, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61166).

Real-time quantitative PCR (RT-qPCR) was performed for linc00675 and EMT marker (E-cadherin, N-cadherin, and Vimentin) mRNAs, with β-actin as an internal control. The total RNA was then converted to cDNA by reverse-transcription using oligodT primers and SuperScript ™ reverse transcriptase (Invitrogen). Quantitative PCR was performed with SYBR green master mix (Roche). Relative expression values were calculated (ΔΔCT method) using β-actin as a normalizer. The primer sequences used in the study are listed in Supplementary Table 1.

**RNA interference**

siRNA oligos targeting linc00675 (CTGATGGGGAGAAAAATCAATT, GTCCGAGAATGGCTGTGATT, and GTTCCAGACTCCATCAATT), and nontargeting siRNAs (UUCUCGAACGUGUCCTGAGTTCCAGACTCCATCAATT) were purchased from Sigma Aldrich. siRNA transfections were done with 80 nmol/L siRNA and Lipofectamine 2000 (Life Technologies) following the manufacturer’s instructions.

**Cell growth assay, cell cycle analysis, and invasion assay**

After transfection, 2 × 10³ cells (SW1990 or Mia PaCa-2) were plated in 96-well plates. A cell proliferation reagent kit (Roche) was used to assess cell proliferation. Transfected cells were assessed every 24 h according to the manufacturer’s instructions. For cell cycle analysis, transfected cells were collected, washed in PBS, stained with propidium oxide using the Cell Cycle Analysis Kit (Beyotime, Haimen, China), and then subjected to FACS analysis. In vitro cell invasion assay was performed using the BD BioCoat™ Matrigel™ Invasion Chamber (Becton Dickinson) according to manufacturer’s instructions, with 3 × 10³ cells...
seeded in the upper chamber. At least three biological replicates of the experiments were performed.

**Western blot analysis**

Cells were washed in PBS and lysed with RIPA buffer (Invitrogen, Carlsbad, CA, United States), and a bicinchoninic acid protein assay kit (Pierce) was used to calculate the protein concentration of each sample. Equivalent amounts of proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes for immunoblotting. The membranes were blocked in 5% fat-free milk for 1 h at room temperature, then incubated with the following primary antibodies: anti-Cyclin A, anti-Cyclin E, anti-Cyclin D1, anti-CDK2 and anti-β-actin (Abcam, Cambridge, MA); anti-Vimentin, Anti-E-cadherin, anti-N-cadherin, and anti-GAPDH (ProteinTech Group, Chicago, IL, United States). GAPDH was used as a loading control. Horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and an ECL chemiluminescence kit (Pierce) were used to detect bound antibody.

**Statistical analysis**

Statistical analyses were performed using SPSS Statistics 17.0 (SPSS Inc). All in vitro experiment quantitative data are presented as the mean ± SD from at least three independent experiments, unless otherwise noted. The differences between two groups were analyzed using a Student’s t-test. The correlation between linc00675 and clinical and pathological characteristics was assessed using Pearson’s χ² test. Survival was evaluated using the Kaplan-Meier method. Cox proportional hazard analysis was performed to calculate the hazard ratio and 95% confidence interval (CI) to evaluate the association between linc00675 and other clinicopathologic factors and survival. All tests performed were two-sided. Differences were considered statistically significant if P < 0.05.

**RESULTS**

**Linc00675 is aberrantly overexpressed in human PDAC cell lines and cancerous tissues**

We conducted an analysis of tissues from eight PDAC cases and four cases of chronic pancreatitis (CP) using a microarray targeting 7419 IncRNAs (Agilent). We discovered that the expression of the long intergenic noncoding RNA linc00675 (LOC100289255) in PDAC tissues was 672 times that in CP tissues (P = 3.69 × 10⁻⁵, Figure 1A). The hybridization signals of another three long noncoding RNAs, HULC, MALAT1, and HOTAIR, which have previously been reported to be upregulated in pancreatic cancer, are also shown in Figure 1A. The expression of linc00675 had the most obvious difference. Next, we investigated whether linc00675 was upregulated in PDAC cell lines and a large cohort of PDAC tissues. As shown in Figure 1B and 1C, RT-qPCR revealed that expression of linc00675 was significantly higher in tumor tissues compared with matched adjacent non-tumor tissues (P < 0.001). We also found that the expression of linc00675 in each PDAC cell line was significantly higher than in the HPDE6 cell line (Figure 1D).

**Association between linc00675 expression and overall survival of PDAC patients**

We assessed the correlation between linc00675 expression and clinical characteristics using expression levels obtained from qRT-PCR data of a cohort of 90 patients (Figure 1B). We found that linc00675 expression level was significantly associated with both lymph node metastasis (P = 0.005) and perineural invasion (P = 0.006) (Table 1). Log-rank analysis demonstrated that overall survival was significantly worse in patients with higher linc00675 expression (P < 0.001) (Figure 2A and B). Further multivariate analysis confirmed that linc00675 expression level was an independent prognostic indicator for overall survival of patients with PDAC (P = 0.009) (Table 2).

**Linc00675 is a potential biomarker for predicting recurrence in PDAC patients**

Because linc00675 showed a significant correlation with lymph node metastasis and perineural invasion, we went on to assess the value of linc00675 in predicting tumor progression after surgery and conducted a ROC (Receiver Operating Characteristic) curve analysis. Results showed that for predicting tumor progression within one year, the area under the ROC curve was 0.893 (P < 0.0001, Figure 2C); and for predicting progression within six months, the area under the ROC curve was 0.928 (P < 0.0001, Figure 2D). These findings suggested that linc00675 has potential diagnostic value in predicting recurrence in PDAC patients after radical surgical resection.

**Linc00675 regulates proliferation of pancreatic cancer cells**

To further examine whether linc00675 functions in pancreatic cancer progression, in vitro studies were conducted. We knocked down linc00675 expression in SW1990 and MIA PaCa-2 cells using small interfering RNAs; the most effective siRNA that showed more than 70% knockout efficiency was selected for the following test (Figure 3A). Linc00675 depletion resulted in decreased tumor cell proliferation in both the pancreatic cancer cell lines SW1990 and MIA PaCa-2, as determined by MTT assay (Figure 3B). Further flow cytometry analysis showed that linc00675 knockdown induced S phase arrest in both SW1990 and MIA PaCa-2 cells (Figure 3C and D). The expression of Cyclin E, CyclinA, Cyclin D1 and CDK2, which are markers of S phase arrest, was analyzed by Western blot. In both linc00675 knockdown-treated
SW1990 and MiaPaca-2 cells, Cyclin E and Cyclin A, which are responsible for G1/S transition and S phase progression, respectively, were significantly upregulated, whereas the levels of Cyclin D1 and CDK2, which are suppressed in the S phase, were found to be significantly reduced (Figure 3E). The latter findings are consistent with S phase arrest via reduced expression of linc00675 in PDAC cell lines.

**Table 2** Multivariate analysis of clinicopathological factors for overall survival

| Variable                | Univariate | Multivariate |
|-------------------------|------------|--------------|
|                         | P value    | HR | 95% CI | P value |
| T stage                 | 0.031      | 1.812 | 1.008-3.258 | 0.047 |
| N stage                 | 0.026      | 2.016 | 1.112-3.657 | 0.021 |
| Perineural invasion     | 0.016      | 2.611 | 1.246-5.471 | 0.011 |
| Linc00675 expression    | 0.013      | 4.620 | 1.233-18.336 | 0.009 |

HR: Hazard ratio.

**Linc00675 regulates invasion ability and expression of epithelial-mesenchymal transition-related genes in pancreatic cancer cells**

Cell invasiveness is closely correlated with cancer metastasis. We therefore examined whether linc00675 knockdown affects invasiveness of pancreatic cancer cells. A Matrigel invasion assay showed that linc00675 knockdown significantly inhibited invasiveness of SW1990 and MIA PaCa-2 cells (Figure 4A). Since EMT is closely related to cell invasiveness, we also examined whether the suppression of linc00675 can affect the expression of epithelial-mesenchymal transition (EMT)-related genes. Both PCR (Figure 4B) and Western blot analyses (Figure 4C) showed that suppression of linc00675 in pancreatic cells resulted in decreased expression of mesenchymal markers.
Figure 2 Overall survival of patients with pancreatic ductal adenocarcinoma based on linc00675 expression status and ROC curves of pancreatic ductal adenocarcinoma patients based on linc00675 for predicting recurrence. A: Pancreatic ductal adenocarcinoma (PDAC) patients were equally divided into two groups based on linc00675 mRNA levels, and then Kaplan-Meier survival curves were employed for comparing overall survival between two groups; B: Overall survival of patients with PDAC was evaluated via Kaplan-Meier survival curves based on whether linc00675 was increased in tumor tissues compared with paired non-cancerous tissue; C: ROC curves of PDAC patients based on linc00675 for predicting recurrence within one year; D: ROC curves of PDAC patients based on linc00675 for predicting recurrence within 6 mo.
Figure 3  Effect of linc00675 knockdown on pancreatic ductal adenocarcinoma growth in vitro. A: Knockout efficiency of siRNA targeting Linc00675 was confirmed by quantitative real-time-polymerase chain reaction in SW1990 and MIA PaCa-2 cell lines, \( P < 0.001, \) NC vs siLINC00675, Student’s t-test; B: Effects of knockdown of linc00675 on the proliferation of SW1990 and MIA PaCa-2 cells were assessed with MTT assay; C: Cell cycle of SW1990 and MIA PaCa-2 was analyzed by flow cytometry 48 h after transfection; D: Effect of knockdown of linc00675 on percentage of cells in G1-G0, S, and G2-M phase was examined quantitatively, \( P < 0.05, \) NC vs siLINC00675, Student’s t-test; E: Cells were untreated, or transfected with linc00675, then the expression of CyclinA, CDK2, CyclinE and CyclinD1 was detected by Western blot. Data are represented as the mean ± SD from three independent experiments.
Figure 4  Effect of linc00675 knockdown on pancreatic ductal adenocarcinoma cell invasiveness in vitro. A: Representative images of transwell assay after linc00675 knockdown in pancreatic cancer cell line SW1990 and MIA PaCa-2; B: Quantitative real time-polymerase chain reaction analysis of E-cadherin, N-cadherin and Vimentin was performed in SW1990 and MIA PaCa-2 cells at 72 h after transfection; \(^{c}P < 0.001\), NC vs siLINC00675, Student’s t-test; C: Western blot analysis of E-cadherin, N-cadherin and Vimentin was performed in SW1990 and MIA PaCa-2 cells at 72 h after transfection. Data are represented as the mean ± SD from three independent experiments.
Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant digestive tumor with extremely poor prognosis. Long intergenic noncoding RNAs (lincRNAs) have key roles in the regulation of multiple biological processes, including development, differentiation and carcinogenesis. There is, therefore, a need to explore the potential of lincRNAs as markers of diagnosis and prognosis and to investigate their biological functions to improve the outcome of PDAC patients.

**Research frontiers**
Recently, lincRNAs have been found to play critical roles in cancer initiation, progression and metastasis. LincRNA expression has been associated with patient survival and may be useful in outcome prediction and the design of anticancer treatments. Several lincRNAs have been implicated in pancreatic tumorigenesis; however, the role of linc00675 in PDAC is still unknown. In this study, the authors demonstrate that linc00675 was highly expressed in PDAC tissues compared with adjacent normal tissues. Increased expression of linc00675 in PDAC tissues positively correlated with poor survival and tumor progression. These results indicate that linc00675 could be a potential prognostic factor for PDAC patients.

**Innovations and breakthroughs**
This is the first study to report that linc00675 is overexpressed in PDAC tissues. The prognostic value of linc00675 in patients with PDAC is supported by functional experiments. We modulated its expression in SW1990 and MiaPaca-2 cell lines and found that suppression of linc00675 could reduce cell proliferation and invasion ability, which was consistent with clinical findings. Interestingly, we found that knockdown of linc00675 resulted in S phase arrest in pancreatic cancer cells. Gemcitabine, a chemotherapy agent, exerts its cytotoxic effect mainly by targeting tumor cells in S phase, which remains a standard therapy in PDAC patients. Some tumor suppressor genes and molecules were identified to increase gemcitabine sensitivity in pancreatic cancer cells through S-phase arrest. Because the patients analyzed in this study were receiving gemcitabine-based chemotherapy after surgery, and linc00675 showed S phase arrest in pancreatic cancer cells, it will be interesting to explore whether linc00675 contributes to increased gemcitabine sensitivity.

In summary, we found strong expression of linc00675 in patients with PDAC, and suggest that linc00675 may serve as an oncogenic lincRNA that promotes pancreatic cancer cell growth and progression. Further study is required to completely define the function of linc00675, its utility in guiding patient management and its potential as a therapeutic target.
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compared with adjacent normal tissue. The overexpression of linc00675 positively correlates with poor survival and short-term recurrence in patients with PDAC and in functional experiments was shown to promote pancreatic cancer cell growth and progression.

Applications
This study showed that the linc00675 expression level may be useful as a predictor of prognosis in pancreatic cancer.

Terminology
Linc00675 serves as an oncogenic lincRNA that promotes pancreatic cancer cell growth and progression. Since linc00675 is associated with the malignancy of PDAC, it may serve as a therapeutic target.

Peer-review
This is an interesting study with valuable information regarding the expression and clinical impact of linc00675 in pancreatic ductal adenocarcinoma.

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P- Reviewer: Park JY, Ritchie S, Stanojevic GZ, Wongkham S S- Editor: Wang JL L- Editor: Wang TQ E- Editor: Liu XM
