Long Noncoding RNA MALAT1 Promotes Aggressive Pancreatic Cancer Proliferation and Metastasis via the Stimulation of Autophagy

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Abstract

Recently, pancreatic ductal adenocarcinoma (PDAC) has emerged as one of the most aggressive malignant tumors with the worst prognosis. Previous studies have demonstrated that long noncoding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is increased in pancreatic cancer and is identified as a diagnostic biomarker. Nonetheless, the molecular mechanism of elevated MALAT1 levels and tumor aggressiveness remains unknown. In this study, MALAT1 was found to be highly expressed in PDAC tissues, and elevated expression was associated with poorer prognoses. In addition, MALAT1 was positively correlated with the expression of LC3B mRNA. Furthermore, several molecules involved in cellular autophagic flux were modulated following the downregulation of MALAT1, including LC3, P62, and LAMP-2. Mechanistically, we found that MALAT1 interacted with RNA binding protein HuR, and silencing of MALAT1 greatly enhanced the posttranscriptional regulation of TIA-1 and had further effects on inhibiting autophagy. MALAT1 was speculated to regulate tumorigenesis via HuR–TIA-1–mediated autophagic activation. Hence, we investigated the biological properties of MALAT1 in terms of tumor proliferation and metastasis by promoting autophagy in vitro. In brief, these data demonstrate that MALAT1 could facilitate the advanced progression of tumors in vivo. Our study highlights the new roles of MALAT1 on protumorigenic functioning and anticancer therapy via activating autophagy in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies. The overall 5-year survival of PDAC ranges from 3% to 5%, even with curative tumor resection and chemotherapy (1). The grave prognosis associated with PDAC is primarily due to delayed diagnoses and a lack of effective treatment, which emphasizes the importance of identifying PDAC in earlier stages and establishing better therapeutic targets (2).

Long noncoding RNA (lncRNA) is a class of ncRNA with a length of more than 200 NTs. So far, a large number of lncRNAs have been recognized by high-throughput transcriptome analysis (HTTA). They play important regulatory roles in the expression of nearby genes, the activation and localization of proteins, and the organization of small RNAs (3). LncRNAs are also critical regulators in tumor-related apoptosis, autophagy, and tumor metastasis (4–6). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as noncoding nuclear-enriched abundant transcript 2 (NEAT2), is highly conserved among mammals and is strongly expressed in the nucleus. MALAT1 was first identified as a sign of metastasis in lung cancer (7). Similarly, early reports demonstrated that MALAT1 was highly expressed in pancreatic cancer hepatocellular carcinoma, prostate cancer, and other malignancies (8–10). Few studies have confirmed the strong association between MALAT1 and poor pancreatic cancer prognosis (11). However, the molecular mechanism for its cancer-promoting effect is yet to be defined.

Autophagy is an evolutionarily conserved catabolic process and mechanism for the degradation of cellular proteins, which is functional in cell growth regulation and intracellular homeostasis (12, 13). Autophagy seems complicated and has both protumorigenic and tumor-suppressive roles in pancreatic cancer. It has also been described as a moderator of cellular invasion and metastasis by impacting cell biological phenotypes (14–16). Increasing evidence suggests that lncRNAs regulate the process of autophagy by means of interacting with RNA-binding proteins (RBP), as well as by suppressing the induction of miRNAs (17). However, the existence of this regulatory effect in pancreatic cancer is still under investigation.

In this study, we correlated the clinical significance of MALAT1 expression with the level of autophagy in PDAC samples. Then, we explored the roles of MALAT1 in tumor progression and found that MALAT1 upregulated cellular autophagy in pancreatic cancer cell lines. Furthermore, our study also demonstrated that MALAT1 directly interacts with HuR and alters the biological process of autophagy via enhancing the regulation of TIA-1 posttranscriptionally. Our study eventually suggested that MALAT1 may accelerate the proliferation and metastasis of cancer cells through the...
stimulation of autophagy. MALAT1 functioned as a protumorigenic oncogene in pancreatic cancer in vitro and in vivo.

**Materials and Methods**

**Cell culture**

Bxpc-3 and Panc-1 and human pancreatic ductal epithelial (HPDE) cells were purchased from the ATCC from 2010. All cell lines were authenticated by short tandem repeat (STR) and genotyped upon re-expansions and tested within 6 months of authentication. Aspc-1, CFPAC, and SW1990 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and used within 6 months after the order. Aspc-1, Bxpc-3, and Panc-1 were cultured in RPMI1640 medium (HyClone), and CFPAC, SW1990, and HPDE were cultured in DMEM (Gibco), all supplemented with 10% FBS (Gibco), 1% penicillin, and streptomycin at 37°C.

**Patients and specimens**

This study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. The 52 PDAC specimens used in this experiment were obtained from patients who underwent pancreaticoduodenectomy for pancreatic head cancer in the Department of Pancreatic and Biliary Surgery (The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China) from January 2008 to January 2010. All patients underwent standard resection without receiving chemotherapy or radiotherapy preoperatively or postoperatively. The patients’ detailed clinicopathologic characteristics are listed in Supplementary Table S1.

**RNA isolation, reverse transcription, and quantitative real-time PCR**

RNA isolation and the PCR amplification conditions were followed as previously described (18). Quantitative real-time (qRT-PCR; SYBR Green Assay, Roche Diagnostics GmbH) was performed on Applied Biosystems 7500 Real-Time PCR System. The relative expression levels of mRNAs were calculated and quantified using the 2ΔΔCT method after normalization for the expression of the control, and GAPDH served as the endogenous control. The primer sequences were designed by Primer 5.0 and purchased from Invitrogen.

**Electron microscopy**

Electron microscopy was performed as described previously (18, 19). Fresh tissues were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide buffer. The tissues were embedded in resin, and thin sections were cut. The sectioned grids were stained with a saturated solution of uranylacetate and lead citrate. Sections were examined at 80 kV using a JEOL 1200EX transmission electron microscope.

**GFP-mRFP-LC3 staining**

The GFP-mRFP-LC3 lentivirus was purchased from Genechem. Pancreatic cancer cell lines cultured on coverslips were transfected with negative control and GFP-mRFP-LC3 lentiviral vector, and were then selected with puromycin for a week. The stably transfected cells were cultured in dishes and transfected with si-MALAT1 for 6 hours. The cells were viewed under a fluorescence microscope. The green dots indicate autophagosomes, whereas the red dots indicate both autophagosomes and autolysosomes.

The yellow dots that result from merging the red and green channels indicate autolysosomes, while the red dots that do not overlay with green dots are indicative of autophagosomes. The number of GFP and mRFP dots was determined by manual counting of the fluorescent puncta in five high-power fields (40×, Olympus).

**RNA pull-down assay**

The full-length MALAT1 fragment cleaved by the EcoRI enzyme was excised from the vector pCRII-TOPO. RNA transcript probes were synthesized using a MEGAscript T7 kit (Ambion), following the protocol provided by the manufacturer. A biotinylated RNA probe synthesized in a 20 μL MEGAscript transcription reaction by adding 1.25 μL 20 mmol/L biotinylated UTP, Biotin-16-UTP (Roche). The synthesized RNA pull-down assay was purified using an RNAeasy Protect Mini kit (Qiagen). Whole cells were grown to 90% confluence and washed three times with PBS. Then, the cells were resuspended in CHAPS buffer (10 mmol/L Tris-HCl pH 7.4, 1 mmol/L MgCl2, 1 mmol/L EGTA, 0.5% CHAPS, 10% glycerol, 0.1 mmol/L PMSE, 5 mmol/L L-2-ME) and incubated 30 minutes on ice for lysis. The cell lysates were obtained by centrifugation at 10,000 × g for 10 minutes at 4°C. The supernatants were collected and the protein concentration was determined using the Bio-Rad protein assay (Bio-Rad). The reaction mixture contained 200 μg of cell extracts and 3 μg of biotinylated MALAT1 5′-UTR RNA probe. The reaction mixture’s final volume was adjusted to 100 μL with RNA mobility shift buffer (5 mmol/L HEPES, 40 mmol/L KCl, 0.1 mmol/L EDTA, 2 mmol/L MgCl2, 2 mmol/L dithiothreitol, 1 U RNasin and 0.25 mg/mL heparin). The mixture was incubated for 15 minutes at 30°C and then added to 400 μL of Streptavidin MagneSphere Paramagnetic Particles (Promega) for binding at room temperature for 10 minutes. Then, the RNA–protein complexes were washed seven times with the RNA mobility shift buffer without heparin. After washing, 30 μL of 2×SDS-PAGE sample buffer was added to the reaction mixture. The retrieved proteins were detected using standard Western blot analysis.

**RNA-binding immunoprecipitation**

The cells (1 × 10^7) were lysed with 500 μL of lysis buffer (10 mmol/L Tris-HCl pH 7.4, 1 mmol/L MgCl2, 1 mmol/L EGTA, 0.5% CHAPS, 10% glycerol, 0.1 mmol/L PMSE, 5 mmol/L L-2-ME) for 20 minutes on ice. The cell lysates were collected after centrifugation. The RNA–protein immunocomplexes were formed by incubating 500 μL of cell lysates with 5 μg of anti-TIA-1 (Santa Cruz Biotechnology), anti-HuR (ProteinTech), or isotype control IgG (Sigma) at 4°C for 2 hours and were brought down by 50 μL of protein A/G Plus agarose beads. After the beads were washed, the complexes were incubated with 20 μL of RNase-free DNase I and further incubated with 0.1% SDS and 0.5 mg/mL Proteinase K to remove DNA or proteins. Finally, qRT-PCR analyses of the RNA isolated from the immunoprecipitation (IP) material were further assessed. The primers to amplify MALAT1 are described in the Supplementary Information.

**Immunofluorescence**

Tumor cells transfected with Lv-MALAT1 lentivirus and the negative control vector were seeded on 24-well plates. The cells were fixed with 4% paraformaldehyde for 30 minutes and were permeabilized with 0.5% Triton X-100 for 20 minutes. After incubation for 2 hours with anti-TIA-1 (Santa Cruz Biotechnology, anti-HuR (ProteinTech), or isotype control IgG (Sigma) at 4°C for 2 hours, the complexes were incubated with 20 μL of RNase-free DNase I and further incubated with 0.1% SDS and 0.5 mg/mL Proteinase K to remove DNA or proteins. Finally, qRT-PCR analyses of the RNA isolated from the immunoprecipitation (IP) material were further assessed. The primers to amplify MALAT1 are described in the Supplementary Information.
Biotechnology) and anti-HuR (ProteinTech), the cells were washed with PBS for three times. Then, the cells were incubated with secondary antibodies for 1 hour (Beyotime), and 4′,6-diamino-2-phenylindole (DAPI, Beyotime) was added to stain the cell nuclei. Finally, the cells were detected by laser scanning confocal microscope (40×, Olympus).

Colony formation assay

The colony formation assay was performed as described previously (18). Five hundred cells transfected with Lv-MALAT1 and negative control lentiviral vectors were cultured in 6-well plates. The chloroquine (CQ, Sigma) groups and Lv-MALAT1 plus CQ groups were exposed to media with 10 μmol/L CQ immediately. The medium was changed every 3 days. After day 14, the colonies were counted after fixation in 4% paraformaldehyde for 10 minutes with 1% crystal violet staining. The colonies were counted manually in five fields (10×, Olympus).

EdU retention assay

Proliferation cells were stained with EdU using the Cell-Light EdU DNA Cell Proliferation Kit (RIBOBio). The cells of the Lv-MALAT1 and negative control groups were seeded in 6-well plates and exposed to media with 10 μmol/L CQ for 24 hours. The cells were treated with 10 μmol/L EdU for 2 hours at 37°C. The cells were fixed with 4% paraformaldehyde for 30 minutes and treated with 0.5% Triton X-100 for 20 minutes. Next, the cells were exposed to 100 μL 1 × Apollo reaction cocktail for 30 minutes and stained with Hoechst 33342 for 30 minutes. The cells were visualized with a fluorescent microscope (10×, Olympus).

Wound healing assay, migration, and invasion assays

Cells cultured in 6-well plate were allowed to form a confluent monolayer for 24 hours, followed by the treatment with 10 μg/mL Mitomycin C (Sigma) for 2 hours. The monolayer was scratched with a sterile pipette tip (200 μL). The cells were washed twice with PBS and incubated in serum-free RPMI1640 medium. The cells were photographed after 0, 24, and 48 hours. The wound areas were photographed with a microscope (10×, Olympus). The percentage of wound closure was estimated by Imagel software.

Migration and invasion assays were performed as described previously (20). The cells were plated in 24-well BioCoat Matrigel Invasion Chambers (Corning). The cells on the top surface of the filter were carefully removed with a cotton swab after 48 hours. The cell numbers on each membrane were counted in five high-power fields using a microscope (20×, Olympus).

Western blot assay

Western blot analysis was performed as described previously (21–23). Whole-cell lysates with approximately 40 μg of proteins were resolved on 10% and 12% SDS-PAGE and were subjected to Western blot assay using the antibodies listed in the Supplementary Information. After appropriate secondary antibody incubation, the bands were visualized with the Molecular Imager System (Bio-Rad) using an enhanced chemiluminescence method (Thermo Scientific).

Orthotopic tumor model

Female nude mice between the ages of 4 to 6 weeks were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. This study protocol was approved by the Institutional Review Board of the First Affiliated Hospital of Harbin Medical University. First, The Bxpc-3-Luc cells were transfected with Lv-MALAT1 and the negative control vector. A total of $5 \times 10^6$ cells in 200 μL PBS were injected into the left flank of nude mice. Two weeks after injection, the primary Lv-MALAT1 and negative control group tumors were harvested and cut into 1 mm³ pieces. Then, two groups of mice were anesthetized with intraperitoneal injections of 0.5% pentobarbital (100 g/mL). The pieces of tumors from different groups were translocated into the pancreatic tail and fixed by 5% Porel suture. The peritoneum and abdominal wall were carefully closed with individual surgical sutures. The animals were imaged weekly using the small-animal imaging system (Berthold Technologies). The numbers of visible metastatic lesions more than 1 mm³ in the gut, mesentery, liver, and spleen were recorded 5 weeks after xenograft procedures. Finally, all animals were euthanized and their pancreases were removed.

Immunohistochemical staining

The immunohistochemical staining protocol has been described previously (19, 22, 23). The paraffin-embedded tissue sections (5 mm) were immunostained with anti-LC3B (Cell Signaling Technology), anti-MMP3 (Santa Cruz Biotechnology), anti-MUC4 (Santa Cruz Biotechnology), anti-CD31 (Santa Cruz Biotechnology), and anti-Ki-67 (Santa Cruz Biotechnology). The numbers of positive cells were counted in five high-power fields using a microscope (Olympus).

Statistical analysis

Statistical analysis was performed with SPSS 19.0 software (IBM). The relationships between MALAT1 and LC3B mRNA levels, the clinicopathologic parameters, and overall survival (OS) were analyzed by Pearson analysis, univariate analysis, multivariate analysis, and the Kaplan–Meier survival analysis. ANOVA and a Student t-test were used to evaluate statistical significance. The data are shown as the mean ± SD, and differences are considered significant when *, P < 0.05; **, P < 0.01; ***, P < 0.001; and non-significant when P > 0.05.

Results

The upregulation of MALAT1 is associated with poor prognosis in PDAC patients

Previously, MALAT1 expression was correlated with clinical characteristics and the prognosis of pancreatic cancer patients. Thus, 52 patients who underwent pancreaticoduodenectomy for PDAC originating from the pancreatic head were included in our studies. The expression of MALAT1 in PDAC tissues was compared with normal tissues (10/52 patients) by qRT-PCR assays (Fig. 1A). The clinical association analysis indicated that MALAT1 expression was significantly associated with TNM stage and distant metastasis in PDAC (Fig. 1B; Supplementary Table S2). According to the Kaplan–Meier analysis, we demonstrated that patients with high-MALAT1 expression levels may have a shorter OS than those with lower levels (Fig. 1C). To confirm the independent prognostic significance of MALAT1, a multivariate analysis was performed to establish the relationship between MALAT1 expression and clinical and pathologic characteristics. The results showed that higher MALAT1 expression and elevated carbohydrate antigen 19-9 (CA19-9) levels in the serum were independent prognostic factors for pancreatic head cancer (Supplementary Table

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MALAT1 Promotes Cancer Proliferation and Metastasis

The expression of MALAT1 in PDAC tissue samples and pancreatic cancer cell lines. A, the expression of MALAT1 in 10 normal tissue samples and 52 PDAC tissue samples was detected by qRT-PCR assays ($P = 0.0001$). The ratio of each mRNA relative to GAPDH mRNA is listed below each blot. B, comparison of MALAT1 mRNA expression of 52 PDAC patients in stages I and II and stages III and IV ($P < 0.0001$). C, Kaplan–Meier analyses of overall survival (OS) for MALAT1 expression in PDAC from two independent cohorts ($P = 0.0029$). D, comparison of MALAT1 expression in Aspc-1, SW1990, Panc-1, Bxpc-3, CFPAC, and HPDE. *** $P < 0.001$.

The upregulation of MALAT1 is relevant to elevated autophagic activation in vitro and in vivo

Historically, autophagy has been shown to be associated with poor clinical outcomes in pancreatic cancer, and the inhibition of autophagy has been shown to attenuate pancreatic cancer growth in vitro and in vivo (24, 25). Our group has extensively studied the impact of autophagic activity on protumorigenic roles via the INK pathway in pancreatic cancer (18). In this study, electron microscopy was performed to assess autophagosome formation in PDAC, and our findings show that the levels of autophagy were consistently higher compared with those in adjacent tissues (Fig. 2A). This result was also supported by Western blot analyses (Fig. 2B). Overall, our data demonstrated that the elevated activation of autophagy might be positively associated with tumor progression in PDAC. To determine the correlation between MALAT1 expression and autophagy, the expression of MALAT1 and LC3B mRNA in 46 PDAC samples was quantitatively analyzed by qRT-PCR assays. As shown in Fig. 2C, LC3B mRNA levels were positively related to the elevation of MALAT1 in PDAC tissues ($r = 0.5736$, $P < 0.001$).

To estimate the relationship between MALAT1 and autophagic flux in vitro, siRNAs and lentiviral vectors were used to limit MALAT1 expression in Bxpc-3 and Panc-1 cells (Supplementary Fig. S1). Western blot assays were carried out to detect the autophagosome-associated lipidated isoform of LC3, which reflects the increased abundance of autophagosomes in the cytoplasm. Our results showed that LC3B II/I levels were significantly reduced while P62 levels were highly elevated in Lv-MALAT1 groups, compared with the control and negative control groups (Fig. 2D and E). Meanwhile, we evaluated autophagic activation via IHC in vivo, and found that LC3B expression was downregulated in the Lv-MALAT1 group compared with the negative control group in the pancreatic orthotopic tumor model (Fig. 2F and Supplementary Fig. S2). Autophagy is executed as a homeostatic mechanism for maintaining cellular integrity and stability (26). A group of genes required for the regulation of autophagy were also associated with the pathogenesis and therapy of various tumors. Autophagy-related genes (ATG), including Atg6 (BECLIN1), Atg5, and Atg7, are involved in complex mechanisms of regulating pancreatic cancer progression (27). Thus, Western blot assays were subsequently assessed to analyze the expression of BECLIN1, Atg5, and Atg7 among different groups. As shown in Fig. 2G, none of these ATGs had noteworthy changes with the reduction of MALAT1 expression. Altogether, we speculate that elevated MALAT1 expression is associated with autophagic activation. However, the upregulation of MALAT1 may not participate in the initial stages of autophagy.

Silencing of MALAT1 blunts autophagic flux

To test the hypothesis that the lack of MALAT1 might blunt the autophagic flux in pancreatic cancer, a scaffold protein P62 which is degraded by autophagic activation was measured in Bxpc-3 and Panc-1 cell lines (28). In addition, lysosomal-associated membrane protein-2 (LAMP-2), a lysosome marker that indicates the proper fusion of autophagosomes with lysosomes and involves in the degradation of autophagic vesicles, was also gauged (12). As shown in Fig. 2H and I, the level of P62 was upregulated while LAMP-2 expression was lowered in the Lv-MALAT1 groups. These data revealed that the knockdown of MALAT1 might depress autophagy by inhibiting the fusion of autophagosomes and lysosomes. As autophagic flux is a dynamic process, it is imperative to distinguish between the enhanced autophagosome formation and the decreased autophagosome clearance. The GFP-mRFP-LC3 staining could be utilized to localize and assess autophagic flux, which is not limited by pH changes in the cellular environment. The GFP signal is sensitive to the acidic condition of the lysosome lumen, whereas mRFP is much more stable. Therefore, we generated a lentiviral vector carrying GFP-mRFP-LC3 to...
assay autophagic flux, autophagosome biogenesis, maturation, and lysosomal degradation. The colocalization of both GFP and mRFP fluorescence indicates an autophagosome or phagophore that has not fused with a lysosome. As shown in Fig. 2F and 5, both the GFP/RFP and RFP dots were significantly reduced in the Lv-MALAT1 groups, indicating that the silencing of MALAT1 decreased the incorporation of LC3 into both autophagosomes and autolysosomes. Clearly, these data demonstrated that the loss of MALAT1 reduced the accumulation of autophagosomes as well as inhibited the autophagic flux in the cytoplasm, which may limit pancreatic cancer progression.

MALAT1 directly interacts with HuR
To decipher the molecular mechanism between MALAT1 and autophagy, we focused on two RBPs: TIA-1 and HuR. TIA-1 (T-cell intracellular antigen-1) acts on pre-mRNA splicing and inhibits mRNA translation by binding to the 3’ UTR of mRNA. Recent studies revealed that TIA-1 functions as a tumor suppressor gene and that TIA-1 depletion may enhance autophagic activation (29, 30). HuR, a member of the ELAVL family, has been reported to contribute to the stabilization of ARE-containing mRNAs. HuR may determine the impact of TIA-1 expression via binding with TIA-1 3’ UTR (31, 32). As shown in Fig. 3A and B, the knockdown of MALAT1 had no significant influence on TIA-1 accumulation while the expression levels of HuR and LC3B II/I were greatly declined. Furthermore, RNA pull-down assay and ribonucleoprotein immunoprecipitation (RIP) analyses were performed to investigate the interaction between MALAT1 and two RBPs. These no evidence suggesting that MALAT1 could bind with TIA-1, whereas direct interaction was found between MALAT1 and HuR (Fig. 3C and D).

MALAT1 regulates autophagic flux through modulating TIA-1 function
TIA-1 and HuR are both major regulators of mRNA stability and translation. In some instances, self-regulatory schemes were reported for specific mechanisms, providing a concept of post-transcriptional control in the RBPs family. To test whether the regulation of autophagy was mediated by TIA-1, a series of Western blot analyses were performed. As shown in Fig. 3E and F, Supplementary Fig. S3A–C and Supplementary Fig. S4A, the depletion of TIA-1 alone resulted in higher expression levels of LC3B II/I compared with the negative control and Lv-MALAT1 groups, while Lv-MALAT1 blocked the effect of si-TIA-1 on enhancing LC3B II/I expression. We simultaneously found that silencing TIA-1 had no impact on HuR expression compared with the negative groups. Furthermore, cotransfection of Lv-MALAT1 and si-HuR were performed to explore the relationship between HuR and TIA-1 (Supplementary Fig. S3D–F). Our results demonstrated that the accumulation of TIA-1 was increased in HuR-silenced cells, which depleted LC3B II/I expression. Interestingly, knockdown both of MALAT1 and HuR suggested that the elimination of MALAT1 could not repress autophagic activation without HuR assistance (Fig. 3G and H and Supplementary Fig. S4B). HuR served as a critical mediator for the process of MALAT1-mediated autophagy.

To gain a more mechanistic perspective of the modulated expression between HuR and TIA-1, the pEGFP-HuR plasmid was constructed to increase the intracellular HuR expression (Supplementary Fig. S5). We found that overexpression of HuR retained TIA-1 accumulation and then induced LC3B II/I expression, while the overexpressed HuR had no effects on MALAT1 expression (Fig. 4A and B and Supplementary Fig. S6). These findings indicated that the abundance of HuR promoted autophagic activation in pancreatic cancer cells. For additional analyses of the relationship between TIA-1 and HuR, immunofluorescent (IF) staining was performed. The abovementioned results were reconfirmed afterwards. Regardless of the absence of HuR, MALAT1-silenced cells did not improve the expression of TIA-1, but enhanced its capabilities posttranscriptionally. In addition, the collocalization of TIA-1 and HuR was exhibited by IF staining assays, providing rational evidence for their interaction in the cytoplasm (Fig. 4C). Indeed, all of these results indicated that a lack of MALAT1 inhibited autophagic activation through the suppression of HuR. Downregulation of MALAT1 inhibited autophagic activation through enhancing the regulation of TIA-1 at posttranscriptional level. The posttranscriptional regulation of TIA-1 relied on the interaction between MALAT1 and HuR. MALAT1 had no effect on the expression of TIA-1.

MALAT1 promotes tumor proliferation and metastasis through the stimulation of autophagy in vitro
Autophagy acts as a dynamic regulator of tumorigenesis via different molecular mechanisms, including control of tumor cell metabolism, promotion of tumor cell survival, regulation of cellular invasion and metastasis, and drug resistance (33, 34). Hence, we investigated whether the effects of MALAT1 on tumorigenesis were critically mediated by the autophagy process. The autophagy inhibitor, CQ, which blocks the fusion of autophagosome with lysosome by raising the intralysosomal pH, was selected. Simultaneously, we confirmed that CQ could not regulate MALAT1 expression in the two cell lines (Supplementary Fig. S7). Colony formation assays demonstrated that the Lv-MALAT1 groups and the CQ groups yielded fewer and smaller colonies compared with the negative control groups, while CQ could not enhance the function of Lv-MALAT1 on inhibiting tumor proliferation (Fig. 5A). In addition, MALAT1-silenced cells showed lower proliferative capacity determined by EdU retention assays compared with the negative control.
and CQ groups. Inhibition of autophagy could not synergize with Lv-MALAT1 on blocking DNA replication (Fig. 5B). To explore whether the elimination of MALAT1 limited pancreatic cancer metastasis with the assistance of autophagic activation, wound-healing assay and Transwell assays were carried out. Our data demonstrated that MALAT1 depletion limited cancer cells migration and invasion, and that the suppression of autophagy could not cooperate with Lv-MALAT1 on tumor metastasis in vitro (Fig. 5C and D and Supplementary Fig. S8). Moreover, the expression of matrix metalloprotein 3 (MMP-3) and Mucin 4 (MUC4) was evaluated and confirmed the results described above (Fig. 5E and Supplementary Fig. S9). Further study revealed that MALAT1 had no impact on tumor angiogenesis (Supplementary Fig. S10). All in all, these data indicated that MALAT1 could promote pancreatic cancer proliferation and metastasis via the stimulation of autophagy.

The downregulation of MALAT1 inhibits tumor proliferation and metastasis in orthotopic tumor model

A xenograft tumor model was developed to further assess the tumor-promoting effect of MALAT1 and its in vivo effect on the progression of pancreatic cancer. The animals were imaged weekly and the volume of tumors was recorded at 35 days (Fig. 6A). Compared with the negative control groups, the average volume of tumors in the Lv-MALAT1 group increased more slowly (Fig. 6B). Next, the metastasis nodes were evaluated in the gut, mesentery, liver, and spleen, and less nodes were found in Lv-MALAT1 group (Fig. 6C–E). Furthermore, the proliferative marker Ki-67 was detected in pancreatic orthotopic tumor via immunohistochemical staining, and presented at lower levels in the Lv-MALAT1 group (Fig. 6F). We then focused on the expression of MMP-3 and MUC4, which was measured in the aforementioned studies. The IHC staining

Figure 4.
Overexpression of HuR-activated autophagy via lessening TIA-1 aggregation. A and B, the overexpression of HuR inhibited TIA-1 expression and facilitated autophagic activation. C, the immunofluorescence staining exhibited the collocation and expression of TIA-1 and HuR in the cytoplasm (bars, 20 μm). ***, P < 0.01; ****, P < 0.001.
results suggested that MMP-3 and MUC4 expression were reduced as a result of MALAT1 depletion (Fig. 6G). Our data suggest that the lack of MALAT1 inhibits tumor proliferation and metastasis in vivo.

**Discussion**

MALAT1 serves as an oncogenic lncRNA determining the malignancy phenotypes of various cancers. It has been identified as an indicator of poor prognoses in lung, renal, bladder, and glioma cancers, as well as the invasion of the esophagus and colon. In previous studies, MALAT1 was identified as a target regulated by different proteins and miRNAs to restrain a subset of tumor proliferation, invasion, and metastasis (35–39). MALAT1 is restrained in the nucleus and its 3’-end can be modified by RNase P and RNase Z cleavage, which yield an additional tRNA-like cytoplasmic miscRNA (40). It also accommodates alternative splicing of a subset of pre-mRNAs by adjusting serine/arginine splicing factor activity. Taken altogether, these results provide a potential new approach for investigating the fundamental role of MALAT1 in tumorigenesis and cancer development.

Previous studies have shown that the overexpression of MALAT1 is related to the poor prognoses in pancreatic cancer patients (11). However, the mechanisms for MALAT1 in cancer development are not clearly understood. In our study, 52 PDAC tissue samples were selected to reflect a typical scenario, as almost 90% of pancreatic cancer present with the pathologic characteristic of PDAC. On the other hand, all selected cancers originated from the pancreatic head, which maximized homogeneity in both metastasis and recurrence. MALAT1 was measured with a higher discrepancy between the PDAC tissues and normal tissues. Ongoing efforts are aimed at testing the correlation between elevated MALAT1 levels and the clinical and pathologic features of PDAC patients. It is plausible that the upregulation of MALAT1 was significantly associated with PDAC TNM stage and distant metastasis. Further study revealed that increased MALAT1 expression and elevated CA19-9 levels in the serum were independent predictors of short OS as assessed by Kaplan–Meier analyses and Cox proportional hazard regression analyses. Our findings, together with other groups, suggest that MALAT1 is involved in PDAC pathogenesis and is a specific predictor for poor prognoses.

A previous study reported that MALAT1 is upregulated in pancreatic cancer cells, and that the induction of MALAT1...
facilitates cell growth, migration, and invasion in vitro (10). Besides, our findings highlight the multiformity and complexity of MALAT1 in gene regulation and autophagocytosis in pancreatic cancer. First, the correlation was observed in different layers of interactions between MALAT1 and LC3B mRNA in PDAC tissues. LC3 reveals the specific labeling of autophagosome membranes in addition to the cytoplasmic labeling, which could be incorporated into protein aggregates independent of autophagy (41). Two forms of LC3, LC3 I and LC3 II, are widely used to monitor the process of autophagy. LC3 I is cytosolic, whereas LC3 II is membrane bound. The enhancement in the amount of LC3 II corresponds to the elevation of autophagosome formation. Our study provides evidence that MALAT1 could exert a complex influence on autophagy during pancreatic cancer development.

Our study suggests that silencing of MALAT1 limits the formation of autophagosomes in vitro and in vivo, which introduces new insights into the molecular underpinning of MALAT1 on modulating autophagic activation. As an alternative to the difficult genetic approach of autophagy in tumor progression, autophagy-related genes, BECLIN1, Atg5, and Atg7 were initially detected. The biochemical properties of BECLIN1 are observed in two fundamentally important cell biological pathways: autophagy and apoptosis (42). Therefore, the undifferentiated differentiation of BECLIN1 between different groups indicated that MALAT1-mediated autophagy might be independent of the
process of apoptosis. Autophagy is needed for the clearance of ubiquitin-positive aggregates. Atg5 and Atg7 function as two key components in ubiquitin-like systems of autophagy execution. Both are not associated with LC3 processing but appear to be specifically involved in autophagosome formation from the late endosome and the trans-Golgi (43). Our study elucidated that MALAT1-mediated autophagic activation and autophagosome formation are not indispensable for Atg5 and Atg7. Consistently, autophagosomes immediately fuse with lysosomes and finally become autolysosomes during the last stage of autophagy (44). P62 has been suggested to act as a chaperone during the degradation of autophagosomes (28). LAMT-2 is essential for the degradation of autophagosomal content via the proper fusion of lysosomes with autophagosomes in the last stage of autophagic flux. Importantly, our study found that P62 was notably elevated while LAMT-2 was decreased in the Lv-MALAT1 groups. Meanwhile, MALAT1 silencing remarkably reduced the degradation of LC3 and colocalization in pancreatic cancer cells. All of these data support the notion that MALAT1 does not influence the formation of autophagosomes or the fusion of autophagosomes and lysosomes, but the degradation of autophagosomes.

In addition, we observed that MALAT1 activated autophagy via altering HuR expression and TIA-1 function. TIA-1 functioned as ancient DNA/RNA trans-acting regulator to broaden the transcriptome and proteome diversity (45, 46). It also triggered a series of biological processes, including cell invasion, migration, apoptosis, and autophagy (5, 27, 28, 47). HuR genetically and functionally synergizes with negative posttranscriptional modulator TIA-1 to inhibit the biosynthesis of specific mediators (33). The silencing of MALAT1 reduced the expression of HuR and LC3B II/I, while the decreased expression of MALAT1 did not appear to be involved in the significant elevation of TIA-1. In this regard, the interaction between MALAT1 and HuR provides experimental support to examine whether the translational activation of autophagy was imposed by HuR and its synergy with TIA-1. In the absence of TIA-1, autophagy activity was increased, while MALAT1 silencing could not abolish the rapid enhancement mediated by TIA-1 degradation. On the other hand, the expression of TIA-1 was enhanced in the absence of HuR, whereas the process of autophagy was impeded. It is possible that overexpression of HuR downregulated restored TIA-1 protein levels and conversely enhanced LC3B II/I expression. These findings support the concept that MALAT1 inhibition enhanced the effect of TIA-1 via the interaction between MALAT1 and HuR. Downregulation of MALAT1 has no effect on the TIA-1 expression, but the lower level of HuR caused by MALAT1 silencing enhanced the regulation of TIA-1 at the posttranscriptional level. In brief, HuR acts as an endogenous messenger between MALAT1 and TIA-1 in the cytoplasm.

In the past decade, several genetic links providing increasing evidence that autophagy plays multiple tumorigenic functions in pancreatic cancer have emerged. Our finding suggested that a lack of MALAT1 blocks pancreatic cancer proliferation, invasion, and migration in vitro and in vivo. Further research revealed that MALAT1 silencing generates these biological behaviors by inhibiting the degradation of autophagosomes, and CQ could not block the effect of MALAT1 on tumor proliferation and metastasis in vitro. In closing, we speculated that MALAT1-silenced cells strongly lowers the protumorigenic function through reducing autophagy. Targeting MALAT1 may be a potential therapeutic approach to prevent pancreatic cancer proliferation and metastasis (Fig. 6H).

In summary, we define the role of a lncRNA, MALAT1, as both a predictive marker and therapeutic target in pancreatic cancer. MALAT1 promotes tumor proliferation and metastasis through activating autophagy. MALAT1 is increased to interact with HuR and stimulate its expression. The upregulation of HuR promotes autophagic activation through regulating TIA-1 effect in posttranscriptional level. Our findings provide greater insights into the function of MALAT1 in the aggressive progression of pancreatic cancer, as well as contribute to the understanding of the oncogenic process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by all authors.

Authors' Contributions

Conception and design: L. Li, H. Chen, Y. Gao, Y.-W. Wang, X.-W. Bai, B. Sun Development of methodology: L. Li, H. Chen, Y. Gao, Y.-W. Wang, G.-Q. Zhang, S.-H. Pan, X.-W. Bai, B. Sun Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Li, G.-Q. Zhang, S.-H. Pan, R. Kong, G. Wang, B. Sun Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Li, H. Chen, Y. Gao, L. Ji, Y.-H. Jia, B. Sun Writing, review, and/or revision of the manuscript: L. Li, Y. Gao, G. Wang, B. Sun Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Li, Y.-W. Wang, S.-H. Pan, B. Sun Study supervision: S.-H. Pan, X.-W. Bai

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