Original research paper

Long non-coding RNA *TINCR* suppresses growth and epithelial-mesenchymal transition by inhibiting Wnt/*β*-catenin signaling pathway in human pancreatic cancer PANC-1 cells: Insights from *in vitro* and *in vivo* studies

ABSTRACT

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There is increasing evidence that long non-coding RNAs (lncRNAs) play a crucial role in the development and progression of malignant tumors, particularly pancreatic cancer. In this study, the influence of the lncRNA *TINCR* on the behavior of human pancreatic cancer cells was investigated with the aim of deciphering its role in growth, migration, and invasion. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate *TINCR* expression in pancreatic cancer cells. Ectopic expression of *TINCR* in PANC-1 cells was induced to evaluate the effects on cell viability and apoptosis, examining the apoptotic genes Bax and Bcl-2. Migration and invasion assays were used to measure the impact of *TINCR* on these cellular processes. *In vivo* studies using a xenograft mouse model examined the effects of *TINCR* on tumor growth, epithelial-to-mesenchymal transition (EMT) markers, and the Wnt/β-catenin signaling pathway. PANC-1 cells showed strikingly low *TINCR* expression compared to other pancreatic cancer cell lines. Ectopic *TINCR* expression reduced the viability of PANC-1 cells primarily by inducing apoptosis, as evidenced by increased Bax and decreased Bcl-2 expression. Overexpression of *TINCR* significantly increased the percentage of apoptotic cells. It also decreased the migration and invasion ability of PANC-1 cells, as demonstrated in wound healing and transwell assays. In addition, overexpression of *TINCR*-suppressed proteins is associated with the Wnt/β-catenin signaling pathway in PANC-1 cells. In the xenograft mouse model, overexpression of *TINCR* inhibited tumor growth, EMT markers, and proteins associated with the Wnt/β-catenin pathway.
This study sheds light on the tumour-suppressive role of *TINCR* in PANC-1 cells and suggests its potential as a therapeutic target. These results shed light on the molecular mechanisms underlying the impact of *TINCR* on pancreatic cancer and offer promising opportunities for innovative therapeutic strategies to improve outcomes in this serious malignancy.

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INTRODUCTION

Pancreatic cancer is a lethal malignancy causing a significant number of human deaths annually (1). The global burden of pancreatic cancer is increasing at an alarming rate and has more than doubled over the past two decades (2). With a poor 5-year survival rate, novel strategies need to be developed for the management of pancreatic cancer (3). Treatment options for pancreatic cancer include surgery, chemotherapy, radiation therapy, and emerging targeted therapies (4). Surgical resection offers the best chance for cure but is feasible for only a minority of patients. Chemotherapy, including regimens like FOLFIRINOX and gemcitabine/nabpaclitaxel, extends survival but is rarely curative (5). Radiation therapy complements other treatments, while targeted therapies like poly (ADP-ribose) polymerase (PARP) inhibitors and immunotherapy show promise in clinical trials (6). Continuous research efforts are necessary to advance early detection, discover new therapeutic targets, and innovate treatment strategies, offering hope to patients affected by this devastating disease (7, 8).

In recent years, non-coding RNAs (ncRNAs) have garnered significant attention in cancer research, with a particular focus on their role in tumorigenesis, including pancreatic cancer (9). Among ncRNAs, long non-coding RNAs (lncRNAs), a subclass characterized by their extended nucleotide sequences exceeding 200 bases, have emerged as key regulators of fundamental cancer hallmarks (10). It is well-established that lncRNAs exert diverse control over various biological processes by modulating the expression of target genes at transcriptional, post-transcriptional/translational, or epigenetic levels (11). Importantly, dysregulated lncRNA expression has been closely linked to the initiation and progression of tumors (12), as lncRNAs play pivotal roles in modulating critical aspects of cancer cell behavior, such as proliferation, apoptosis, migration, invasion, and microenvironment remodeling (13, 14). Additionally, lncRNAs contribute to the maintenance of stemness in various human cancers (15). Given the central role of lncRNAs in cancer regulation, accumulating evidence suggests that lncRNA-based cancer therapeutics may soon transition from a theoretical concept to a tangible reality.

TINCR, an acronym for Terminal differentiation-induced non-coding RNA, is a type of RNA molecule that does not code for proteins. It is located on chromosome 19p13.3 and has been identified as having a significant role in several cancer types, such as breast cancer, lung cancer, gastric cancer, and liver cancer. This non-coding RNA's involvement in various cancers highlights its importance in understanding and potentially targeting cancer-related processes (16, 17). Notably, *TINCR* demonstrates varying effects on the progression and development of different types of human cancers, highlighting its context-dependent characteristics (18). Despite these findings, our understanding of the precise impact of *TINCR* on the biological characteristics of pancreatic cancer cells remains limited. This knowledge gap emphasizes the importance of conducting a comprehensive investigation into *TINCR*'s role within the context of pancreatic cancer. In accordance with these observations, our study is dedicated to a thorough exploration of the involvement of the long non-coding RNA *TINCR* in controlling the growth and metastasis of human pancreatic cancer. Our research initiative seeks to unravel the intricate mechanisms through which *TINCR* influences the pathophysiology of pancreatic cancer.

EXPERIMENTAL

Cell lines

Pancreatic cancer cell lines like PaCa-2 (RRID:CVCL_0428), PL45 (RRID:CVCL_3567), PANC-1 (RRID:CVCL_0480), and BxPC-3 (RRID:CVCL_0186) were obtained from the

American Type Culture Collection USA. The human normal pancreatic ductal epithelial cell line (H6C7) was obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (China). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) and streptomycin-penicillin (100 U mL^{-1}). The cells were maintained in a humidified CO_2 incubator at 37 °C with 5 % CO_2.

Chemicals and kits

Dulbecco's modified Eagle's medium was purchased from Gibco. Lipofectamine 2000 was purchased from Invitrogen (USA).

SYBR PCR master mix was purchased from Takara, Japan. PcDNA3.1 overexpression vector was purchased from Addgene, USA. Trizol reagent, Revert Aid First Strand cDNA synthesis kit, Cell Counting Kit-8 assay kit, EdU solution, Annexin V-FITC Apoptosis Detection Kit, propidium iodide, medetomidine, midazolam, and butorphanol were all obtained from Sigma Aldrich, USA.

Transfection

The overexpression vector for *TINCR* was constructed using pcDNA3.1. *TINCR* (NM_001396408.1) was amplified by PCR from cDNA extracted from PANC-1 cells, employing specific primers: *TINCR* forward primer 5'-<u>CGGATCC</u>AGAGCTGGAGCCG-GAGCCGGGC-3' (with BamHI restriction site) and *TINCR* reverse primer 5'-<u>TGAATTC</u>-TAGTTCCAAGCTGGGTGATCC-3' (with an EcoRI restriction site). PCR was performed in Veriti 96 well thermocycler (ThermoFisher Scientific, USA), with program conditions: 95 °C for 5 min, 30 cycles of (denaturation: 95 °C for 30 sec, annealing: 63 °C for 60 sec, and extension: 72 °C for 1 min), final extension: 72 °C for 10 min, and hold temperature at 4 °C. The resulting PCR product was separated on a 1.2 % agarose gel and purified using a Gel Purification Kit (Qiagen, USA). Subsequently, the purified product underwent digestion with BamHI and EcoRI restriction enzymes and was again purified using the Gel Purification Kit. The doubly digested amplicon was then ligated with the BamHI/EcoRI-digested pcDNA3.1 vector using T4 DNA ligases, resulting in the generation of the pcDNA-TINCR construct. The transfection procedure was carried out in a serum-free medium using Lipofectamine 2000, following the manufacturer's recommended protocol. Approximately 6 hours after the initial transfection, the cells were replenished with a fresh complete medium. Following this medium change, the cells were allowed to incubate for approximately 48 hours. After this incubation period, the cells were harvested and collected for subsequent analysis. To serve as a control group for comparison, cells transfected with the pcDNA3.1 vector alone (without the *TINCR* insert) were included. These cells, designated as the negative control cells, provided a reference point for evaluating the effects of *TINCR* over-expression in the experimental group.

Expression analysis via qRT-PCR

To analyze the expression of the target gene, qRT-PCR was employed. Firstly, total RNA was extracted from the cell samples using Trizol reagent, following the manufacturer's guidelines for optimal RNA isolation. Isolated RNA (100 ng) was reversely transcribed using the Revert Aid First Strand cDNA synthesis kit according to the manufacturer's instructions. For

the quantification of gene expression, a qRT-PCR approach was adopted. In this method, the SYBR PCR master mix was used in conjunction with the QuantStudio 3.0 PCR system from Applied Biosystems. In order to standardize and normalize the data, the GAPDH gene was utilized as an internal control in the expression study. The qRT-PCR primers used were: e-cadherin (forward 5′-GTCAGTTCAGACTCCAGCCC-3′ and reverse 5′-AAATTCACTCT-GCCCAGGACG-3′); *α*-catenin (forward 5′-CAACCCTTGTAAACACCAAT-3′ and reverse 5′-CCTTCTCCAAGAAATTCTCA-3′); vimentin (forward 5′-TCTACGAGGAGGAGATGC-GG-3′ and reverse 5′-GGTCAAGACGTGCCAGAGAC-3′); fibronectin (forward 5′-CCCACC-GTCTCAACATGCTTAG-3′ and reverse 5′-CTCGGCTTCCTCCATAACAAGTAC-3′); GAPDH (forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse: 5′-TCCACCCTGTTGCTGTA-3′). The reaction conditions were: 94 °C for 10 minutes; 35 cycles of 94 °C for 15 s, 55 °C for 40 seconds, and 72 °C for 20 seconds. The relative expression levels of *TINCR* were calculated using the widely accepted $2^{-\Delta\Delta Ct}$ method (19).

CCK-8 assay

To evaluate the viability of PANC-1 cells following transfection with either the pcD-NA-*TINCR* construct or the control vector (pcDNA3.1), a Cell Counting Kit-8 (CCK-8) assay was employed. Initially, a total of 10^5 PANC-1 cells were seeded into each well of a 96-well plate. These cells were then allowed to incubate for a 24-hour period at a temperature of 37 °C to facilitate adherence and growth. Subsequently, the CCK-8 solution was introduced into each well, and the incubation continued at 37 °C for an additional hour. This solution is known to contain a colorimetric reagent that becomes metabolically active in living cells. Following the incubation period, the optical density at 570 nm (OD_{570}) was measured for each well using a microplate reader (ELx808™, Agilent, USA). This OD_{570} reading served as an indicator of cell viability. Essentially, a higher OD_{570} value indicates a greater number of viable cells, while a lower value suggests reduced cell viability.

EdU assay

To delve into the impact of *TINCR* overexpression on the proliferation of PANC-1 cancer cells, we employed an EdU Apollo *in vitro* flow cytometry kit in line with the manufacturer's recommended procedures. Initially, the transfected cells (2×10^3 cells/well) were cultured in a 12-well plate and allowed to incubate for a 24-hour period at 37 °C. This incubation period was essential for facilitating cell adherence and establishing the experimental conditions. Subsequently, the cells were exposed to an EdU (5-Ethynyl-2′-deoxyuridine) solution at a concentration of 50 μmol L^{-1}. EdU is a thymidine analog that is incorporated into the DNA of actively dividing cells during DNA synthesis. The cells were allowed to incubate with the EdU solution for 2 hours. Following the incubation with EdU, the cells underwent a series of processing steps. They were first washed with phosphate-buffered saline (PBS) and then fixed using a 4 % paraformaldehyde solution. To visualize the EdU-labeled cells, they were stained using Apollo staining solution from San Francisco, CA, for a duration of 25 minutes. Additionally, the cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole) to label the cell nuclei. The final step involved visualizing the cells under a fluorescence micro-scope (Evos FL, ThermoFisher Scientific, USA) to examine the nuclear fluorescence. This allowed for the identification and quantification of cells that had incorporated EdU, indicating active DNA synthesis and, by extension, cell proliferation.

Annexin V/PI staining assay

To assess the impact of *TINCR* overexpression on apoptosis in PANC-1 cancer cells, a series of experiments were conducted, specifically utilizing an Annexin V/PI staining assay. This assay was employed to distinguish between early and late apoptotic cells, as well as non-apoptotic cells. Initially, PANC-1 cells (2×10^3 cells/well) were transfected with pcDNA-*TINCR*, and the negative control cells were cultured in DMEM for a 24-hour period within a 12-well plate, maintaining a temperature of 37 °C. Following the incubation period, the cells were harvested and subjected to a thorough washing regimen, involving three washes with phosphate-buffered saline (PBS) to ensure the removal of extraneous cellular debris. The Annexin V-FITC/PI double staining procedure was carried out according to the manufacturer's instructions, employing the Annexin V-FITC Apoptosis Detection Kit. Subsequently, the stained cells were analyzed using BD FACSCanto™ II Flow Cytometry System, BD (USA). Flow cytometry data were processed and analyzed using FlowJo version 6.0 software (BD), which facilitated the quantification of the relative percentage of apoptotic cells within the experimental and control groups.

Wound healing assay

To investigate the migratory behavior of PANC-1 cancer cells overexpressing *TINCR* in comparison to control cells, a wound-healing assay was conducted. This assay allowed for the evaluation of cell migration by tracking the closure of a scratched "wound" over time. Initially, 5×10^5 PANC-1 cells transfected with pcDNA-*TINCR* or the control vector were seeded into each well of a six-well plate. Following cell seeding, a 24-hour incubation period at 37 °C was allowed to promote cell adherence and growth. Subsequently, the serum-free medium was carefully aspirated to create a consistent and linear "wound" on the cell monolayer . This was achieved by gently scratching the cell surface with a sterile pipette tip. The resulting scratch, representing the wound, was then immediately documented using a light microscope, with images captured for reference. The plated cells were then incubated at 37 °C for an additional 24 hours. During this incubation period, cell migration and proliferation would contribute to the gradual closure of the wound. After the incubation, the wound area was re-evaluated and photographed under the microscope (CKX53, Olympus, USA). By comparing the initial and final images of the scratch, the extent of wound closure could be quantified. This provided valuable information about the migratory capacity of PANC-1 cells in response to *TINCR* overexpression, as evidenced by the degree of wound closure.

Transwell assay

We conducted a Transwell assay to investigate the invasive potential of transfected PANC-1 cancer cells. In this assay, we utilized Matrigel-coated Transwell chambers with 8 µm pores. Approximately 2.5×10^4 transfected cells were seeded in the upper chamber, containing serum-free medium, while the lower chamber contained only serum-free medium. Following 24-hour incubation at 37 °C, cells that had successfully invaded through the Matrigel and reached the lower chamber were fixed with 70 % ethanol. Subsequently, these cells were stained with 0.2 % crystal violet and examined under a microscope (CKX53, Olympus, USA) to quantify their invasiveness. This assay provided crucial insights into the ability of these cells to invade and penetrate barriers resembling the extracellular matrix, a critical aspect of cancer cell behavior.

Western blotting

To extract proteins from the transfected PANC-1 cancer cells, we employed the RIPA lysis and extraction buffer, obtained from Thermo Fisher Scientific. The protein content of each extract was quantified using the Bradford assay. Next, 35 μg of protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were subsequently transferred onto activated polyvinylidene difluoride (PVDF) membranes. To ensure specific antibody binding and minimize non-specific interactions, the PVDF membranes were blocked using 5 % skimmed milk powder for a duration of 1 hour. Following blocking, the PVDF membranes were incubated overnight at 4 °C with primary antibodies: anti-Bcl-2 antibody (Sigma Aldrich, SAB4500003, 1:1000 dilution, USA) or anti-Bax antibody (Sigma Aldrich, SAB4502548, 1:1000 dilution, USA) or anti-E-cadherin (CST, 3195, 1:800 dilution, USA), anti-alpha catenin (Sigma Aldrich, C2081, 1:1000 dilution), anti-vimentin (Abcam, ab137321, 1:700, USA), anti-fibronectin (Sigma Aldrich, F3648, 1:1000), anti-β-catenin (Abcam, ab6302, 1:1000), anti-p-GSK3β (CST, 9336, 1:1000) and anti-GAPDH (Sigma Aldrich, G9545, 1:1000) was used as a housekeeping control. Following the washing step, horse radish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibodies (CST, 7074 1:4000 dilution) was used and it was detected using the ECL chemiluminescence method. Finally, protein bands were visualized using UVP ChemStudio Western Blot Imaging Systems (Analytik Jena, USA) and subsequently analyzed using ImageJ software (version 1.48u; National Institutes of Health (USA). GAPDH was employed as an endogenous control to normalize protein expression levels.

In vivo *xenograft growth assay in nude mice*

All animal experiments were conducted in strict accordance with institutional guidelines for the ethical care and use of laboratory animals. The experimental protocol was duly reviewed and approved by the Liaoning Cancer Hospital & Institute (Approval Number: LCH/31/2019-23).

The mice were housed in the Cancer Hospital of China Medical University, maintaining specific pathogen-free conditions. Environmental parameters included a controlled temperature of 23 ± 2 °C, relative humidity of 55 ± 10 %, and a 12-hour light/12-hour dark cycle. Food and water were provided ad libitum, and the mice's health was monitored at least once daily after tumor injection.

Male BALB/c nude mice, aged 4–6 weeks, were randomly separated into two groups, each comprising 8 animals:

Group I: Nude mice were subcutaneously injected with 2×10^6 transfected cells (PANC-1 empty vector) on one side of the posterior flank.

Group II: Mice were subcutaneously injected with 2×10^6 transfected cells (*TINCR*-PANC-1) on one side of the posterior flank.

At the end of the experimental period, on day 25, all surgical procedures were performed under anesthesia induced by medetomidine (0.3 mg kg^{-1}), midazolam (4.0 mg kg^{-1}), and butorphanol (5.0 mg kg^{-1}). Euthanasia of mice was conducted using CO_2 inhalation at the study's conclusion. Tumor volumes were assessed by measuring two perpendicular diameters with calipers and calculated using the formula $V = (a \times b)/2$, where *a* represents the small diameter and *b* the large diameter. Finally, xenograft tumors were homogenized

to create a uniform mixture, and then centrifuged to separate solid cellular components from the liquid supernatant. This supernatant was subsequently employed for Western blot analysis to gauge specific protein expression levels.

Statistical analysis

The obtained results were presented as mean values with positive standard deviation error bars (mean + SD). To ascertain the statistical significance of our findings, we conducted rigorous statistical analyses utilizing GraphPad Prism 7.0 (USA). Unpaired Student's *t*-tests determined differences between two experimental cell groups. For comparisons involving three or more groups, a one-way ANOVA followed by Tukey's post hoc test was applied. In our presentation of the results, significance levels were indicated as follows: * $p < 0.05$ for statistically significant results and ** $p < 0.01$ for highly significant findings.

RESULTS AND DISCUSSION

Downregulation of TINCR expression in PANC-1 cells

LncRNAs have emerged as intriguing regulators of various biological processes, despite their inability to code for proteins (20). Accumulating evidence has underscored their impact on key aspects of human health and disease, with cancer being a prominent area of investigation (21). Dysregulation of lncRNAs has been implicated in the initiation and progression of multiple pathological conditions, including various cancers. Particularly in the context of cancer, lncRNAs have been identified as influential players, modulating critical cellular behaviors such as proliferation, invasion, and metastasis (22).

In our study, we delved into the role of the long non-coding RNA *TINCR* in the context of pancreatic cancer. Notably, previous research has linked *TINCR* to cancer suppression in other malignancies, such as melanoma and breast cancer, shedding light on its potential as a tumor suppressor (23, 24).

We aimed to investigate the expression levels of *TINCR* in various pancreatic cancer cell lines, including PaCa-2, PL45, PANC-1, and BxPC-3, in comparison to H6C7, a human normal pancreatic ductal epithelial cell line. This was accomplished through qRT-PCR analysis.

The qRT-PCR results yielded compelling findings, indicating a noteworthy downregulation of *TINCR* expression in all the examined pancreatic cancer cell lines when compared to H6C7 (Fig. 1a). This downregulation was found to be statistically significant ($p = 0.04$), underscoring the potential importance of *TINCR* in the context of pancreatic cancer progression. Remarkably, among the tested pancreatic cancer cell lines, PANC-1 exhibited the lowest levels of *TINCR* transcript expression. This finding led us to select PANC-1 cells for further experimentation due to their pronounced downregulation of *TINCR* and its potential implications in the context of pancreatic cancer biology.

In summary, the qRT-PCR analysis unveiled a consistent downregulation of *TINCR* expression in PANC-1 cells, highlighting its potential relevance as a key regulatory molecule in pancreatic cancer and underscoring its candidacy for further investigations in subsequent experiments.

Suppression of pancreatic cancer cell proliferation and viability by TINCR overexpression

TINCR exhibits aberrant expression patterns across various tumor types, suggesting a pivotal role in influencing either oncogenic or tumor-suppressive pathways, thereby influencing tumor initiation and progression. In-depth investigations have unveiled distinct associations between *TINCR* and breast cancer, where it emerges as an oncogenic player (25). On the other hand, *TINCR* exerts inhibitory effects on both proliferation and invasion in lung cancer by modulating the miR-544a/FBXW7 axis (26). Here, we endeavored to investigate the influence of *TINCR* on the proliferation and viability of PANC-1 cells.

To investigate the impact of *TINCR* on the proliferation and viability of PANC-1 cells, we initiated *TINCR* overexpression in PANC-1 cells, and the success of this overexpression was confirmed through qRT-PCR analysis (Fig. 1b). Subsequently, we sought to determine whether the upregulation of *TINCR* had any influence on the viability and proliferation of
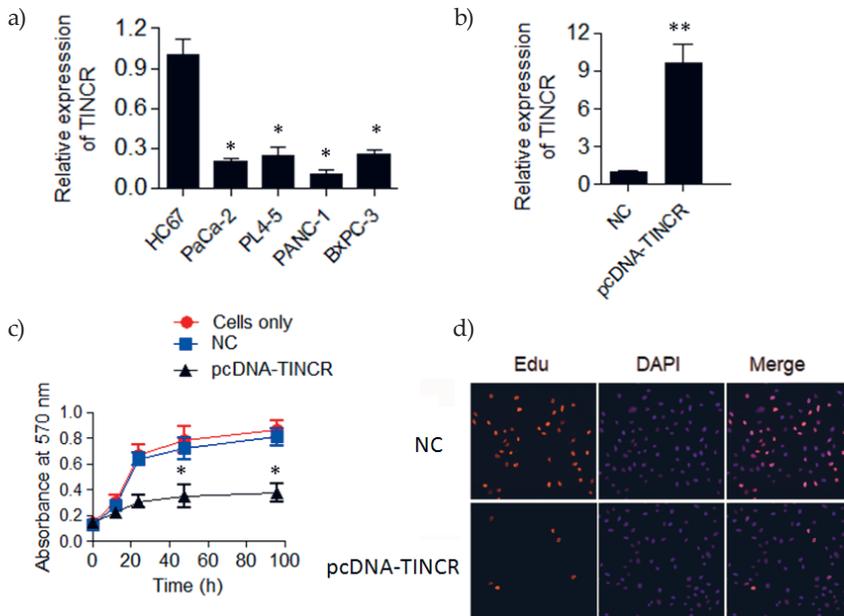
Fig. 1. *TINCR* expression and its impact on pancreatic cancer cell proliferation. a) Expression levels of long non-coding RNA *TINCR* were analyzed in various pancreatic cancer cell lines (PaCa-2, PL45, PANC-1, and BxPC-3) compared to the human normal pancreatic ductal epithelial cell line, H6C7, using qRT-PCR. Results demonstrate significant downregulation of *TINCR* expression in all pancreatic cancer cell lines; b) *TINCR* overexpression in PANC-1 cells confirmed by qRT-PCR; c) cell Counting Kit-8 (CCK-8) assay was performed to assess the impact of *TINCR* overexpression on the proliferation of PANC-1 cancer cells. *TINCR* overexpression led to a significant reduction in cell proliferation compared to the corresponding negative control cells; d) EdU incorporation assay showed a marked decrease in the incorporation of EdU into PANC-1 cancer cells overexpressing *TINCR*, indicating a loss of cell viability. *$p < 0.05$ and **$p < 0.01$, compared to control. Data are representative of at least three independent experiments.

PANC-1 cells. To accomplish this, we conducted a Cell Counting Kit-8 (CCK-8) assay, a widely used method for assessing cell viability. The results of the CCK-8 assay revealed a noteworthy and statistically significant decrease in the viability of PANC-1 cancer cells overexpressing *TINCR* in comparison to their corresponding negative control cells (Fig. 1c). This decline in viability strongly suggests that *TINCR* exerts a suppressive effect on the growth of PANC-1 cells.

Furthermore, we examined the incorporation of EdU (5-ethynyl-2'-deoxyuridine), a thymidine analog, into PANC-1 cancer cells, as it serves as a marker for actively proliferating cells. Importantly, the overexpression of *TINCR* was found to substantially reduce the incorporation of EdU into PANC-1 cancer cells (Fig. 1d). This decline in EdU incorporation provides additional evidence of diminished cell proliferation associated with *TINCR* overexpression.

In summary, our findings suggest that *TINCR* plays a pivotal role in inhibiting the proliferation and viability of PANC-1 cells. These results shed light on the potential therapeutic relevance of *TINCR* in pancreatic cancer management.

TINCR-induced apoptosis in PANC-1 cells

In our quest to unravel the underlying mechanisms responsible for the tumor-suppressive effects of *TINCR* in PANC-1 cells, we turned our attention to the intricate process of cell apoptosis. To investigate this, we employed the Annexin V-FITC/PI staining method, a robust technique for discerning apoptotic events within the cellular context. The results of our Annexin V-FITC/PI staining revealed a compelling story. The overexpression of *TINCR* in PANC-1 cells had a profound and statistically significant impact ($p = 0.02$) on the induction of apoptosis, with both early and late apoptotic cells displaying a remarkable increase in comparison to control cancer cells (Fig. 2a). This shift towards heightened apoptosis was indicative of *TINCR*'s potent pro-apoptotic role in these cells.

Furthermore, a closer examination of the apoptotic cell percentage underscored the significance of *TINCR* overexpression. Notably, the apoptotic cell percentage exhibited a substantial increase following *TINCR* overexpression when compared to control conditions, reinforcing the notion that *TINCR* exerts a pro-apoptotic influence on PANC-1 cancer cells. Complementing these findings, our Western blotting analysis provided molecular insights into the apoptotic process. PANC-1 cancer cells that upregulated *TINCR* displayed a marked increase ($p < 0.03$) in the expression of the pro-apoptotic protein Bax, while the expression of the anti-apoptotic protein Bcl-2 was notably repressed when compared to negative control cells (Fig. 2b). This shift in the balance of pro- and anti-apoptotic proteins further substantiated the role of *TINCR* in driving apoptotic pathways within PANC-1 cells.

In summary, our comprehensive examination of apoptosis in PANC-1 cells unveiled a pivotal role for *TINCR* in enhancing apoptosis, as evidenced by the increased percentages of apoptotic cells and the molecular alterations observed in Bax and Bcl-2 expression. These findings shed light on the potential mechanisms through which *TINCR* exerts its tumor-suppressive effects in pancreatic cancer.

Impact of TINCR on the migration and invasion of PANC-1 cells

Metastasis is a pivotal hallmark of cancer, contributing significantly to disease progression and treatment resistance (27). In our study, we extended our investigation to
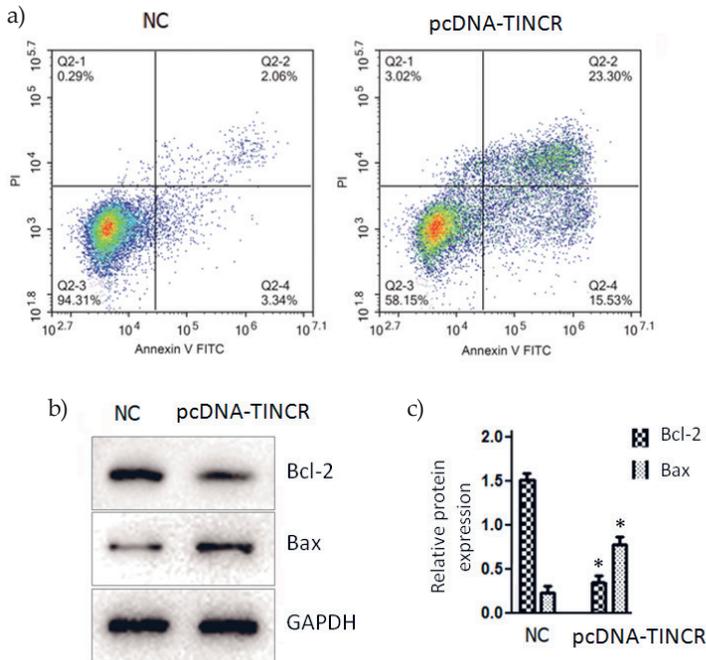
Fig. 2. *TINCR* induces apoptosis in PANC-1 cells. a) Apoptosis analysis was conducted using Annexin V-FITC/PI staining in PANC-1 cells overexpressing *TINCR* and control cells. *TINCR* overexpression increased the percentage of both early and late apoptotic cells compared to the control, with the apoptotic cell percentage rising upon *TINCR* overexpression compared to control cells; b) Western blot analysis of PANC-1 cancer cells overexpressing *TINCR* and control cells revealed upregulation of Bax protein expression and marked repression of Bcl-2 protein expression in *TINCR*-overexpressing cells compared to the negative control cells; c) densitometry analysis of Western blot. *$p < 0.05$, compared to control. Data represent at least three independent experiments.

explore the influence of *TINCR* on the metastatic potential of PANC-1 cells. Metastasis is intricately linked to cell migration and invasion, both of which are essential for cancer cells to navigate through the body and establish secondary tumors (28, 29). Our results revealed that *TINCR* overexpression led to a substantial reduction in the rates of metastasis *in vitro*. Additionally, *TINCR* upregulation triggered notable changes in the expression of EMT markers, a process often associated with enhanced metastatic capability. These findings suggest that *TINCR* may act as a regulator of pancreatic cancer metastasis, which is consistent with recent research implicating *TINCR* in breast cancer metastasis (30).

Our investigation extended to the evaluation of how *TINCR* influences the migratory and invasive properties of PANC-1 cells in an *in vitro* setting. To accomplish this, we employed a wound-healing assay to assess cell migration and a Transwell invasion assay to evaluate cell invasion.

In the wound-healing assay, we observed a noteworthy reduction ($p = 0.04$) in the migration of PANC-1 cancer cells when *TINCR* was overexpressed (Fig. 3a). Specifically,

the percentage of cell migration was significantly diminished by over 70 % in PANC-1 cells that were overexpressing *TINCR* compared to the negative control cells. This substantial decrease in cell migration underscored the potent inhibitory effect of *TINCR* on the migratory capacity of these PANC-1 cells.

Furthermore, our investigation into cell invasion yielded equally compelling results. The Transwell invasion assay demonstrated a significant reduction in the invasive potential of PANC-1 cancer cells when *TINCR* was upregulated (Fig. 3b). More specifically, PANC-1 cells that overexpressed *TINCR* exhibited an invasion rate that was merely one-fourth that of the negative control cells. This marked decrease in invasive capacity provided robust evidence of *TINCR*'s inhibitory influence on the invasiveness of PANC-1 cells.

In summary, our findings underscore the pivotal role of *TINCR* in impeding both the migration and invasion of PANC-1 cells. The substantial reductions observed in cell migration and invasion rates upon *TINCR* upregulation emphasize its potential significance in modulating the aggressive behavior of PANC-1 cells, with potential implications for the suppression of metastasis.

TINCR inhibits EMT in PANC-1 cells

In our exploration of the role of *TINCR* in pancreatic cancer, we sought to understand its influence on the expression of key markers associated with EMT, a fundamental process in cancer progression. Our investigation involved the assessment of transcript levels of specific EMT markers. The results unveiled a compelling pattern of gene expression changes associated with *TINCR*.
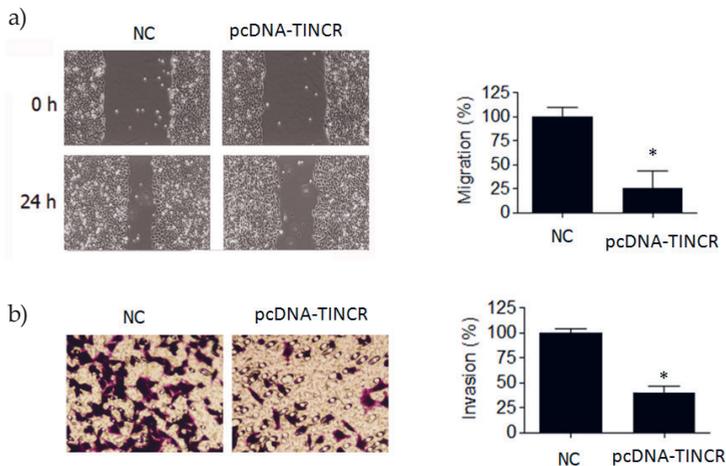
Fig. 3. *TINCR* inhibits metastasis of PANC-1 cells. a) Wound-healing assay was conducted to assess the effect of *TINCR* overexpression on the migration of PANC-1 cells. *TINCR* overexpression significantly reduced cell migration, with over 70 % decrease in cell migration observed in PANC-1 cells overexpressing *TINCR* compared to negative control cells; b) Invasion assay demonstrated a significant reduction in the invasion capability of PANC-1 cancer cells upon *TINCR* upregulation. PANC-1 cells overexpressing *TINCR* exhibited only one-fourth of the invasion rate of negative control cells. * $p < 0.05$, compared to control. Data are representative of at least three independent experiments.
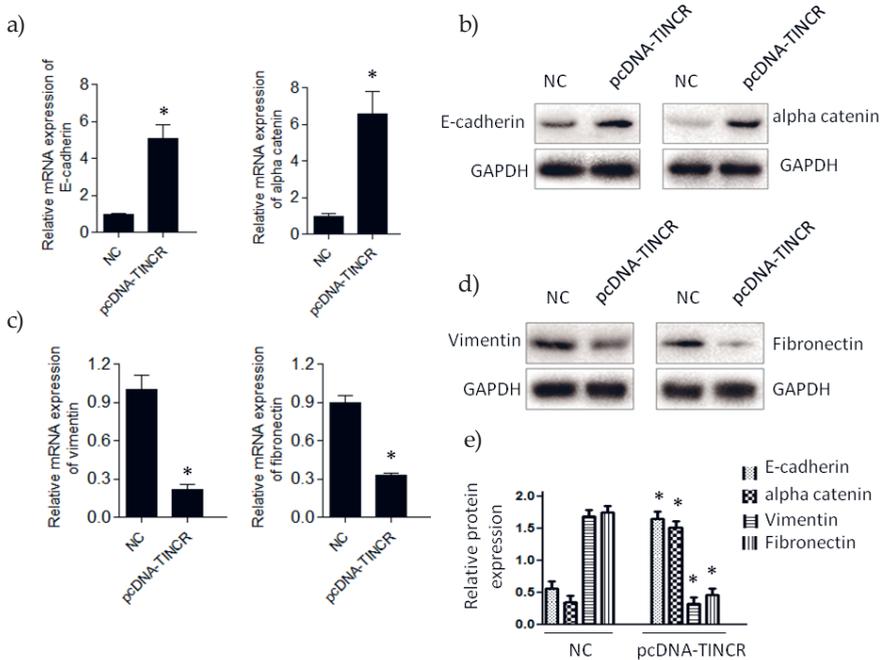
Fig. 4. *TINCR* modulates epithelial-to-mesenchymal transition (EMT) marker expression. a) and b) qRT-PCR and Western blotting analysis of epithelial (E-cadherin and α-catenin) markers in PANC-1 cells overexpressing *TINCR* compared to the control cells; c) and d) qRT-PCR and Western blotting analysis of mesenchymal markers (vimentin and fibronectin) in PANC-1 cells overexpressing *TINCR* compared to the control cells; e) densitometry analysis of Western blot. *$p < 0.05$, compared to control. Data represent at least three independent experiments.

Firstly, we observed a notable increase in the expression of E-cadherin and α-catenin (Fig. 4a,b). E-cadherin is a critical cell adhesion molecule associated with epithelial characteristics, and α-catenin is a protein involved in adherens junctions, both of which are indicative of an epithelial phenotype. This downregulation suggests that *TINCR* promotes the retention of epithelial features within PANC-1 cells. Conversely, the expression levels of vimentin and fibronectin exhibited a significant decrease (Fig. 4c,d). Vimentin and fibronectin are markers typically associated with the mesenchymal state, which is characterized by increased motility and invasiveness. The increase in their expression levels suggests that *TINCR* inhibits the acquisition of mesenchymal traits in these cells.

In summary, our findings indicate that *TINCR* plays a pivotal role in promoting an epithelial phenotype and restraining the mesenchymal features typically associated with EMT in PANC-1 cells. These observations provide valuable insights into the potential mechanisms through which *TINCR* influences cancer progression and metastasis in this context.

The inhibition of the Wnt/β-catenin signaling pathway by TINCR

While our research has confirmed that *TINCR* can induce EMT in PANC-1 cells, the specific signaling pathways involved have remained unclear. To investigate *TINCR's*

potential impact on signaling cascades, we focused on the Wnt/β-catenin pathway. The decision to investigate the Wnt/β-catenin pathway in the context of *TINCR*-induced EMT is underpinned by its known involvement in orchestrating key events during cancer progression (31). Several studies have highlighted the Wnt/β-catenin pathway as a crucial mediator of EMT. Using Western blot experiments, we systematically examined the expression patterns of key proteins in the Wnt/β-catenin pathway, specifically β-catenin and p-GSK3β. Elevated *TINCR* expression was associated with a notable increase in p-GSK3β levels and a simultaneous decrease in β-catenin protein expression (Fig. 5). In summary, our findings indicate that *TINCR* can inhibit the Wnt/β-catenin signaling pathway, highlighting its role in inhibiting the EMT process in PANC-1 cells. This contributes significantly to our understanding of the complex molecular dynamics at play in these cells.

TINCR prevents tumor growth, induces apoptosis, and inhibits Wnt/β-catenin in mice

Our study unveils the multifaceted impact of *TINCR* overexpression in a pancreatic cancer mice model, shedding light on potential therapeutic strategies. *TINCR* emerges as a potent modulator, impeding tumor growth and reducing tumor size, suggesting its promise as a therapeutic target for pancreatic cancer (Fig. 6). Notably, *TINCR* demonstrates its ability to promote apoptosis, as evidenced by increased pro-apoptotic protein Bax levels and decreased anti-apoptotic protein Bcl-2 levels, indicating its role in initiating programmed cell death – an essential mechanism to curtail cancer cell survival. Furthermore, our research reveals *TINCR*'s involvement in inhibiting the Wnt/β-Catenin signaling pathway, a key regulator of cell proliferation and cancer progression, presenting a potential avenue for targeted therapies.

In our *in vivo* xenograft growth assay in nude mice, key outcomes include substantial inhibition of tumor growth and consistent reduction in tumor size in the *TINCR*-overexpressing Panc-1 cell group compared to the control group. Enhanced activation of the
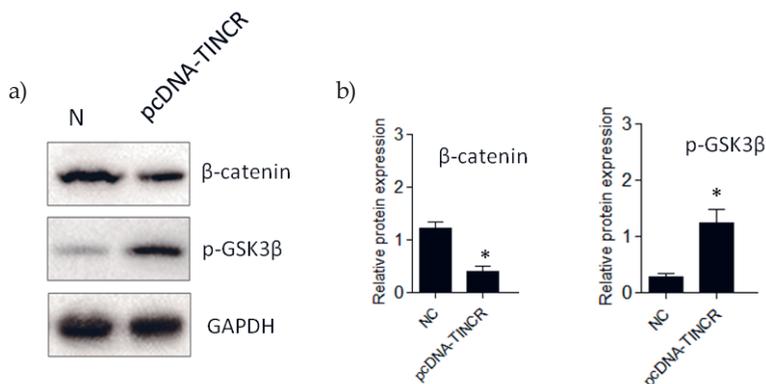
Fig. 5. *TINCR*-mediated inhibition of the Wnt/β-catenin signaling pathway in PANC-1 cells. a) Western blot analysis of key proteins in the Wnt/β-catenin pathway. Elevated *TINCR* expression correlated with increased p-GSK3β levels, while simultaneously decreased β-catenin protein expression; b) and c) densitometry analysis of Western blot. * $p < 0.05$, compared to control. Data represent at least three independent experiments.

Wnt/β-Catenin signaling pathway, as indicated by increased p-GSK3β levels and decreased β-catenin protein expression in the *TINCR*-overexpressing group, further supports the involvement of *TINCR* in modulating critical signaling pathways. Additionally, *TINCR* demonstrates an influence on epithelial-mesenchymal transition (EMT) markers, promoting the preservation of epithelial traits and hindering the acquisition of mesenchymal characteristics in PANC-1 cells.
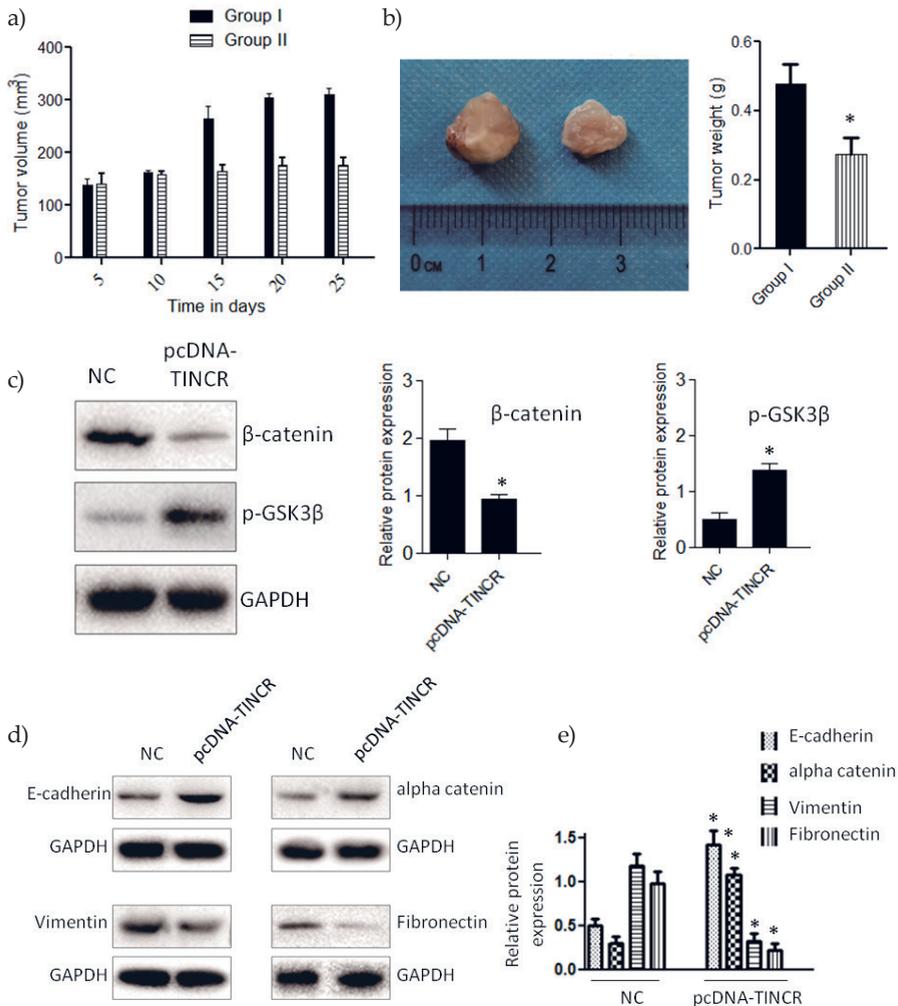
Fig. 6. Impact of *TINCR* on pancreatic cancer progression in mice. a) Tumor growth inhibition: The effect of *TINCR* on tumor growth in mice; b) tumor size reduction: The influence of *TINCR* on tumor size during the study; c) Wnt/β-Catenin inhibition: *TINCR*'s role in modulating the Wnt/β-Catenin signaling pathway in mice; d) inhibition of EMT markers: The impact of *TINCR* on EMT markers in pancreatic cancer; e) densitometry analysis of Western blot. *$p < 0.05$, compared to control. Data represent at least three independent experiments.

These findings provide compelling evidence for the therapeutic potential of *TINCR* in pancreatic cancer. The observed effects on tumor growth, apoptosis, Wnt/β-Catenin signaling, and EMT present a comprehensive understanding of *TINCR*'s impact, paving the way for innovative therapeutic strategies in pancreatic cancer management. Further exploration into *TINCR*-based interventions holds promise for the development of targeted therapies aimed at improving outcomes for pancreatic cancer patients.

CONCLUSIONS

In summary, our study highlights the noteworthy downregulation of lncRNA *TINCR* in PANC-1 cells. The overexpression of *TINCR* emerged as a potent inhibitor of cancer cell proliferation, primarily through the induction of apoptosis. Furthermore, our investigation revealed a significant decrease in the migratory and invasive capabilities of PANC-1 cells following *TINCR* upregulation. Furthermore, *TINCR* inhibits tumor growth, triggers apoptosis, and inhibits the Wnt/β-Catenin pathway in mice. These findings pave the way for further research into *TINCR*'s mechanisms and its potential as a targeted therapy for pancreatic cancer. Understanding *TINCR*'s nuanced role could lead to more effective strategies against this challenging malignancy, offering hope to patients.

REFERENCES

1. P. Rawla, T. Sunkara and V. Gaduputi, Epidemiology of pancreatic cancer: Global trends, etiology and risk factors, *World J. Oncol.* **10**(1) (2019) 10–27; https://doi.org/10.14740/wjon1166

2. G. Lippi and C. Mattiuzzi, The global burden of pancreatic cancer, *Arch. Med. Sci.* **16**(4) (2020) 820–824; https://doi.org/10.5114/aoms.2020.94845

3. A. McGuigan, P. Kelly, R. C. Turkington, C. Jones, H. G. Coleman and R. S. McCain, Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes, *World J. Gastroenterol.* **24**(43) (2018) 4846–4861; https://doi.org/10.3748/wjg.v24.i43.4846

4. E. G. Chiorean and A. L. Coveler, Pancreatic cancer: Optimizing treatment options, new, and emerging targeted therapies, *Drug Des. Devel. Ther.* **9** (2015) 3529–3545; https://doi.org/10.2147/DDDT.S60328

5. D. R. Principe, P. W. Underwood, M. Korc, J. G. Trevino, H. G. Munshi and A. Rana, The current treatment paradigm for pancreatic ductal adenocarcinoma and barriers to therapeutic efficacy, *Front. Oncol.* **11** (2021) Article ID 88377 (25 pages); https://doi.org/10.3389/fonc.2021.688377

6. J. Hunia, K. Gawalski, A. Szredzka, M. J. Suskiewicz and D. Nowis, The potential of PARP inhibitors in targeted cancer therapy and immunotherapy, *Front. Mol. Biosci.* **9** (2022) Article ID 1073797 (24 pages); https://doi.org/10.3389/fmolb.2022.1073797

7. Y. You, X. Lai, Y. Pan, H. Zheng, J. Vera, S. Liu, S. Deng and L. Zhang, Artificial intelligence in cancer target identification and drug discovery, *Signal Transduct. Target Ther.* **7**(1) (2022) Article ID 156 (24 pages); https://doi.org/10.1038/s41392-022-00994-0

8. S. Dimitrov-Markov, J. Perales-Paton, B. Bockorny, A. Dopazo, M. Munoz, N. Banos, V. Bonilla, C. Menendez, Y. Duran, L. Huang, S. Perea, S. K. Muthuswamy, F. Al-Shahrour, P. P. Lopez-Casas and M. Hidalgo, Discovery of new targets to control metastasis in pancreatic cancer by single-cell transcriptomics analysis of circulating tumor cells, *Mol. Cancer Ther.* **19**(8) (2020) 1751–1760; https://doi.org/10.1158/1535-7163.MCT-19-1166

9. J. Yi, S. Li, C. Wang, N. Cao, H. Qu, C. Cheng, Z. Wang, L. Wang and L. Zhou, Potential applications of polyphenols on main ncRNAs regulations as novel therapeutic strategy for cancer, *Biomed. Pharmacother.* **113** (2019) Article ID 108703 (8 pages); https://doi.org/10.1016/j.biopha.2019.108703

10. A. Vafadar, Z. Shabaninejad, A. Movahedpour, S. Mohammadi, S. Fathullahzadeh, H. R. Mirzaei, A. Namdar, A. Savardashtaki and H. Mirzaei, Long non-coding RNAs as epigenetic regulators in cancer, *Curr. Pharm. Des.* **25**(33) (2019) 3563–3577; https://doi.org/10.2174/1381612825666190830161528

11. L. Statello, C. J. Guo, L. L. Chen and M. Huarte, Gene regulation by long non-coding RNAs and its biological functions, *Nat. Rev. Mol. Cell Biol.* **22**(2) (2021) 96–118; https://doi.org/10.1038/s41580-020-00315-9

12. H. Sun, Z. Huang, W. Sheng and M. D. Xu, Emerging roles of long non-coding RNAs in tumor metabolism, *J. Hematol Oncol.* **11** (2018) Article ID 106 (16 pages); https://doi.org/10.1186/s13045-018-0648-7

13. J. C. de Oliveira, L. C. Oliveira, C. Mathias, G. A. Pedroso, D. S. Lemos, A. Salviano-Silva, T. S. Jucoski, S. C. Lobo-Alves, E. P. Zambalde, G. A. Cipolla and D. F. Gradia, Long non-coding RNAs in cancer: Another layer of complexity, *J. Gene Med.* **21**(1) (2019) e3065; https://doi.org/10.1002/jgm.3065

14. Y. H. Lin, M. H. Wu, C. T. Yeh and K. H. Lin, Long non-coding RNAs as mediators of tumor microenvironment and liver cancer cell communication, *Int. J. Mol. Sci.* **19**(12) (2018) Article ID 3742 (21 pages); https://doi.org/10.3390/ijms19123742

15. Z. Ma, Y. Y. Wang, H. W. Xin, L. Wang, F. Arfuso, A. Dharmarajan, A. P. Kumar, H. Wang, F. R. Tang, S. Warrier, V. Tergaonkar and G. Sethi, The expanding roles of long non-coding RNAs in the regulation of cancer stem cells, *Int. J. Biochem. Cell Biol.* **108** (2019) 17–20; https://doi.org/10.1016/j.biocel.2019.01.003

16. U. Sharma, T. S. Barwal, A. Malhotra, N. Pant, Vivek, D. Dey, A. Gautam, H. S. Tuli, K. M. Vasquez and A. Jain, Long non-coding RNA *TINCR* as potential biomarker and therapeutic target for cancer, *Life Sci.* **257** (2020) Article ID 118035; https://doi.org/10.1016/j.lfs.2020.118035

17. S. Li, J. Li, H. Li, M. Gao, N. Li, Y. Wang, L. Tong, M. Song and Z. Yin, Clinicopathological and prognostic significance of *TINCR* in caner: A meta-analysis, *Pathol. Res. Practice* **215**(10) (2019) Article ID 152596; https://doi.org/10.1016/j.prp.2019.152596

18. S. Ghafouri-Fard, S. Dashti, M. Taheri and M. D. Omrani, *TINCR*: An lncRNA with dual functions in the carcinogenesis process, *Non-coding RNA Res.* **5**(3) (2020) 109–115; https://doi.org/10.1016/j.ncrna.2020.06.003

19. K. J. Livak and T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* **25**(4) (2001) 402–408; https://doi.org/ 10.1006/meth.2001.1262

20. E. Ji, C. Kim, W. Kim and E. K. Lee, Role of long non-coding RNAs in metabolic control, *Biochim. Biophys. Acta Gene Regul. Mech.* **1863**(4) (2020) Article ID 194348; https://doi.org/10.1016/j.bbagrm.2018.12.006

21. M. E. Forrest and A. M. Khalil, Review: Regulation of the cancer epigenome by long non-coding RNAs, *Cancer Lett.* **407** (2017) 106–112; https://doi.org/10.1016/j.canlet.2017.03.040

22. Q. Huang, J. Yan and R. Agami, Long non-coding RNAs in metastasis, *Cancer Metastasis Rev.* **37**(1) (2018) 75-81; https://doi.org/10.1007/s10555-017-9713-x

23. X. Han, Y. Jia, X. Chen, C. Sun and J. Sun, lncRNA *TINCR* attenuates the proliferation and invasion, and enhances the apoptosis of cutaneous malignant melanoma cells by regulating the miR-424-5p/LATS1 axis, *Oncol. Rep.* **46**(5) (2021) Article ID 238 (11 pages); https://doi.org/10.3892/or.2021.8189

24. Y. Wang, L. Zhou, J. Lu, B. Jiang, C. Liu, J. Guo and G. G. Xiao, Research progress on long non-coding RNAs and their roles as potential biomarkers for diagnosis and prognosis in pancreatic cancer, *Cancer Cell Int.* **20** (2020) Article ID 457 (12 pages); https://doi.org/10.1186/s12935-020-01550-y

25. D. Lu, S. Di, S. Zhuo, L. Zhou, R. Bai, T. Ma, Z. Zou, C. Chen, M. Sun, J. Tang and Z. Zhang, The long noncoding RNA TINCR promotes breast cancer cell proliferation and migration by regulating OAS1, *Cell Death Discov.* **7**(1) (2021) Article ID 41 (16 pages); https://doi.org/10.1038/s41420-021-00419-x

26. X. Liu, J. Ma, F. Xu and L. Li, TINCR suppresses proliferation and invasion through regulating miR-544a/FBXW7 axis in lung cancer, *Biomed. Pharmacother.* **99** (2018) 9–17; https://doi.org/10.1016/j.biopha.2018.01.049

27. M. Esposito, S. Ganesan and Y. Kang, Emerging strategies for treating metastasis, *Nat. Cancer* **2**(3) (2021) 258–270; https://doi.org/10.1038/s43018-021-00181-0

28. N. M. Novikov, S. Y. Zolotaryova, A. M. Gautreau and E. V. Denisov, Mutational drivers of cancer cell migration and invasion, *Br. J. Cancer* **124**(1) (2021) 102–114; https://doi.org/10.1038/s41416-020-01149-0

29. J. Winkler, A. Abisoye-Ogunniyan, K. J. Metcalf and Z. Werb, Concepts of extracellular matrix remodelling in tumour progression and metastasis, *Nat. Commun.* **11** (2020) Article ID 5120 (19 pages); https://doi.org/10.1038/s41467-020-18794-x

30. D. Lu, S. Di, S. Zhuo, L. Zhou, R. Bai, T. Ma, Z. Zou, C. Chen, M. Sun, J. Tang and Z. Zhang, The long noncoding RNA *TINCR* promotes breast cancer cell proliferation and migration by regulating OAS1, *Cell Death Discov.* **7**(1) (2021) Article ID 41 (16 pages); https://doi.org/10.1038/s41420-021-00419-x

31. Y. Zhang and X. Wang, Targeting the Wnt/beta-catenin signaling pathway in cancer, *J. Hematol. Oncol.* **13** (2020) Article ID 165 (16 pages); https://doi.org/10.1186/s13045-020-00990-3