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ORIGINAL ARTICLE

High Expression of Long Noncoding RNA HOTAIRM1 is Associated with the Proliferation and Migration in Pancreatic Ductal Adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an incurable malignancy. Long noncoding RNA (LncRNA) HOTAIRM1 (HOX antisense intergenic RNA myeloid 1) has been shown to play important roles in the progression of several type cancers. However, the exact role of HOTAIRM1 in PDAC development remains largely unknown. This study aims to evaluate the potential function of HOTAIRM1 in the development and progress of PDAC. HOTAIRM1 expression was measured by RT-qPCR in forty seven paired human PDAC tissues and five PDAC cell lines. SW1990 and PANC-1 cells were transfected with siHOTAIRM1 to achieve HOTAIRM1 silence. MTT assay and colony formation assay were used to detect the effect of HOTAIRM1 knockdown on cell proliferation. The impact of HOTAIRM1 silence on cell cycle and apoptosis was assessed by flow cytometry assay. Transwell migration assay was performed to explore the influence of HOTAIRM1 downregulation on the migratory potential of PDAC cells. Western blot assay was applied to determine the expression changes of cell cycle, apoptosis, and migration-related genes before and after downregulating HOTAIRM1. HOTAIRM1 expression was abnormally upregulated in PDAC tissues and cells when compared with the control samples, and was positively associated with the expression of KRAS gene mutation. In vitro functional experiments, HOTAIRM1 expression was significantly downregulated by transfection with siHOTAIRM1 in SW1990 and PANC cell lines. HOTAIRM1 knockdown attenuated cell proliferation by inducing cell cycle arrest at G0/G1 phase, promoted cell apoptosis, and inhibited cell migration in PDAC cells by regulating related-genes expression. In conclusion, HOTAIRM1 plays a critical role in PDAC progression, which may be a novel diagnostic and rational therapeutic target for the treatment of pancreatic ductal adenocarcinoma.

Keywords HOTAIRM1 · Pancreatic ductal adenocarcinoma · Proliferation · Migration

Introduction

Pancreatic cancer, one of the most aggressive malignancies around the world currently, has become the fourth leading reason of death induced by cancers in developed countries [1]. Pancreatic ductal adenocarcinoma (PDAC) is the major form of pancreatic cancer, which derives from the epithelial cells of pancreatic duct [2]. As an incurable disease, PDAC is characterized by a highly malignant phenotype and is associated with a relatively poor prognosis [3]. Despite great efforts have been made in past years, the overall 5-year survival rate of PDAC patients remains much less than 5% [4]. It is urgent to make clear the potential pathogenesis of PDAC development, as well as discover novel effective diagnostic and therapeutic approaches of PDAC.

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The key role of noncoding genome in both normal development and diseases has been confirmed by increasing evidence [5]. Long noncoding RNAs (LncRNAs) are defined as a class of endogenous cellular RNAs longer than 200 nucleotides but lacking protein coding function [6], which participates in various biological processes, such as cell cycle control, cell differentiation and apoptosis, transcriptional and translational control, epigenetic silencing, and splicing regulation [7–10]. Recently, abundant lncRNAs have been revealed to act as oncogenes or tumor suppressor depending the physiological conditions, most of which have shown promise as cancer biomarkers and potential therapeutic targets in various cancer subtypes [11–13]. In pancreatic, a large amount of differentially expressed lncRNAs have been found to possess pro-oncogenic or tumor-suppressive activities, such as LOC285194 [14], ENST00000480739 [14], HULC [15], MALAT1 [16], and HOTAIR [17], emphasizing the potential regulatory function of lncRNAs in pancreatic cancer development. Therefore, identifying the lncRNAs involved in PDAC progression may help yield novel prognostic biomarkers or therapeutic targets.

HOTAIRM1 (HOX antisense intergenic RNA myeloid 1) is a novel lncRNA identified by Zhang et al. [18], which locates on chromosome band 7p15 between HOXA1 and HOXA2. HOTAIRM1 acts as a myeloid lineage-specific regulator of HOXA genes, and is associated with the regulation of gene expression during myelopoiesis [18]. Recently, HOTAIRM1 has been shown to be abnormally expressed in leukemia and several solid tumors, as well as play an important role in the various cellular activities in these cancers [19–21]. Yet, little is known about HOTAIRM1 expression and its underlying role in PDAC development.

In the present study, we observed that lncRNA HOTAIRM1 was significantly upregulated in PDAC tissues and cell lines. Knockdown of HOTAIRM1 could inhibit cell proliferation and migration, induce cell cycle arrest, and promote apoptosis in PDAC cells. Our study preliminarily reveals the important role of HOTAIRM1 in the development and progression of PDAC, which may promise as a novel potential therapeutic target in the treatment of PDAC.

Table 1 Clinical characteristics of pancreatic ductal adenocarcinoma patients

Clinical characteristics	Number of cases ($n = 47$)
Age (year-old)	
≤ 60	25
> 60	22
Gender	
Male	30
Female	17
Tumor size (cm)	
≤ 4	19
> 4	28
Histological differentiation	
Well/Moderate	33
Poor	14
T classification	
T1/T2	31
T3/T4	16
Lymph-node metastasis	
Yes	18
No	29
Smoking status	
Non-smoker	23
Current	18
Former	4
Unknown	2
Drinking status	
Non-drinker	22
Current	17
Former	3
Unknown	5
Diabetes	
Yes	29
No	18
Chronic pancreatitis	
Non-CP	11
ICP	24
ACP	9
HCP	1
Other	2
Fibrosis	
Yes	35
No	12
KRAS mutations[a] in codons 12 and 13	
Wild-type (GGT)	5
G12 V (G<u>T</u>T)	16
G12D (G<u>A</u>T)	19
G12R (C<u>G</u>T)	7
Other (G13D, Q61R, Q61H)	0

[a] Base changes are underlined; CP = chronic pancreatitis, ICP = idiopathic chronic pancreatitis, ACP = alcoholic chronic pancreatitis, HCP = hereditary chronic pancreatitis

Methods

Clinical Specimens

PDAC tumor tissues and the adjacent tissues were obtained from 47 patients who had undergone surgical resection of the pancreas at General Hospital of Ningxia Medical University between 2014 and 2016. Clinical characteristics of these patients were shown in Table 1, these characteristics included age, gender, tumor size, histological differentiation, T classification, lymph-node metastasis, smoking status, drinking status, diabetes, chronic pancreatitis, fibrosis, and KRAS mutations in codons 12 and 13. Written informed consent was obtained from each participant before sample collection, and this study was approved by Ethic Committees of General Hospital of Ningxia Medical University. All patients were diagnosed with PDAC by pathological examination. None of them had received any anticancer treatments before their surgery. The collected fresh tissues samples were snap-frozen in liquid nitrogen immediately after surgical removal, then were stored at −80 °C for further use.

Cell Culture

Human normal pancreatic ductal epithelial cell line HPDE6-C7, and human PDAC cell lines including AsPC-1, SW1990, PANC-1, and Capan-2, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM by the supplier with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified chamber with 5% CO_2.

siRNA Transfection

The small interfering RNA (siRNA) targeting HOTAIRM1 were constructed by Shanghai GenePharma Co. Ltd. (Shanghai, China), and then transfected into human PDAC cells, SW1990 and PANC-1, using the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Sunnyvale, CA, USA) according to the manufacturer's protocol. The non-specific siRNA served as a negative control. The knockdown efficiency of HOTAIRM1-siRNA was detected by real-time PCR.

Mutation Detection for KRAS

KRAS gene mutation analysis was performed according to the previously described method [22]. Briefly, DNA was first extracted from the tumor tissues using the QIAampDNA Mini kit (Qiagen, Hilden, Germany) and then were amplified at 94 °C for 5 min, followed at 94 °C by 30 cycles of 1 min, at 55 °C for 1 min, and 72 °C for 1 min, and final at 72 °C for 10 min. After purification of PCR products, the sequencing reactions were carried out under the following conditions: 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C. The sequencing data were obtained from the ABI PRISM 3100 DNA Analyzer (Applied Biosystems) and were analyzed to compare variations using sequencing software (Applied Biosystems, Foster, CA, USA). Primers ($5' \rightarrow 3'$) used for KRAS and KRAS wild type were as follows: F: TTATGTGTGACATGTTCTAAT, R: AGAATGGT CCTGCACCAGTAA; F: GGTGGAGTATTTGATAGTGT ATTAACC, R: AGAATGGTCCTGCACCAGTAA.

MTT Assay

After seeding the cells into 96-well culture plates at a density of 5×10^3 cells/well, we tested the proliferation ability of SW1990 and PANC-1 cells transfected with siHOTAIRM1 at the given time points of 0, 24, 48, 72, 96, and 120 h (on day 1, 2, 3, 4 and 5). Briefly, a total of 20 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) reagent (Sigma-Aldrich, Shanghai, China) was added to the medium and the cells were incubated at 37 °C. Four hours later, the supernatant was discarded and cells were oscillated in 150 μL/well dimethyl sulfoxide for 15 min. The growth curves of cells were obtained by measuring the absorbance of each well at 595 nm (595 OD) using a microplate reader (ELx808 Bio-Tek Instruments, City, ST, USA).

Colony Formation Assay

After transfection for 24 h, SW1990 and PANC-1 cells were trypsinized, centrifuged, and seeded in 24-well culture plate at 500 cells for each condition. Two weeks later, colonies were washed using PBS, fixed in paraformaldehyde for 15 min, and stained with 0.1% crystal violet for 1 h. After washing three times in ddH_2O and air-dried overnight, the colonies containing more than 100 cells were counted using a microscope (Olympus, Tokyo, Japan).

Cell Cycle Analysis

Flow cytometry assay was used to confirm the cell cycle distribution [23]. After transfection for 48 h, all cells were collected by trypsinization, washed three times using PBS, and then fixed in 70% ethanol at 4 °C overnight. Following, the cells were harvested by centrifugation and re-suspended in PBS containing 100 μg/ml RNase A and 40 μg/ml PI. After incubating in the dark at 4 °C for 30 min, the cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). The proportion of the cells in G0/G1, S, and G2/M phases were analyzed using FlowJo V10 software (Becton–Dickinson, San Jose, CA, USA).

Cell Apoptosis Analysis

The infected and uninfected cells were collected by centrifugation at 1500 rpm for 5 min. After washing twice with pre-cooling PBS, the cells were stained with Annexin V-FITC for 30 min and propidium iodide (PI) for 5 min. Finally, the samples were subjected to a fluorescence-activated cell-sorting (FACS) flow cytometer (BD Biosciences, San Jose, CA, USA).

Transwell Migration Assay

Cell migration assay was conducted using the 24-well BD transwell chambers with 8-μm pore size polycarbonate membrane (Corning Incorporated, Los Angeles, CA, USA). SW1990 or PDAC cells transfected with HOTAIRM1-siRNA or control-siRNA were suspended in serum-free DMEM medium (BD Biosciences, Franklin Lakes, NY, USA), and then seeded in the upper chamber of each insert. For the assay, medium containing 20% fetal bovine serum was added in the lower chamber as chemo-attractant. After 12 h, the migrated cells were fixed with methanol, and then stained with 0.5% crystal violet solution overnight. The cells in the

upper layer of the membrane were discarded and the cells in the lower layer were washed off using 33% acetic acid. Three visual fields were randomly selected from each membrane to count the number of migrated cells using an inverted microscope. The quantification of migrated cells was achieved by measuring the absorbance at 570 nm.

Quantitative Real-Time PCR

Quantitative real-time PCR (RT-qPCR) was performed to evaluate HOTAIRM1 level. The expression of GAPDH was used as an internal control. Total RNA was extracted from the PDAC tissues, the corresponding adjacent tissues, and the pancreatic cell lines using TRIzol reagent (Invitrogen, USA). Next, cDNA was synthesized with a reverse transcription kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For real-time quantitative PCR, each sample were amplified and analyzed on a 7300 Real-Time PCR System (Thermo Fisher Scientific, Sunnyvale, CA, USA) using SYBR green agent (Applied Biosystem, Foster City, CA, USA). The cycling conditions were as follows: 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Relative gene expression was quantified using $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

Immunoblotting was performed to evaluate the protein expression of CDK1, Cyclin D1, P21, Bax, Bad, Bcl-2, E-cadherin, N-cadherin, and Vimentin in PDAC samples according to the previous description with minor modification [24]. Briefly, tissues or cells were washed in PBS and then lysed with RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The concentration of extracted protein was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The collected protein samples were subjected to SDS-PAGE gel and then transferred onto PVDF membrane. After blocking in fat-free milk, the membranes were probed with the primary antibodies at 4 °C overnight, and then incubated with HRP-conjugated secondary antibody for 2 h at room temperature. ECL chemiluminescence kit (Haigene, Harbin, China) was used to visualize the signals. Antibody against GAPDH was used as an internal control.

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). All data were presented as the mean ± SEM from at least three independent experiments. The Student's *t* test was used for comparisons between groups. $p < 0.05$ was deemed as statistically significant.

Results

HOTAIRM1 is Significantly Upregulated in PDAC Tissues and Cells

First, we detected the expression of HOTAIRM1 in PDAC tumor tissues and the corresponding adjacent tissues by real-time PCR. Based on the comparison of 47 groups of specimens, we observed that HOTAIRM1 was abnormally upregulated in PDAC tumor tissues compared with their paired non-tumor tissues ($p < 0.001$, Fig. 1a). Previous research has confirmed that 95% of PDACs is accompanied by the mutation of KRAS, which is a well-validated driver of PDAC growth and development [22]. Specially, mutations in codon 12 of KRAS occur the most frequently in pancreatic cancer. The KRAS status of AsPC-1, SW1990, PANC-1, and Capan-2 cells is G12D, G12D, G12D and G12 V, respectively [25]. Following, we performed a correlation analysis to explore the association between the status of KRAS mutation and HOTAIRM1 expression. Not surprisingly, a positive correlation between the relative level of HOTAIRM1 and gene expression of G12 V and G12D was found at the tissues level ($p < 0.05$, Fig. 1b). However, such correlation was not found between HOTAIRM1 level and G12R expression, which may be caused by the small sample size. Further, to identify whether HOTAIRM1 was also overexpressed in PDAC cell lines, the expression of HOTAIRM1 in four PDAC cell lines (AsPC-1, SW1990, PANC-1, and Capan-2 cells) and normal human pancreatic ductal epithelium cell (HPDE6–7 cell) was examined. It turned out that HOTAIRM1 expression was remarkably up-regulated in each PDAC cell line compared with normal pancreatic cell line ($p < 0.05$, $p < 0.01$, $p < 0.001$; Fig. 1c). Collectively, these data indicated that the expression of HOTAIRM1 was significantly increased in PDAC tumor tissues and cell lines.

HOTAIRM1 Knockdown Attenuates Cell Proliferation in PDAC Cells

To further identify the potential role of HOTAIRM1 in PDAC development, we next performed a series of in vitro functional studies. First and foremost, SW1990 and PANC-1 cells with the highest expression of HOTAIRM1 were selected to transfect with HOTAIRM1 siRNA to achieve HOTAIRM1 silence, and the efficiency of knockdown was evaluated by real-time PCR assay. We found that the relative HOTAIRM1 expression was significantly reduced in both SW1990 and PANC-1 cells by transfection of siHOTAIRM1 compared with that of the siCon ($p < 0.001$, Fig. 2a), suggesting the successful establishment of HOTAIRM1 knockdown model. The impact of HOTAIRM1 on PDAC cells proliferation were determined by MTT and clone formation assays. As determined by MTT assay, HOTAIRM1 knockdown contributed to a

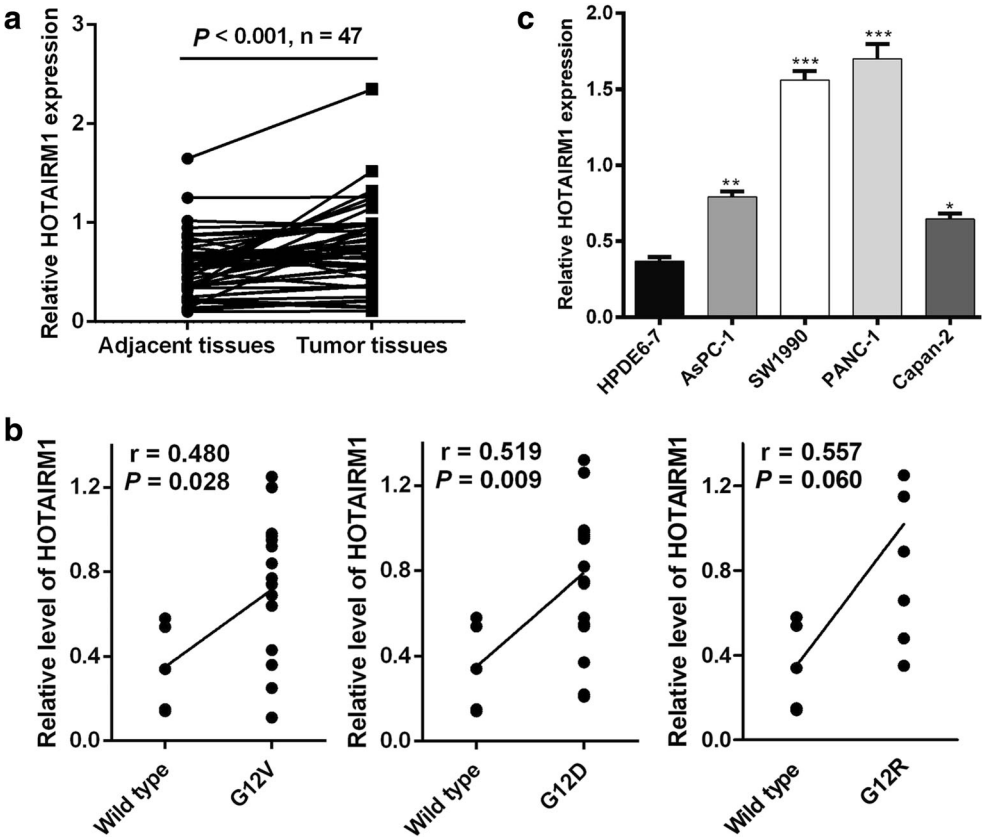

Fig. 1 HOTAIRM1 is significantly upregulated in PDAC tumor tissues and cell lines. **a** The relative HOTAIRM1 expression in PDAC tumor tissues and the adjacent non-tumor tissues determined by RT-qPCR. **b** Analysis of the correlation between the relative level of HOTAIRM1 and the expression of KRAS gene mutation in PDAC tumor tissues. **c** The relative HOTAIRM1 expression in four PDAC cell lines (AsPC-1, SW1990, PANC-1, and Capan-2 cells) and one normal pancreatic cell line (HPDE6–7) measured by RT-qPCR. GAPDH was used as an internal control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

dramatical decrease of tumor cell proliferation at 4 and 5 days post-transfection in both PDAC cell lines ($p < 0.001$, Fig. 2b, c). Meanwhile, the colony numbers presented for siCon and siHOTAIRM1 groups were 114.34 ± 4.85 and 10.21 ± 1.21 for SW1990 cells, and 81.18 ± 2.46 and 9.96 ± 0.82 for PANC-1 cells, respectively (Fig. 2d). Therefore, the number of formative colonies of both PDAC cell lines in siHOTAIRM1 transfected group were significantly less than those in siCon transfected group ($p < 0.001$), confirming the negative effect of HOTAIRM1 knockdown on the colony formation capacity of PDAC cells. Both above methods supported that when HOTAIRM1 was downregulated, PDAC cell proliferation was suppressed.

HOTAIRM1 Knockdown Induces Cell Cycle Arrest at G0/G1 Phase in PDAC Cells

We also performed flow cytometry assay to explore siRNA transfection-induced changes in the cell cycle of PDAC cells. As shown in Fig. 3a, the percentage of cells in G0/G1 phase increased from 48.1% to 67.4% in SW1990 cells ($p < 0.001$); however, the percentage of cells at the S phase decreased from 28.5% to 16.3% ($p < 0.001$). Meanwhile, the similar phenomenon was also occurred in PANC-1 cells ($p < 0.01$, $p < 0.001$; Fig. 3a). These data revealed that HOTAIRM1 knockdown can induce G0/G1 phase arrest and decrease cell population at S phase in SW1990 and PANC-1 cell lines. In consideration of the prior result, we can indirectly conclude that the modulation of HOTAIRM1 on cell proliferation was partly achieved by regulating cell cycle. To investigate the possible mechanism by which HOTAIRM1 regulate PDAC cell cycle, western blot assay was conducted to detect the protein levels of cell cycle regulatory genes, including CDK1, Cyclin D1, and p21. Obviously, a significant reduction of CDK1 and Cyclin D1 protein levels were observed in both SW1990 and PANC-1 cells in response to the knockdown of HOTAIRM1 ($p < 0.01$, $p < 0.001$), while p21 protein level was remarkably upregulated ($p < 0.001$, Fig. 3b), demonstrating that the involvement of HOTAIRM1 in the progress of PDAC cell cycles is achieved by regulating cell cycle related genes, including CDK1, Cyclin D1, and p21.

HOTAIRM1 Knockdown Promotes Cell Apoptosis in PDAC Cells

Following, Annexin V/PI apoptosis assay was performed to explore the apoptosis extent in siHOTAIRM1-transfected PDAC cells. As shown in Fig. 4a, the number of apoptotic tumor cells was increased to a remarkable extent in SW1990 and PANC-1 cells with HOTAIRM1 silence compared with the control cells ($p < 0.001$), with an observed apoptosis rate of 5.76%, 6.16% in the control SW1990 and PANC-1 cells, and of 84.84%, 23.31% in the siHOTAIRM1-transfected

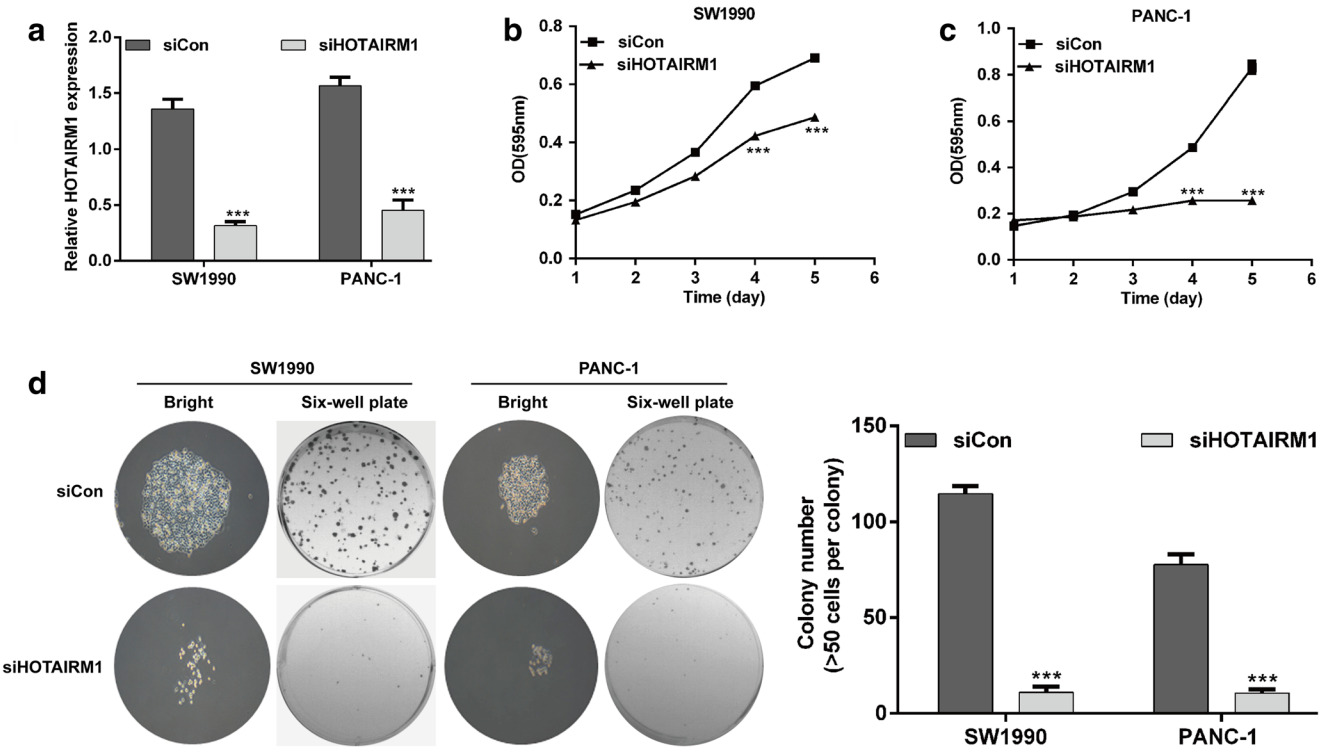

Fig. 2 HOTAIRM1 knockdown attenuates cell proliferation in PDAC cells. **a** Knockdown efficiency of HOTAIRM1 confirmed by real-time PCR in SW1990 and PANC-1 cell lines. **b** The fold change of cell growth rate in SW199 cells transfected with siCon or siHOTAIRM1 for 0, 1, 2, 3, 4, and 5 days detected by MTT assay. **c** The fold change of cell growth rate in PANC -1 cells transfected with siCon or siHOTAIRM1 for 0, 1, 2, 3, 4, and 5 days detected by MTT assay. **d** HOTAIRM1 knockdown reduces the colony number in SW1990 and PANC-1 cells detected by colony formation assay. ***$p < 0.001$

SW1990 and PANC-1 cells, which suggested that downregulation of HOTAIRM1 could promote PDAC cells apoptosis. As we known, cell apoptosis is generally accompanied with the abnormal expression of apoptosis-related targets. Hereon, to further identify the pro-apoptosis mechanism of HOTAIRM1 silence on PDAC cells, the protein expression level of some apoptosis-related genes including Bax, Bad, and Bcl-2 was detected. Compared with the control cells, the protein level of Bax and Bad was markedly upregulated in the cells infected with HOTAIRM1 siRNA ($p < 0.01$, $p < 0.001$), while a significant reduction of Bcl-2 expression ($p < 0.05$, $p < 0.01$; Fig. 4b). Based on these, we can conclude that the knockdown of HOTAIRM1 can promote cell apoptosis by regulating cell apoptosis-related genes, including Bax, Bad, and Bcl-2 in PDAC cell lines.

HOTAIRM1 Knockdown Inhibits Cell Migration in PDAC Cells

Finally, we tried to explore whether downregulation of HOTAIRM1 affect the migratory potential of PDAC cells by transwell migration assay. The results showed that the mean numbers of migrated cells in siCon or siHOTAIRM1 transfected SW1990 cells were 148.23 ± 12.41 and 55.40 ± 3.52, whereas the numbers in PANC-1 cells were 376.50 ± 5.97 and 188.50 ± 5.79, respectively. The number of migrating cells in siHOTAIRM1 group was significantly decreased compared with that in control group significantly ($p < 0.001$, Fig. 5a), suggesting that knockdown of HOTAIRM1 could impair the migration capacity of PANC cells. Also, western blot assay was performed to investigate the protein expression change of migration-related genes including E-cadherin, N-cadherin, and Vimentin in PDAC cells after downregulating HOTAIRM1. Clearly, the expression of E-cadherin was significantly enhanced at the protein level in both SW1990 and PANC-1 cells transfected with siHOTAIRM1 compared with the control cells ($p < 0.001$), while the protein expression levels of N-cadherin and Vimentin were remarkably decreased ($p < 0.001$, Fig. 5b). These data indicated that the suppression of migration capacity in PDAC cells induced by siHOTAIRM1 was achieved by the stimulation of E-cadherin, and the inhibition of N-cadherin and Vimentin.

Discussion

Long noncoding RNAs have been shown to be implicated in many important biological processes, including cell cycle control, epigenetic regulation, and RNA transcription [7–10]. Thus far, abundant lncRNAs exhibit cancer-specific and serve

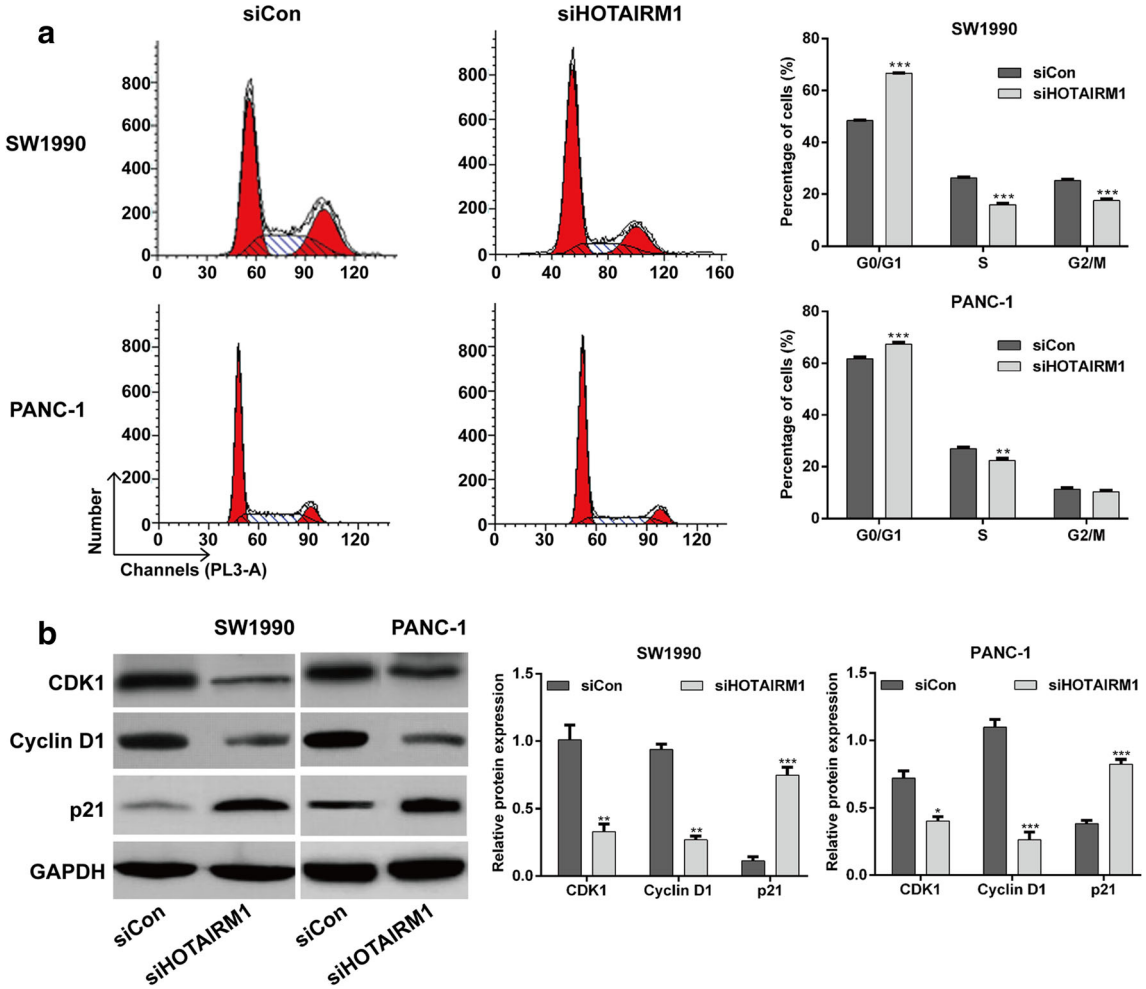

Fig. 3 HOTAIRM1 knockdown induces cell cycle arrest at G0/G1 phase in PDAC cells. **a** HOTAIRM1 knockdown increases cell population at G0/G1 phase but decreases at S phase in SW1990 and PANC-1 cell lines measured by flow cytometry analysis. **b** HOTAIRM1 knockdown reduces the protein expression of CDK1, Cyclin D1, and enhances the expression of p21 determined by western blotting. GAPDH was used as an internal control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

as oncogenes or tumor suppressors in various types of human cancers, which are closely associated with the tumorigenesis, progression, drug resistance, and epigenetic modification [5, 26]. Especially, the important roles of lncRNAs in the development of pancreatic cancer have been confirmed by increasing evidences [14–17]. HOTAIRM1 (HOX antisense intergenic RNA myeloid 1) is a lincRNA located in the HOXA genomic cluster [18], which is found to be aberrantly upregulated or downregulated in some solid tumors. The important roles of HOTAIRM1 in tumor cell proliferation and migration have also been supported by prior studies [19–21]. However, to our knowledge, few researches have paid attention to the potential role of HOTAIRM1 in PDAC development thus far.

In the present study, an obvious higher expression level of HOTAIRM1 in PDAC tissues and cell lines was confirmed when compared to the control samples, which implied that HOTAIRM1 may be participated in the occurrence and development of PDAC. Our finding was consistent with a previous study that revealed a significant augment of HOTAIRM1 expression in PDAC samples compared with the non-tumor tissues using microarray analysis [19]. In fact, dysregulation of HOTAIRM1 expression has been found in some other cancer types. For instance, Wan et al. [27] found that the expression level of HOTAIRM1 in colorectal cancer (CRC) was less than that in matched adjacent tissues. Also, the study of Su et al. [28] indicated that HOTAIRM1 was significantly overexpressed in the basal-like subtype of breast cancer. PDAC progression also refers to the accumulation of both germline and somatic gene mutations, such as KRAS, p16, p53 and SMAD4 [29, 30]. Of which, KRAS-activating mutations play a critical role in PDAC growth and development because that mutated KRAS can contribute to the constant activation of cancer-relevant signaling pathways, such as RAS, Wnt, Notch, and Hedgehog pathway [31–35]. KRAS has several types of mutation status, which exerts different impact on clinical outcome in pancreatic cancer [36]. Mutations in codon 12 of KRAS occur the most frequently in pancreatic cancer [25]. A specific mutation G12D

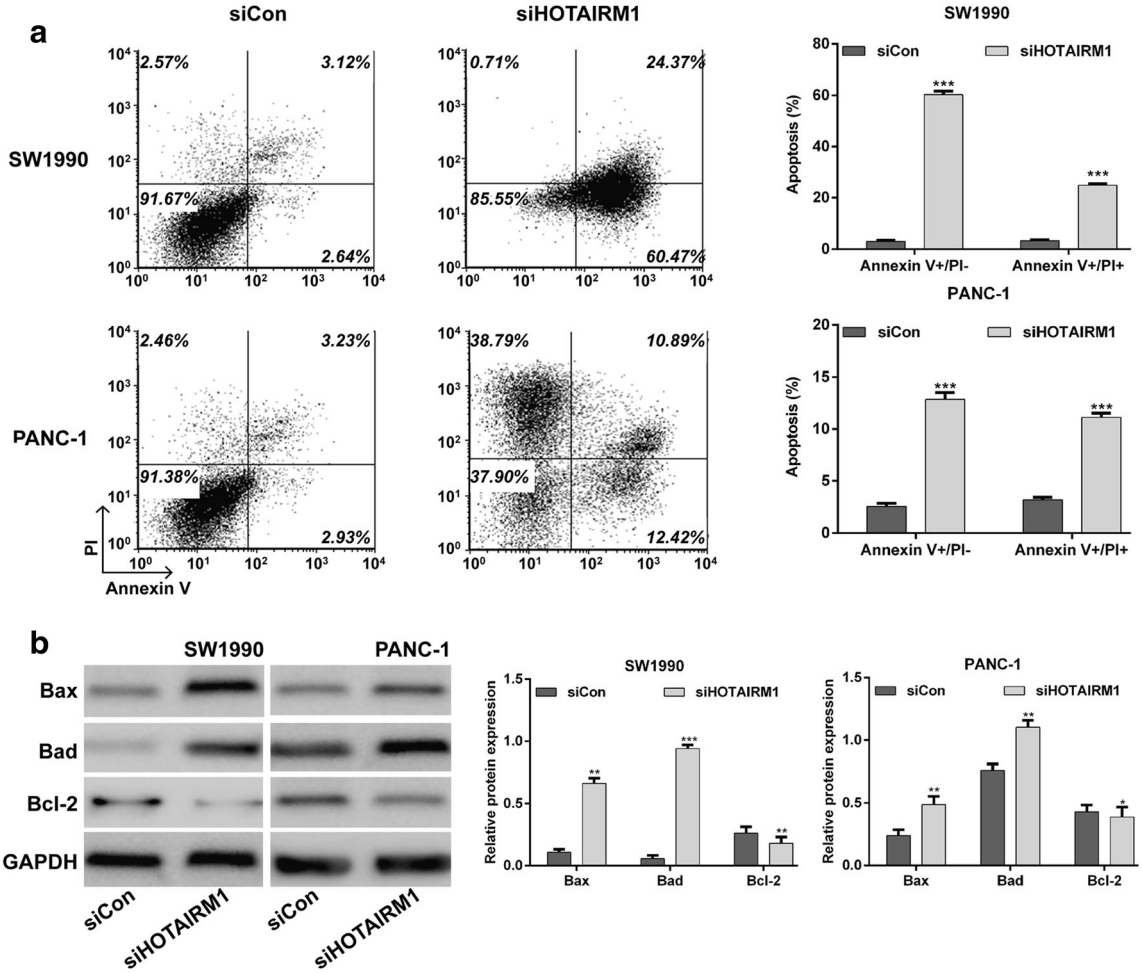

Fig. 4 HOTAIRM1 knockdown promotes cell apoptosis in PDAC cell. **a** HOTAIRM1 knockdown increases the number of apoptotic cells in SW1990 and PANC-1 cell lines measured by flow cytometry analysis. **b** HOTAIRM1 knockdown increases the protein expression of Bax and Bad, and reduces Bcl-2 protein level determined by western blotting. GAPDH was used as an internal control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

demonstrated significant association with shorter OS (overall survival) and DFS (disease free survival) in pancreatic cancer [36]. This study revealed a positive relationship between the relative level of HOTARM1 and the gene expression of G12 V and G12D in PDAC tissues, which may indicate that HOTARM1 participates in the regulatory progress of KRAS mutation induced-PDAC development.

To further confirm the impact of HOTAIRM1 on PDAC development, a series of in vitro functional studies were conducted in the following experiments. First, we transfected siHOTAIRM1 into PANC cell lines SW1990 and PANC-1 to obtain the successful HOTAIRM1 knockdown model. As we known, cell proliferation is an important component of tumor progression [37]. Therefore, MTT and clone formation assays were first performed to evaluate the impact of HOTAIRM1 on PDAC cells proliferation. We found that HOTAIRM1 silence contributed to a dramatical decrease of tumor cell proliferation and the number of formative colonies compared with the control group, implying the critical role of HOTAIRM1 in the viability and proliferation of PDAC cells. These data indicated that HOTAIRM1 knockdown might be responsible for the inhibition of PDAC growth and subsequently block PDAC development. Our result was diametrically opposed with the phenomenon previously shown in colorectal cancer that HOTAIRM1 downregulation significantly accelerated cancer cell proliferation [21]. The cell cycle is a continuous progress consisting of four phases, namely G0/G1, S, G2, and M phases [38]. A grow list of anti-tumor drugs has been reported to induce cell death by arresting the cell cycle at different phases [39–41]. Thus, we speculated that the inhibition of HOTAIRM1 knockdown on cell proliferation in PDAC cells is achieved by influencing cell cycle distribution. To verify this, cell cycle analysis was performed by flow cytometry. As expected, we found that the downregulation of HOTAIRM1 induced a large amount of cell population arrest at G0/G1 phase in both SW1990 and PANC-1 cell lines, while cell population at S phase decreased significantly. These findings suggested that the change of cell proliferation induced by

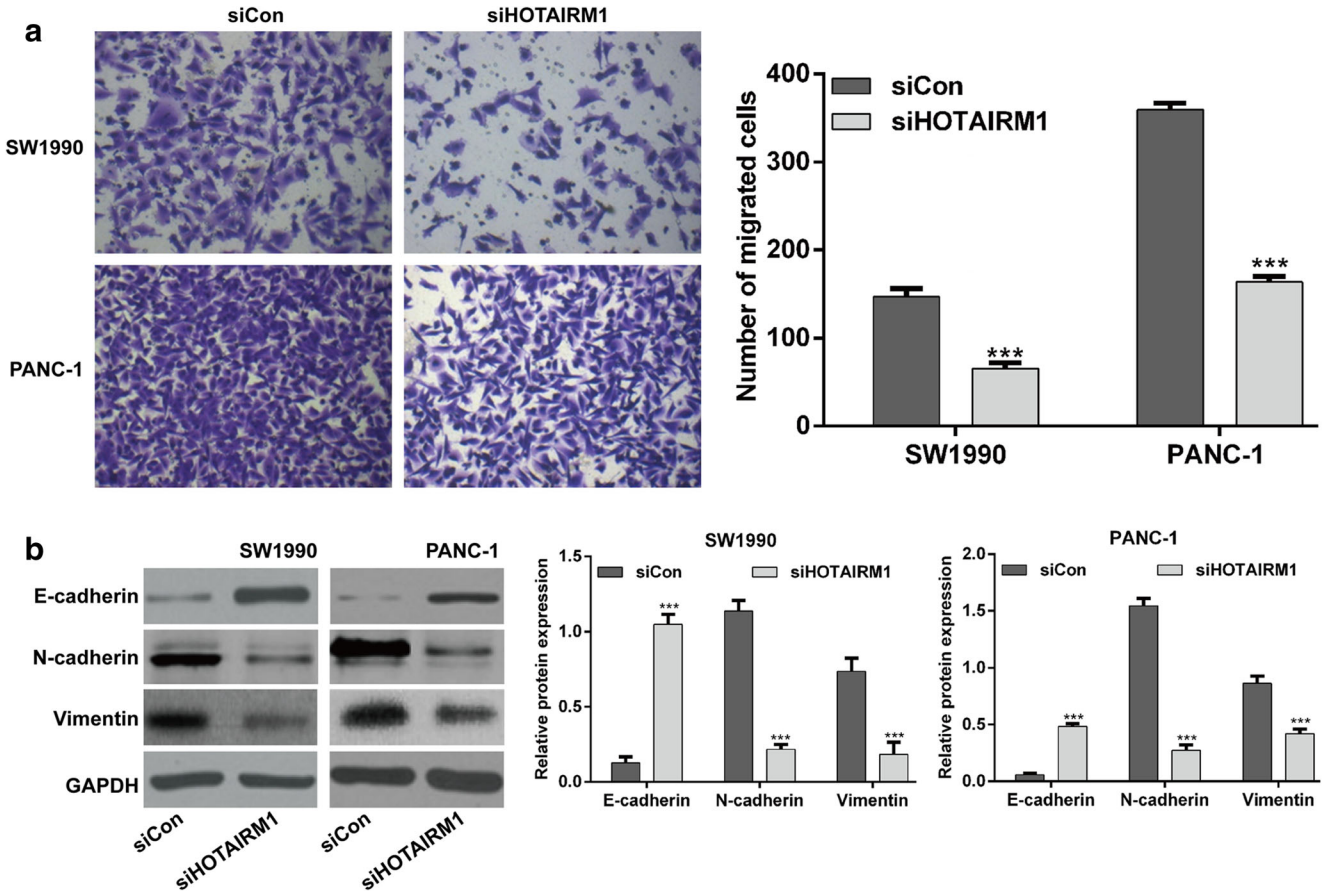

Fig. 5 HOTAIRM1 knockdown inhibits cell migration in PDAC cells. **a** The migration ability of SW1990 and PANC-1 cell lines transfected with siCon or siHOTAIRM1 assessed by transwell migration assay. **b** HOTAIRM1 knockdown increases the protein expression of E-cadherin, and decreases the expression of E-cadherin and Vimentin determined by western blotting. GAPDH was used as an internal control. ***$p < 0.001$

HOTAIRM1 was achieved by regulation of cell cycle distribution. The effect of HOTAIRM1 on cancer cell cycle progression has been explored by the study of Zhang et al. [42], who found that HOTAIRM1 knockdown could preserve NB4 human promyelocytic leukemia cells remain at S phase during myeloid maturation. The data of cell cycle arrest was consistent with the result of western blot analysis, which showed that HOTAIRM1 knockdown reduced the protein expression of CDK1 and cyclin D1 but increased p21 protein expression in both SW1990 and PANC-1 cells. Cyclin D1 and p21 are two major regulatory proteins that control G1 checkpoint in the cell cycle. Cyclin D1 is G1-S transition promoter; p21 is an important CDK inhibitor, which acts as a G1 gatekeeper and can induce cell cycle progression arrest at G1 phase [43, 44]. Based on these, it can be concluded that the inhibition of PDAC cell proliferation caused by the downregulation of HOTAIRM1 could be at least contributed to the arrest of cell cycle.

Apoptosis, namely the inactivation of programmed cell death, is central to the tumor development [45]. In the result of cell apoptosis assay, we found that HOTAIRM1 silence is accompanied with a higher apoptosis rate of PDAC cells, suggesting the promotion of the downregulation of HOTAIRM1 on PDAC apoptosis. Our observation was supported by Wan et al. [27], who paid attention to the role of HOTAIRM1 in the apoptosis of colorectal cancer cells. The above phenomenon was also reflected in the protein expression changes of several famous apoptosis-related genes, including pro-apoptosis protein Bax and Bad, as well as anti-apoptosis protein Bcl-2. Compared with the control cells, the protein level of Bax and Bad was markedly upregulated in the cells infected with HOTAIRM1 siRNA, while Bcl-2 expression was significantly reduced. Therefore, the promotion of HOTAIRM1 knockdown on PDAC cell apoptosis is achieved by regulating cell apoptosis-related genes including Bax, Bad, and Bcl-2. We also performed the transwell migration assay to explore whether downregulation of HOTAIRM1 affects the migratory potential of PDAC cells. We observed that the number of migrating cells in siHOTAIRM1 group was much more than that in control group, suggesting the negative effect of HOTAIRM1 downregulation on cancer cell migration. To further explore the effect of HOTAIRM1 on the migration capacity of cancer cells, western blot analysis was used to examine the content of E-cadherin, N-cadherin, and Vimentin in siHOTAIRM1 transfected PDAC cells. E-cadherin is a

famous cell-cell adhesion protein, which functions as a tumor-suppressor in tumor invasion of many malignancies [46]. The development of cancers could interfere and destroy E-cadherin adhesion system via varying routes, then contribute to the migration of cancer cells [47]. N-cadherin is associated with an enhanced invasive potential in tumor cells [48]. The important stimulative effect of Vimentin in cell migration of various cancer cells has been confirmed by a grow list of evidence [47, 49]. This finding further confirmed that HOTAIRM1 downregulation can suppress the migration of PDAC cells by the regulation of E-cadherin, N-cadherin, and Vimentin expression level.

Taken together, HOTAIRM1 is significantly up-regulated in PDAC tissues and cell lines, and its knockdown correlates with the suppression of cell proliferation and migration, as well as the enhancement of cell apoptosis by regulating the related-genes expression. The exact mechanism of HOTAIRM1 involving PDAC development remains to be further explored in future study. Overall, our study reveals that HOTAIRM1 could be a potential therapeutic target of PDAC, which may lay the foundation for molecular treatment of PDAC at genetic level.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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