Iodine-125 inhibits invasion and metastasis of non-small cell lung cancer cells by downregulating the long noncoding RNA AK213263

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Abstract

Background:The interstitial implantation of iodine-125 (^{125}I) by thoracotomy represents a new minimally invasive method for the treatment of lung cancer. Plasmacytoma variant translocation1 (AK213263), which is a long non-coding RNA (lncRNA), is increased in non-small cell lung cancer (NSCLC) tissues. AK213263 promotes the proliferation of NSCLC cells and is, therefore, an important indicator for early diagnosis and accurate prognosis of NSCLC. However, the detailed molecular mechanism by which ^{125}I and AK213263 affect the pathophysiology of NSCLC remains vague. **Methods:** A total of thirty-one pairs of NSCLC tissue and corresponding adjacent healthy lung tissue were collected from Second Hospital of Tianjin Medical University between March 2015 and September 2016. Using transwell and wound healing assays, together with western blotting and xenograft mouse model, we demonstrated that ^{125}I significantly inhibits NSCLC cell invasion and metastasis by downregulating AK213263. **Results:**we show that AK213263 was significantly downregulated by ^{125}I treatment in the NSCLC cell lines tested. In a xenograft mouse model, small interfering AK213263 (si-AK213263) effectively reversed the effect of ^{125}I in reducing tumor size. **Conclusion:** ^{125}I inhibits NSCLC cell invasion and metastasis by downregulating AK213263. We elucidated the molecular mechanism underlying the oncogenic role of AK213263, which provides a lncRNA-directed target for improved treatment of NSCLC.

Introduction

Lung cancer is the primary source of malignant tumors that result in death; it is estimated that lung cancer malignancies cause 1.59 million deaths per year worldwide [1, 2]. About 85% of lung cancers are non-small cell lung cancers (NSCLC) including squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. In recent years, great progress has been made in the diagnosis and treatment of NSCLC, but the 5-year survival rate is still less than 20% due to poor or delayed prognosis and cancer drug resistance. Unfortunately, 57% of lung cancers are diagnosed at an advanced stage because early stages of the disease are typically asymptomatic [6, 7]. Up to 80% of patients with NSCLC have missed the opportunity for surgery at the time of diagnosis. For these patients, radiotherapy and chemotherapy are very important treatments [8, 9, 10]. In recent years, Iodine-125 (^{125}I) brachytherapy has been widely used, with good results, to treat patients whose tumors cannot be surgically removed or for patients who have refused surgery. However, the mechanism by which ^{125}I affects NSCLC is unclear which limits its use. Therefore, it is essential to determine the molecular mechanisms that cause the aggressive behavior of lung cancer and to identify new biomarkers that are necessary for early disease detection, prevention, and treatment. Long non-coding RNAs (lncRNAs) are comprised of more than 200 nucleotides. They participate in multiple aspects of cellular biology such as regulating gene expression, chromatin structuring, genomic imprinting, developmental processing, epigenetic control, and embryonic stem cells [11, 12, 13, 14]. Increasing evidence shows that various lncRNAs are involved in the proliferation, migration, and invasion behavior of NSCLC cells. For instance, MIAT enhances proliferation and metastasis of NSCLC by activating MMP9 [15]. SNHG12 leads to multi-drug resistance by muting miR-181a and activating the MAPK/Sug pathway in NSCLC [16]. The expression levels of HOTTIP are directly

related to the progression of NSCLC and predicts patient outcome [17]. Epigenetic silencing of p21 expression by SNHG20 leads to the proliferation and migration of NSCLC cells [18], and AK213263 knockdown increases radiosensitivity in NSCLC by sponging miR-195 [19].

In this study, we first determined the expression levels of a novel lncRNA—AK213263—in NSCLC tissues compared to its expression levels in paired adjacent tissues and NSCLC-derived cell lines. Then we determined the expression levels of AK213263 in NSCLC tissues before and after radiotherapy. Using loss- and gain-of-function assays, we evaluated the effects of AK213263 on the biological functions of NSCLC cells, including cell proliferation, the cell cycle, and migration and invasion during radiotherapy.

Materials And Methods

Patients and tissues samples

A total of thirty-one pairs of NSCLC tissue and corresponding adjacent healthy lung tissue were collected from Second Hospital of Tianjin Medical University between March 2015 and September 2016. All tissue samples were kept at -80°C. Table 1 shows the clinical-pathological features of patients with NSCLC. Informed consent was signed by each participant according to the recommended protocols. The experiments were approved by the Medical Ethics Committee of the Second Hospital of Tianjin Medical University.

Cell culture

The cell lines NSCLC A549 and H1703 used in this study were purchased from Tongpai Biotechnology Co., Ltd. (Shanghai, China), cultured in a humidified incubator at 37°C with 5% CO₂ in RPMI 1640 medium with 10% fetal calf serum (Invitrogen, Carlsbad, CA).

Cell viability assay

Cells were uniformly added to a 96-well plate at 3×10^4 A549 or H1703 cells per well. All cells were passaged every 4 days and the medium was replaced daily. Cells were used for experiments during the log phase. Cell proliferation was determined 24, 48, 72, and 96 h after transfection. The cell viability of A549 or H1703 cells was calculated with a Cell Counting Kit-8 (CCK8; Boster Biological Technology, Wuhan, China) according to manufacturer instructions.

Colony formation assay

Approximately 24 hours after transfection, we seeded 500 cells into 6-well plates and cultured them for 14 days. Subsequently, cells were fixed in methanol for 15 min and stained with 0.1% crystal violet. The number of colonies was manually counted in ten fields of view and the mean values were calculated.

Transwell cell invasion and metastasis experiment

We determined the migration ability of A549 and H1703 cells in a Transwell Chamber (BD Biosciences). For cell invasion assays, we precoated the upper compartments of the chambers with a matrix gel (BD,

CA, USA) 4 h before seeding the cells. The cells were plated in the upper chambers of Matrigel-coated wells in 24-well plates and incubated for 24 h. We added a chemoattractant comprising 20% fetal bovine serum to the lower chambers to induce cell invasion. The cells attached to the bottom of each membrane were fixed with methanol, stained with Crystal Violet, and counted under an inverted microscope (Nikon, Japan).

Cell cycle analysis

Approximately 24 h after transfection, we harvested 1×10^6 cells and fixed them in ice-cold 75% ethanol. The cells were treated for 30 min with 2 µg/mL bovine pancreatic RNase (Sigma, USA) before cell cycle analysis and subsequently incubated for 20 min in 20 µg/mL propidium iodide (Sigma, USA). We performed cell cycle analysis on a FACSCalibur system (BD Biosciences, NJ, USA).

Western blot assay

We extracted proteins from A549 and H1703 cells. Protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% SDS gel, then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, CA, USA). Nonspecific binding was blocked by incubating the membranes with 5% nonfat milk for 2 h at 20 – 25°C. We incubated the membranes overnight at 4°C with CDK16 and GAPDH (Abcam, Cambridge, England) primary antibodies. After washing three times with a mixture of Tris-buffered saline and Tween 20, we incubated the PVDF membranes with horseradish peroxidase-conjugated secondary antibody for 2 h at 37°C. Finally, we examined the proteins using enhanced chemiluminescence detection kits (Millipore, MA, USA).

Xenograft model

A549 cells were resuspended in RPMI 1640 medium and subcutaneous injection of $3 \times 10^6/100$ µL cells in posterior flanks of nude mice was performed. We measured the length (L) and width (W) of each tumor every 3 d with calipers. After 28 days, we excised the tumors from the sacrificed mice and weighed them.

Statistical analysis

We performed all statistical analyses and created all graphs with the Statistical Package for Social Sciences software (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY). Quantitative data were described by mean $\pm$ standard deviation (SD), one-way analysis of variance was used for comparison between groups, and a student's t-test was used for comparison between two groups. A P-value < 0.05 was set as demonstrating statistical significance.

Results

Up-regulation of AK213263 expression in NSCLC patient tissues

We analyzed the expression levels of AK213263 in NSCLC and adjacent healthy tissues using quantitative PCR (qPCR). The expression of AK213263 was markedly higher in the NSCLC tissues than in the surrounding normal tissue (Fig. 1A). Next, we detected the AK213263 expression levels of nine patients with NSCLC who had received ^{125}I brachytherapy. The results demonstrated that AK213263 expression was significantly reduced after ^{125}I brachytherapy (Fig. 1B). We summarized the clinical features of the 31 patients with primary NSCLC in Table 1. Statistical analyses showed that higher expression levels of AK213263 in NSCLC were significantly associated with tumor size, TNM stage, and lymph node infiltration. The expression levels of AK213263 had no significant correlation with other characteristics such as gender or age. Furthermore, the results of a Kaplan Meier-plotter showed that the higher the expression level of AK213263 in patients with NSCLC, the poorer the prognosis. (Fig. 1C).

AK213263 knockdown reversed the effects of ^{125}I treatment on the proliferation and cell cycle of NSCLC cells

We detected the expression levels of AK213263 in several NSCLC cell lines (Fig. 2A), then chose the A549 and H1703 cell lines for our study. The A549 and H1703 cells were transfected with small interfering RNAs (siRNAs) of AK213263 or control siRNA for 48 h. We then evaluated the AK213263 expression levels in the A549 and H1703 cells to confirm knockdown by the siRNAs. The results showed that si-AK213263-1 and si-AK213263-3 reduced AK213263 expression levels by more than three times (Fig. 2B). We performed a CCK8 analysis every 24 hours and plotted a proliferation curve based on the results. The cell viability of both cell lines at 72, 96 and 120 h increased significantly due to knockdown of AK213263. (Fig. 2C), while colony formation rates were similar in those groups. AK213263 knockdown significantly reversed the inhibitory effect of ^{125}I on the colony formation of A549 and H1703 cells (Fig. 2D). Cell cycle analysis by flow cytometry showed that the S phase cells of A549 and H1703 cells were significantly arrested after ^{125}I treatment. AK213263 knockdown reversed the effect of ^{125}I treatment (Fig. 2E). To further investigate the specific mechanism by which AK213263 regulates the cell cycle of NSCLC, we next examined the expression levels of several proteins. Western blot results indicated that ^{125}I induced S-phase cell cycle arrest by reducing p21, cyclin D3, and CDK2 levels while elevating cyclin A2 levels. AK213263 knockdown reversed this regulatory effect of ^{125}I (Fig. 2F, G).

AK213263 knockdown reversed the effect of ^{125}I on the migration and invasion of NSCLC cells

Transwell and wound healing experiments were carried out to investigate migration and invasion of NSCLC cells. ^{125}I treatment notably decreased the number of migrating and invading A549 and H1703 cells, whereas AK213263 knockdown reversed the inhibitory effect of ^{125}I (Fig. 3A, B). We noticed similar trends in the wound healing experiments. Knockdown of AK213263 reversed the inhibition of ^{125}I on A549 and H1703 wound healing (Fig. 3C).

^{125}I regulates the expression of AK213263 and the PI3K/AKT and Wnt/ β -catenin pathways

We determined expression levels of several proteins related to epithelial-mesenchymal transition (EMT) in order to further explore the mechanism by which ^{125}I inhibits proliferation, migration, and invasion of NSCLC cells. Western blot results indicated that treatment with ^{125}I reduced the expression of vimentin, MMP2, MMP9, and N-cadherin while increasing the expression of E-cadherin and ZO-1. AK213263 knockdown via siRNA transfection significantly reversed this regulatory effect of ^{125}I (Fig. 4A). We confirmed changes in the expression levels of E-cadherin and vimentin by immunofluorescence. AK213263 knockdown reversed the effects of ^{125}I (Fig. 4B). We also detected the expression levels of several proteins involved in the PI3K/AKT and Wnt/ β -catenin signaling pathways. We found that ^{125}I suppressed the phosphorylation of GSK3 β , mTOR, AKT, and β -catenin. As expected, AK213263 knockdown promoted the phosphorylation of mTOR, GSK3 β , AKT, and β -catenin (Fig. 4C).

AK213263 knockdown reverses inhibitory ^{125}I effect on the growth of NSCLC cells

We carried out a xenograft assay to investigate the growth of NSCLC cells *in vivo*. ^{125}I treatment notably reduced the growth of A549 cells, whereas AK213263 knockdown reversed this inhibitory effect (Fig. 5A-C).

Discussion

Metastatic grade III/IV NSCLC recurrent tumors are usually inoperable. In such cases, surgical treatment is inadvisable, either because of widespread metastasis or the poor physical condition of the patient [20]. Instead, palliative radiotherapy and chemotherapy are recommended. However, the efficacy of external beam radiation therapy is minimal and the length of survival time often short because it is difficult to provide adequate doses of radiation without damaging adjacent healthy tissues [21, 22].

A ^{125}I seed implantation is a form of internal radiation therapy that has been widely applied to treat various malignancies, including prostate malignancy, head and neck tumors, and hepatocellular carcinoma [23, 24]. ^{125}I seed implantation provides a sufficient dose of radiation to tumors that are not easy to reach with external radiation. The procedure provides continuous radiation at a far higher total dose and has a smaller effect on the surrounding tissues and organs [25].

^{125}I particles arrested cells in the G2/M phase of the cell cycle and caused apoptosis in Panc-1 cells. Furthermore, anti-epidermal growth factor receptor monoclonal antibody C225 sensitized with irradiation from ^{125}I seeds causes apoptosis in colorectal cancer cells [26, 27]. However, the precise mechanism by which ^{125}I inhibits the growth of NSCLC is still vague. We found that a dose of 6 Gy for ^{125}I radiation therapy significantly inhibited NSCLC cell proliferation and induced arrest of cell cycle in the S phase. ^{125}I treatment also notably attenuated the migration and invasion of NSCLC cells.

EMT is the process by which epithelial cells morphologically transition to a mesenchymal fibroblast phenotype and subsequently migrate. During EMT, epithelial cells lose cell polarity and epithelial

phenotypes such as attachment to the basement membrane. Cells also obtain higher interstitial phenotypes such as migration and invasion, anti-apoptotic ability, and the ability to degrade extracellular matrix [28]. EMT plays an important role in several different processes, including embryonic development, fibrosis, chronic inflammation, and the oncogenesis of lung cancer [29, 30, 31, 32, 33, 34]. Classical EMT is characterized by the loss of epithelial markers, such as E-cadherin and claudin-1, and the upregulation of mesenchymal markers, such as vimentin and N-cadherin [35]. E-cadherin is a phenotypic marker of epithelial cells and plays an important role in cell-cell interactions. Down-regulation of E-cadherin causes a decrease in cell adhesion [36]. Normal mesenchymal cells possess vimentin-specific expression, and vimentin expression is up-regulated in lung cancer cells.

We reported that ^{125}I treatment reduced expression levels of MMP2, vimentin, N-cadherin, and MMP9 while increasing levels of N-cadherin and ZO-1. AK213263 knockdown via siRNA transfection significantly reversed this regulatory effect of ^{125}I .

EMT in NSCLC involves a variety of signaling pathways including TGF- β , c-Jun N-terminal kinase, Wnt/ β -catenin and Slug [37, 38, 39, 40]. The PI3K/AKT/mTOR signaling pathway is essential for cell proliferation, survival, neovascularization, and tumor growth. It takes part in the EMT process and regulates the migration and invasion of NSCLC cells [41, 42, 43]. A large family of secreted lipid-modified glycoproteins, the Wnt proteins, activates intracellular pathways and is involved in the proliferation, differentiation, migration, and apoptosis of cells [44]. The Wnt/ β -catenin pathway has been found to play a crucial role in the development and progression of NSCLC [45, 46]. Activation of the Wnt/ β -catenin pathway promotes the activation of Slug, E47, Snail, Twist, ZEB1, and ZEB2, which are EMT-related transcription factors that promote EMT induction [47]. We investigated alterations to the Wnt/ β -catenin and PI3K/AKT signaling pathways and found that ^{125}I can inhibit the phosphorylation of AKT, mTOR, GSK3 β , and β -catenin. AK213263 knockdown enhanced phosphorylation of AKT, mTOR, GSK3 β , and β -catenin.

Studies on the mechanisms of cancer in humans have found that lncRNA dysregulation plays a crucial role in cancer disease progression and metastasis [48, 49]. For example, studies have found that some lncRNAs are involved in the development and progression of lung cancer such as SCAL1, ANRIL, MALAT1, H19, and HOTAIR [50]. We demonstrated that ^{125}I inhibits NSCLC cell proliferation, invasion, and migration by downregulating AK213263 expression. However, whether ^{125}I regulates the expression of AK213263 at the transcriptional level, through a certain degree of post-transcriptional competition for endogenous RNA (ceRNA), or by directly targeting AK213263, remains unclear. To some up, these findings may broaden our understanding of the potential molecular mechanisms of ^{125}I brachytherapy for sensitization and provide new insights for improving the effectiveness of treatment.

Declarations

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper

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Table

Table legends

Characteristics	Group	Total n=84	AK213263		P value
			High (n=45)	Low (n=39)	
Gender	Male	58	27	31	0.458
	Female	26	14	12	
Age	≥ 60	30	14	16	0.279
	<60	54	29	25	
Smoking	Yes	56	30	26	0.692
	No	28	16	12	
Lymph nodes metastasis	Yes	40	18	22	0.018
	No	44	24	20	
TNM stage	I/II	36	27	9	0.012
	III/IV	48	14	34	

Figures

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