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Original Article

A novel long noncoding RNA IRAIN regulates cell proliferation in non small cell lung cancer

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Abstract: Long non-coding RNAs (lncRNAs) are a novel class of RNA molecules defined as transcripts longer than 200 nucleotides that lack protein coding potential. LncRNA IRAIN has been verified that it is related to acute myeloid leukemia (AML) and breast cancer. However, there was no study to clarify whether it is involved in non-small cell lung cancer (NSCLC). Here, we demonstrated IRAIN as a tumor promoter in NSCLC. Its expression level was remarkably upregulated in NSCLC tissues and connected with tumor size and smoking status. Knockdown of IRAIN suppressed NSCLC cells proliferation in vitro. These data identify IRAIN as a novel promoting gene, which plays a vital role in tumorigenesis of NSCLC.

Keywords: Long non-coding RNA, IRAIN, non-small cell lung cancer, gene therapy, A549

Introduction

NSCLC is the leading cause of cancer-related deaths worldwide [1]. The incidence of lung cancer is still increasing remarkably in China, in both urban and rural areas [2]. No matter how great efforts and progressions have been made in the study of lung cancer in recent decades, the molecular mechanism of lung cancer remains unclear.

Insulin-like growth factor 1 (IGF-1) signaling mediated by IGF1R is an important growth regulatory pathway, enhanced activation of which is thought to play a crucial role in cancer cell proliferation, migration, and apoptosis [3-5]. IGF1R expresses in many types of cancer cells, including NSCLC [6]. Current studies find that IRAIN plays an important role in the regulation of IGF-1 signaling. IRAIN is downregulated both in leukemia cell lines and blood obtained from high-risk AML patients. There is no large open reading frames can be identified using software prediction programs in IRAIN transcript which is a 5.4 kb noncoding RNA. IRAIN is within the IGF1R locus, transcribed from an intronic promoter in antisense orientation as compared to the IGF1R coding mRNA and in a parent-of-origin specific manner. There is a single nucleotide polymorphism (SNP) rs8034564: the 'G' genotype was favorably imprinted over the 'A' genotype imbalanced expressing in the two parental alleles. In addition, it is also downregulated in breast cancer as an imprinted gene [7, 8].

However, the function of IRAIN in other malignancies remains unclear. A large number of studies have demonstrated that the IGF1R pathway is frequently dysregulated in lung cancer. It is necessary to clarify IRAIN whether it is also related to lung cancer. In this study, we characterized the differential expression of IRAIN in a case-control study of lung cancer samples and normal tissue specimens. Moreover, we explored if IRAIN takes part in cancer cell proliferation, apoptosis and migration in A549 cells.

Materials and methods

Patients

47 NSCLC tissues and their adjacent non-tumor tissues were collected from patients in Jiangsu Province hospital from 2013-2014. All speci-

Table 1. Correlation between IRAIN expression and clinicopathological parameters of lung adenocarcinoma patients

Variable	Number (47)	LncRNA IRAIN expression		*P* value
		low	high	
Age (years)				0.450
<60	12	7	5	
≥60	35	16	19	
Gender				0.450
Male	35	16	19	
Female	12	7	5	
Smoking				0.029*
Former/Present	24	8	16	
Never	23	15	8	
Tumor size (cm)				0.002**
<3	11	14	4	
≥3	36	9	20	
Histological type				0.059
Squamous cell ca.	25	9	16	
Adenoca.	22	14	8	
Tumor stage				0.587
I-II	33	17	16	
III	14	6	8	
Grade				0.846
I-II	17	8	9	
III	30	15	15	
Lymph nodes metastasis				0.654
No	25	13	12	
Yes	22	10	12	

*P<0.05, **P<0.01.

mens performed no local or systemic treatment before collecting. This research was approved by the Research Ethics Committee of Jiangsu province Hospital, and written informed consent was obtained from all patients. All tissue samples were excised and immediately stored in liquid nitrogen until detected. Histological type and tumor-node-metastasis (TNM) classifications were determined according to the National Comprehensive Cancer Network (NCCN) criteria. These patients' clinicopathologic characteristics were shown in **Table 1**.

Cell culture

The human pulmonary adenocarcinoma cell line A549 was obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). Cells were incubated at 37.5C with 5% CO_2 in Dulbecco's modified Eagle's medium (HyClone, UT, USA), supplemented with 10% fetal bovine serum (Gibco, Auckland, New Zealand), 100 U/mL penicillin, and 100 mg/mL streptomycin.

Transfection

IRAIN siRNA and its corresponding negative control (NC) (Invitrogen) were transfected into cells for 24 h for loss-of-function experiments using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The NC scrambled oligonucleotide does not encode for any known lncRNA. Transfection efficiency was verified by SYBR Green real-time PCR detection of IRAIN expression. IRAIN siRNA and its corresponding NC sequences were listed in **Table 2**.

RNA isolation, reverse transcription (RT), and quantitative PCR (qPCR)

Total RNA was extracted from frozen tissue samples or cells using TRIzol (Invitrogen) according to the manufacturer's protocols. For reverse transcription, 500 ng total RNA was converted to cDNA using a Reverse Transcription Kit (Takara, Dalian, China) according to the manufacturer protocol. Real-time PCR analysis was conducted with Power SYBR Green (Takara, Dalian, China) in the ABI PRISM 7900HT. The primers were shown in **Table 2**. Ct values were calculated using SDS 2.4 software. IRIAN expression was normalized to that of GAPDH with the 2^-ΔCt method.

Cell proliferation assay

To assess the effect of IRAIN expression on A549 cells proliferation, Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used for assessing cell viability. For this assay, 5 × 10^3 cells/well in 100 UL culture medium were seeded in 96-well plates and incubated for 24 h after transfection. WST-8 (10 UL) was added to each well. The optical density at 450 nm was measured after 1 hour of incubation at 37.0°C using a Tecan Infinite M200 Multimode microplate reader (Mechelen, Belgium). Each experiment was performed 3 times.

Table 2. Sequences of primers for qRT-PCR and IRAIN related sequence

Name	Sequences (5' to 3')
Primers used for mRNA detection	
IRAIN (Forward)	CGACACATGGTCCAATCACTGTT
IRAIN (Reverse)	AGACTCCCCTAGGACTGCCATCT
GAPDH (Forward)	GAAGAGAGAGACCCTCACGCTG
GAPDH (Reverse)	ACTGTGAGGAGGGGAGATTCAGT
Interference sequences	
IRAIN siRNA (Forward)	GGCAACCAUUACUCACCAATT
IRAIN siRNA (Reverse)	UUGGUGAGUAAUGGUUGCCTT
IRAIN siRNA-nc (Forward)	UUCUCCGAACGUGUCACGUTT
IRAIN siRNA-nc (Reverse)	ACGUGACACGUUCGGAGAATT

Cell migration assays

For the cell migration assay, 5 × 10^4 cells after transfection were resuspended in 100 μl serum-free DMEM and seeded in the top portion of a Transwell chamber with 8-mm pores (Millipore). The transwell chambers were placed in the 24-wells. The lower portion of the chamber contained 600 ul 10% FBS DMEM as a chemoattractant. After incubated at 37.0°C in 5% CO_2 for 24 h, the cells on the top of the membrane representing that those cells did not migrate the barrier were removed with cotton swabs. After fixed with 95% methanol, the migrating cells at the bottom of insert were stained with 0.2% Crystal Violet Staining Solution (Beyotime), photographed and counted under ×20 magnification. Five random fields were analyzed for each chamber. Each experiment was repeated 3 times.

Cell cycle and apoptosis assays

For the cell cycle analysis, A549 cells transiently transfected with IRAIN siRNA or NC for 24 h were washed with PBS and fixed with ice-cold 70% ethanol at -20°C. Fixed cells were rehydrated in PBS and stained with PI using the Cycle TESTTM PLUS DNA reagent kit (BD Biosciences) according to the manufacturer's protocol. The percentages of G0/G1, S, and G2/M cells were counted and compared. Experiments were performed in triplicate. APC-conjugated Annexin V (Annexin V-APC) Kit (Cat No: 550474, BD Pharmingen, USA) and Propidium Iodide Staining Solution (Cat No: 556463, BD Pharmingen, USA) were used to detect the percentage of apoptotic cells according to the manufacturer instructions. For the apoptosis analysis, A549 cells transfected with IRAIN siRNA or NC for 24 h were added with Annexin V-APC and PI. Viable, dead, early apoptotic and late apoptotic cells were identified with a flow cytometer. Data were expressed as mean ± SEM of three independent experiments.

Statistical analysis

All values were calculated by the SPSS statistical software (version 13.0, SPSS, IBM, Armonk, NY, USA) and Graphpad 5.0. They were shown as mean ± SD. To explore the correlation between IRAIN expression and clinical pathological features, two-tailed Chi-squared test was employed. Differences between the groups were compared using the Student's t test. Results were considered significant when P values were <0.05.

Results

Increased expression of IRAIN in NSCLC

To explore the role of IRAIN in NSCLC, we analyzed 47 paired clinical NSCLC tissues and their adjacent normal counterparts for IRAIN expression using qRT-PCR. IRAIN expression was significantly increased in tumor samples as shown in **Figure 1A** (P<0.01). Then we assessed its clinical significance by evaluating the correlation between its expression and clinicopathological parameters (i.e., age, gender, smoking status, tumor size, histological type, tumor stage, tumor grade and lymph node metastasis). From the results shown in **Figure 1B** and **1C**, we found that IRAIN expression level in NSCLC was significantly correlated with smoking status (P<0.05) and tumor size (P<0.05). However, there was no association between IRAIN expression and other clinical characters such as age, gender, histological type, tumor stage, grade and lymph node metastasis in NSCLC (**Table 1**).

Knockdown of IRAIN suppressed proliferation of A549 cells

To explore the function of IRAIN in the regulation of cell proliferation, small interfering RNA (siRNA) was transfected into A549 cells. IRAIN expression was decreased by 41.15% in A549 cells with IRAIN siRNA transfection compared

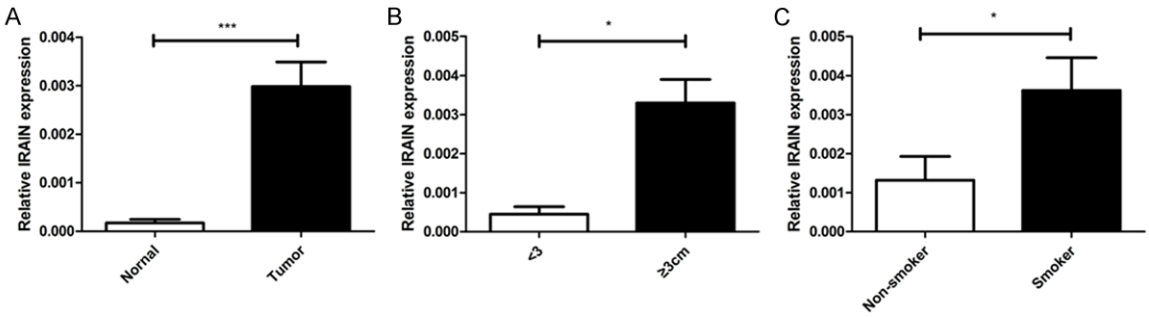

Figure 1. Expression pattern of IRAIN in NSCLC tumor samples compared with adjacent normal tissues, large tumors. (≥3 cm) compared with small tumors (<3 cm), NSCLC patients with smoking history compared with non-smokers (***P<0.0001, *P<0.05).

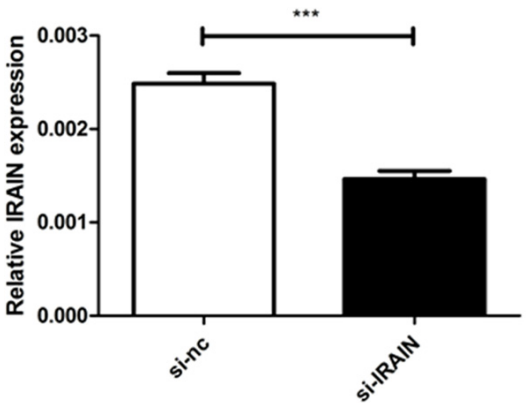

Figure 2. Expression of IRAIN in transfected A549 cells. A549 Cells were transiently transfected with IRAIN siRNA and its corresponding NC, respectively. This effect was examined by SYBR Green real-time PCR and normalized to GAPDH expression. Data represent mean ± SEM from three independent experiments (*P<0.05).

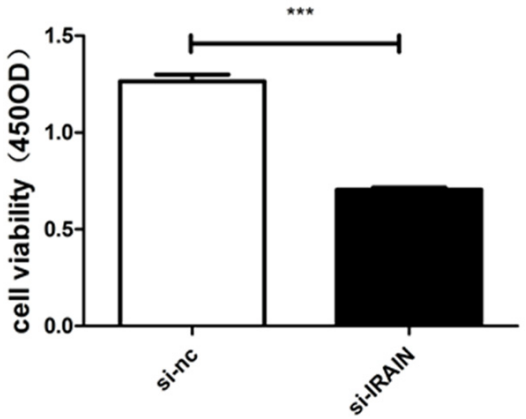

Figure 3. Decreased IRAIN inhibits the proliferation of A549 cells. The effect of IRAIN on A549 cells proliferation was determined by the CCK-8 assay. Data represent mean ± SEM from three independent experiments (***P<0.05).

to the scrambled siRNA at 24 h post-transfection, determined by qRT-PCR (**Figure 2**, P<0.01). The effect of IRAIN expression in cell proliferation was evaluated by CCK-8 assay and inhibited by 44.24% when compared with control cells (**Figure 3**, P<0.01). In addition, the effect of IRAIN on cell cycle and apoptosis was analyzed by flow cytometry. There was significant difference in G0/G1 phase (P<0.05), S phase (P<0.01) and G2/M phase (P<0.01) (**Figure 4**). However, alteration of IRAIN expression did not affect apoptosis of A549 cells (**Figure 5**, P>0.05). Transwell assay was performed to determine whether IRAIN could promote cell migration of A549 cells. As shown in **Figure 6**, knockdown of IRAIN did not decrease cell migration compared with the controls (P >0.05).

Discussion

The concept of functional lncRNA was first introduced over 20 years ago, with the description of the X-inactive specific transcript (XIST), which functions as a X-chromosome suppressor and lacks an open reading frame [9, 10]. In recent years, emerging evidence indicates that lncRNAs are dysregulated and fulfill important functions in the regulation of gene expression, involved in tumorigenesis and other biological progression [11]. lncRNAs expression profiles are altered in many types of cancers. For example, increased expression of HOTAIR has been noted in various primary and metastatic tumors, including breast, liver and the gastrointestinal tract [12-15]. Moreover, Some lncRNAs may play important roles in recurrent tumor, such as ever reported H19, CRNDE, HOTAIRM1 or first reported AC016745.3, XLOC_001711, RP11-128A17.1 in glioma recurrence

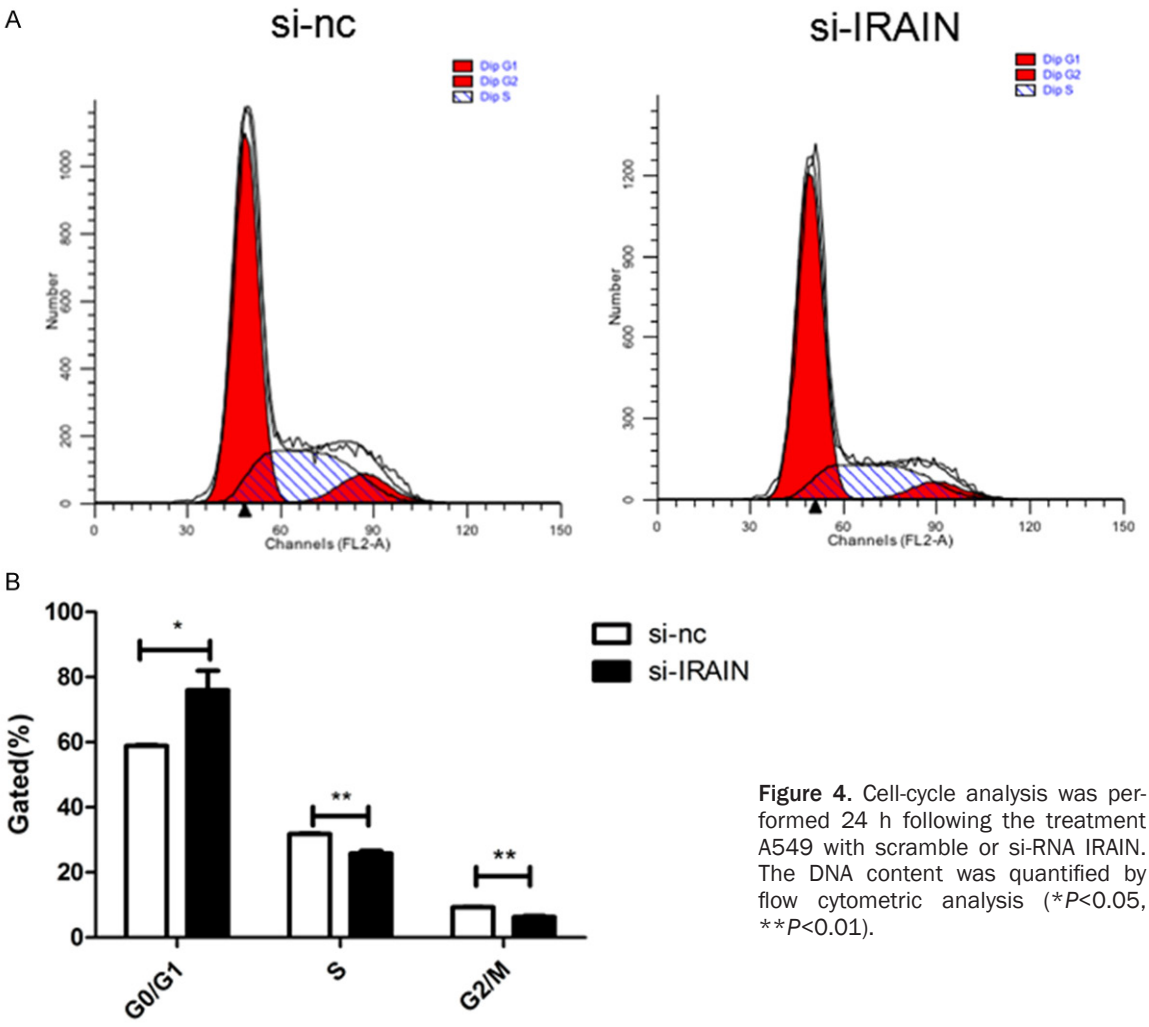

Figure 4. Cell-cycle analysis was performed 24 h following the treatment A549 with scramble or si-RNA IRAIN. The DNA content was quantified by flow cytometric analysis (*P<0.05, **P<0.01).

[16]. Another meta-analysis has demonstrated that the overexpression of lncRNA MALAT1 was an unfavorable prognostic factor for patients' overall survival in NSCLC and pancreatic cancer [17]. Beyond MALAT1, lncRNA TUG1 [18] and PANDAR [19] are also involved in the progression of NSCLC. They are both associated with TNM stage, tumor size, overall survival, as well as growth regulation. Therefore, the identification and investigation of cancer-associated lncRNAs may become new prognostic biomarkers or therapeutic strategies for cancer, including NSCLC.

IRAIN is an antisense noncoding RNA and the full-length transcript is 5.4 kb [7]. It has been identified that IRAIN is related to AML and breast cancer [7, 8]. However, whether it is correlated with NSCLC remains unclear. In this study, we first described the characters of IRAIN in NSCLC tissues and explored its role in NSCLC development and progression in vitro primarily. We discovered that IRAIN was increased in NSCLC tissues and identified the upregulated expression of IRAIN in bigger tumors and smokers. However, our results are contrary to findings from earlier studies in AML and breast cancer. This phenomenon is probably because lncRNAs exhibit dramatically tissue-specific expression patterns than protein-coding genes [20, 21]. According these results, we could infer that IRAIN may present a tissue-specific expression pattern and exhibit important role in NSCLC development and progression. Knockdown of IRAIN inhibited cell proliferation in NSCLC cells. The proliferation of A549 was inhibited when IRAIN was interfered by its knockdown. The effect of IRAIN on decreasing cells growth was exerted by blocking cells in G1 phase.

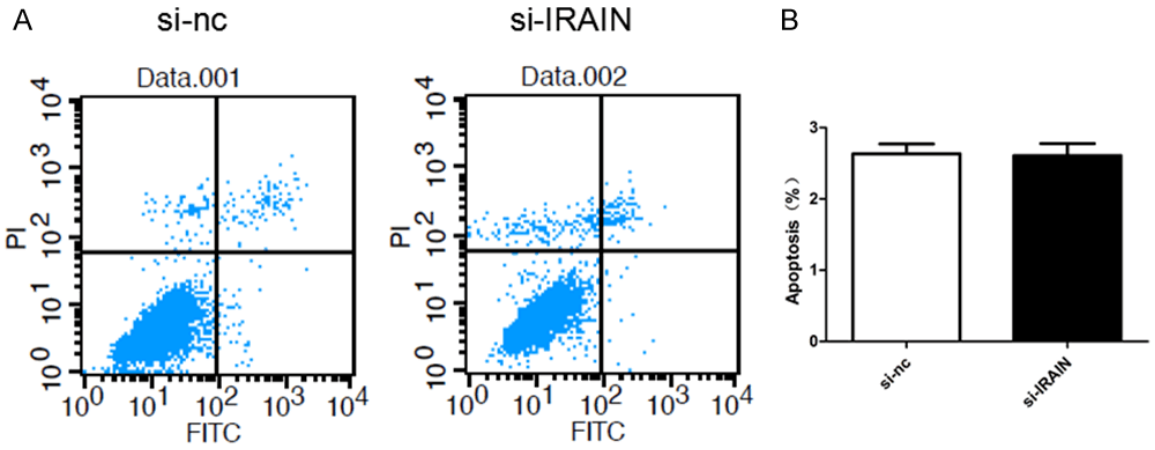

Figure 5. Effect of IRAIN on A549 cell apoptosis. A. Cells were harvested for cell apoptosis analysis by flow cytometry 24 h after transfection. B. Percentage of apoptotic A549 cells. Data represent mean ± SEM from three independent experiments (P>0.05).

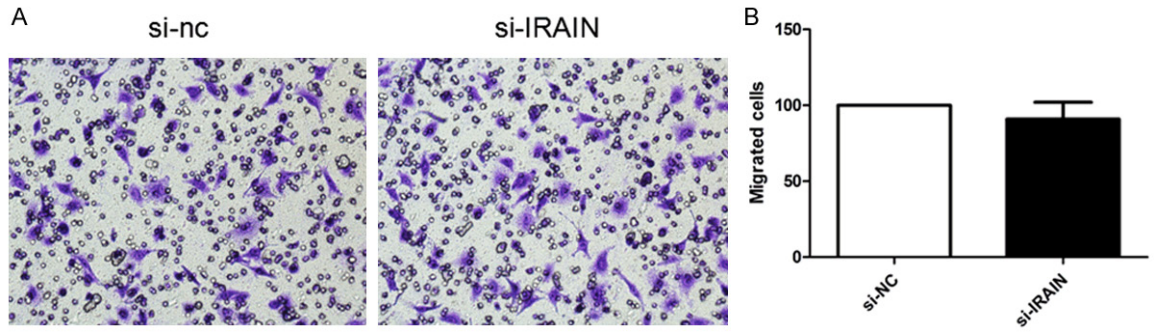

Figure 6. Cell migration assays was employed to evaluate the migratory ability of A549 cells after treatment with IRAIN siRNA and its corresponding NC (P<0.05).

IGF1R is upregulated in breast cancer [22, 23], lung cancer [24], gastrointestinal stromal tumors [25], primary prostate cancer [26]. It is involved in tumorigenesis [27, 28]. IGF1R plays an important role in moderating a broad range of cellular processes, including proliferation, differentiation, survival, and motility [29-31]. In additional, IGF1R participates in radioresistance [32] and drug resistance [33, 34]. It has been identified that IRAIN plays an important role in the formation of an intrachromosomal enhancer/promoter loop of IGF1R. We speculate that the effect of IRAIN in NSCLC is different from that in AML, namely that it actives the pathway of IGF1R to promote the occurrence of NSCLC while it plays a tumor suppressor in AML. Of course it remains to further confirm. These data showed IRAIN as a possible therapeutic target for NSCLC, exerting its anti-tumor effects by regulating IGF1R. The first example of therapeutic targeting of an lncRNA in lung cancer is decreasing the level of MALAT1 by antisense oligonucleotides (ASOs) efficiently reduced lung cancer metastasis in a mouse model [35]. Therefore, IRAIN is also a potential treatment site for gene therapy.

In conclusion, our data indicated that IRAIN was significantly increased in NSCLC tissues and showed significant relation to tumor size and smoking status. Downregulated IRAIN could suppress cell growth of NSCLC cells by blocking cells in G1 phase. IRAIN maybe serve as a therapeutic target for NSCLC in the future, especially for NSCLC with bigger tumor size and/or smoking history.

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Disclosure of conflict of interest

None.

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