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Original Research Article

Suppression of long non-coding RNA H19 inhibits proliferation, cell migration and invasion in human cervical cancer cells

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Abstract

Purpose: *To determine the expression profile of lncRNA H19 in different cervical cancers, and to decipher its function in the growth and metastasis of cervical cancer.*
Methods: *The analysis LncRNA H19 expression was performed using quantitative real time polymerase chain reaction (qRT-PCR). Cell counting kit 8 (CCK8) assay was used to assess the viability of the cells. The cells were transfected with Si-H19 using Lipofectamine 2000 and the metastasis of cells was determined by cell migration and invasion assay. Immunoblotting was used to evaluate the protein expression.*
Results: *The lncRNA H19 expression was considerably enhanced in cervical cancer cells, and was about 2.6 to 5.3 times more in cervical cancer cells relative to non-cancer cells. Inhibition of lncRNA caused significant reduction in cervical cancer cell growth in a time-dependent manner. In addition while silencing of lncRNA inhibited the metastasis of HeLa cells. Cell migration and invasion was about 26 % in Si-H19 transfected cervical cancer cells, relative to 65 % in Si-NC cervical HeLa cells. Similarly, cell invasion was 45 % in Si-H19 cervical HeLa cells relative to the negative control (Si-NC). Inhibition of HeLa cell metastasis was also concomitant with decline of metalloproteinases (MMP)-2 and 9 expression.*
Conclusion: *lncRNA regulates the growth and metastasis of cervical cancer cells. Thus, lncRNA may be an important therapeutic agent for cervical cancer.*

Keywords: *Cervical cancer, lncRNA, Proliferation, Invasion*

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INTRODUCTION

Cervical cancer is among the most commonly detected cancers and is ranked as the 3rd most common type of cancers in women worldwide. Annually, around 0.5 million women are detected with this type of cancer accounting for about 9 % of all the diagnosed cancer cases annually [1]. Although the currently used treatment options such as radical hysterectomy have shown

encouraging results, cervical cancer is still responsible for about 0.3 million annual deaths in the world. Surgery is the only suitable option for cervical cancers detected at early stage. In addition, radiotherapy has been found to exhibit severe adverse effects on the health of the patients [2]. Therefore, it is very important to either explore new drugs or to identify novel targets for the management of cervical cancer.

Long non-coding RNAs (lncRNAs) form a large group of RNA molecules which are more than 200 nucleotides long. These RNA molecules do not code for proteins, and they lack a complete open reading frame [3]. Studies carried out over the last few years suggest that lncRNAs play pivotal functions in a number of biological processes, including regulation of gene expression via DNA methylation [4].The lncRNAs have also been found have an involvement in cancer progression and metastasis [5]. The expression of several lncRNAs is deregulated. Therefore, they are considered as potential therapeutic agents [6]. As per earlier investigations, lncRNA H19 is upregulated in several cancers such as breast and bladder cancers [7]. The expression of H19 decreases in human cells from birth but its expression gets upregulated in cancer cells, and has therefore been implicated in the development of cancers [8,9].

In this study, the expression of lncRNA H19 was investigated in different cervical cancer cell lines. Its influence on the growth and metastasis was also investigated.

EXPERIMENTAL

Cell lines and culture conditions

Human cervical cancer (HeLa, CaSki, MARQ, C33A, C4-1 and SW756) and non-cancerous (HNCF-PI 15) cells were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China and were cultured in RPMI 1640 complete media containing 10% FBS and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), at 37 °C in a humidified chamber.

Assay of lncRNA H19 by RT-PCR

Total RNA was isolated from the cervical cancer cells with the help of RNeasy RNA Isolation Kit following the guidelines of the manufacturer and cDNA was synthesized from it by employing RevertAid cDNA Synthesis Kit (Fermentas). The cDNA samples were subjected to qRT-PCR analysis using SYBR Green Master Mix (Fermentas). For determination of differences in the expressions of lncRNA H19 in different samples, the $^{\Delta\Delta}$-CT method was used. Actin served as internal control.

Suppression of lncRNA H-19

H19-siRNAs (Si-H19) and the negative control siRNA (Si-NC) were obtained from Genepharm (Shanghai, China), and were transfected into cervical cancer using Lipofectamine 2000 reagent (Invitrogen).

Investigation cell proliferation by CCK8 assay

In order to determine the rate of proliferation rate of HeLa cells, CCK-8 assay was carried out. The HeLa cells (both Si-NC and Si-H19) were collected at varied time intervals after transfection, and cell viability was assessed using Cell Numbering Kit-18 (Dojondo, Japan) as per the guidelines of the kit manufacturer.

Cell migration assay

The migration of HeLa (Si-NC and Si-H19) cells was determined with wound healing assay. The cells were grown till confluence and a scratch was made with a scratching device. The cells were then incubated for 48 h again, and the wound healing capacity of Si-NC and Si-H19 transfected cells was evaluated by comparing the widths of the wounds.

Matrigel invasion assay

Invasion was evaluated with the help of Matrigel®-coated invasion chambers. The Si-NC and Si-H19 HeLa cells that reached the lower surface of the membrane were subjected to staining with crystal violet (CV), and the images of CV-stained cells were taken. The crystal violet complexes formed were dissolved in 10 % acetic acid and the cell invasion was determined by measuring the absorbances of the resultant solutions at 600 nm in a spectrophotometer.

Western blotting analysis

Si-NC and Si-H19 transfected HeLa cervical cancels were collected and treated with lysis buffer. The extracts were boiled for 10 min in the presence of loading buffer, followed by separation of cell extracts using 15 % SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and subjected to blocking with skim milk (5%). Membrane incubation with primary antibodies was performed overnight at 4˚C and subsequently incubated with horseradish

peroxidase-linked biotinylated secondary antibodies at 1:1,000 dilution for 2 h. The membranes were then subjected to washing with PBS and the immune-reactive bands were visualized using ECL-PLUSKit. The development of the immune complexes was carried out using an ECL detection kit according to the manual protocol. The bands were analyzed with Ge1GDoc 2000 imaging system.

Statistical analysis

Each test was carried out thrice and the values are presented as mean ± SD. Student's *t*-test was employed for statistical analysis using GraphPad prim 6 software.

RESULTS

lncRNA H19 upregulated in cervical cancer cell lines

The qRT-PCR results showed that lncRNA H19 expression was enhanced in cervical cancer cells as against the non-cancerous cell line HNC-PI 15. The expression of lncRNA H19 was about 2.6 to 5.3 times more in cervical cancer cells as against the non-cancer cells. In addition, the expression of lncRNA H19 was found to be highest MARQ and comparatively lower in CaSki cells. Out of these all the cell lines, HeLa cervical cancer cells were selected for further experimentation (Figure 1).

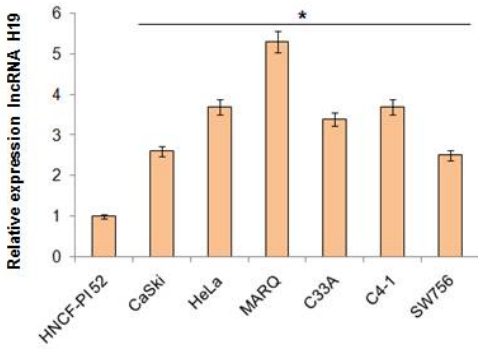

Figure 1: The lncRNA H19 expression in different cervical cancer lines as depicted by qRT-PCR analysis. The values are mean ± SD (n = 3) and (*p < 0.01 vs normal cells used as control)

Silencing of lncRNA H19 inhibits cell proliferation

The expression of lncRNA H19 was considerably suppressed in Si-H19 cervical HeLa cancer cells (Figure 2). Furthermore, it was found that the silencing of lncRNA H19 caused significant suppression of growth of HeLa cells as evident from the CCK8 proliferation assay (Figure 3).

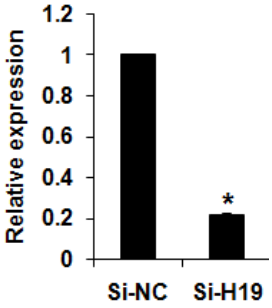

Figure 2: Expression of lncRNA H19 in Si-NC and si-H19 transfected cervical cancer lines as depicted by qRT-PCR analysis. Values are mean ± SD (n = 3) (*p < 0.01, Si-H9 Vs Si-NC)

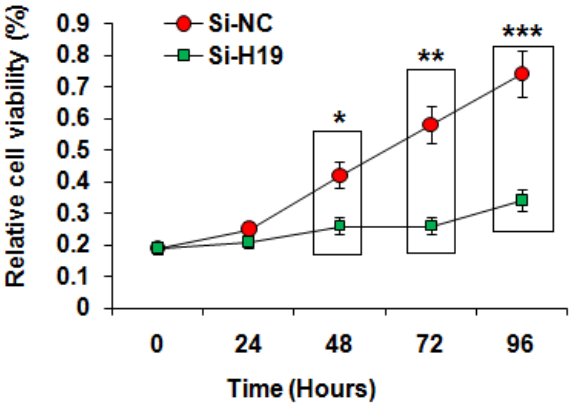

Figure 3: Relative cell viability of Si-NC and si-H19 transfected cervical cancer lines as revealed by CCK8 assay. Values are mean ± SD (n = 3, *p < 0.01, **p < 0.001, ***p < 0.0001; Si-H9 vs Si-NC)

Silencing of lncRNA H19 inhibits cell migration

Further the effect of lncRNA H19 silencing on migration of the HeLa cells was also assessed. The results revealed that the cell migration of Si-H19 HeLa cells was significantly inhibited when compared to the Si-NC cervical HeLa cancer cells used as negative control (Figure 4 A). The percent cell migration was found to be around 26 % in Si-H19 transfected cervical cancer cells as compared to 65 % the Si-NC cervical HeLa cancer cells. Moreover, the inhibition of cell migration was concomitant with suppression of Metalloproteinases MMP-9 and MMP-2 as shown in the western blots (Figure 4 B).

Silencing of lncRNA H19 inhibits cell invasion

The results of Matrigel assay showed that the cell invasion of Si-H19 cancer cells was considerably suppressed as against the negative control (Si-NC). The cell invasion was found to be 45 % in Si-H19 cervical HeLa cancer cells relative to the negative control (Si-NC) (Figure 5).

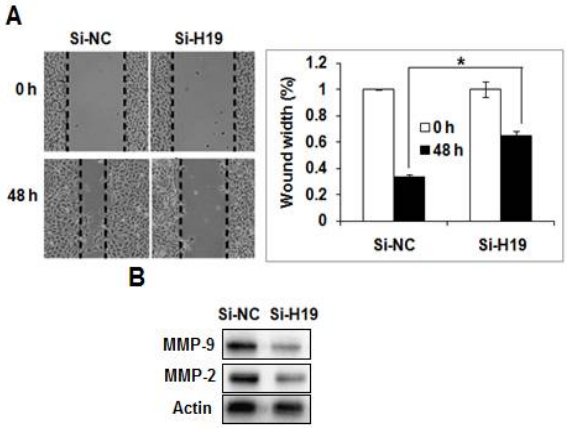

Figure 4: (A) Migration of negative control (NC) and si-H19 transfected cervical cancer lines as depicted by wound healing migration assay. (B) Protein expression of MMP-2 and MMP-9 Si-NC and si-H19 transfected cervical cancer cells. Values are mean ± SD (n = 3, *p < 0.01; Si-H9 Vs Si-NC)

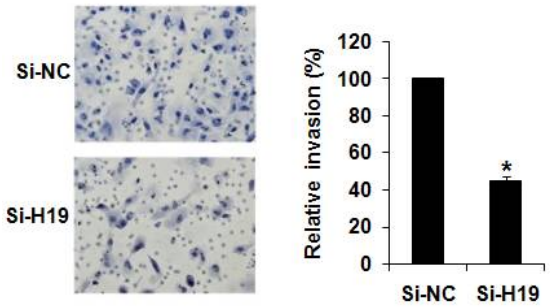

Figure 5: Cell invasion of Si-NC and si-H19 transfected cervical cancer lines as depicted by matrigel assay. Values are mean ± SD (n = 3, *p < 0.01; Si-H19 Vs Si-NC)

DISCUSSION

Cervical cancer is commonly found women and causes considerable mortality and morbidity. The current treatments are inefficient, have side effects and are very costly [1]. In addition, the availability of chemotherapeutic drugs for cervical cancer is also limited [10]. Therefore, need of the hour is to identify novel and prospective therapeutic targets for the treatment of cervical cancer. Long non-coding RNAs have emerged as important RNA molecules that have been reported to be implicated in large number of biological processes [11].Several of the lncRNAs have been reported to be deregulated in cancers and have therefore been considered as key therapeutic targets for cancer treatments [12].

Herein the role of lncRNA H19 in the cervical cancers was investigated. Previous studies have shown that the transcription of lncRNA H19 is deregulated in different types of cancers. Keeping this in view, the expression of lncRNA H19 in six cervical and one normal cell lines was examined. It was found that the expression of lncRNA was 5.6 times more in cervical cancer cells relative to the no-cancerous cells. Similar observations were found in breast cancer cells wherein lncRNAH19 was also upregulated [13]. The qRT-PCR analysis gave first hint about the prospective of lncRNA H19 as therapeutic agent for treating cervical cancer. To decipher the function of lncRNA H19 in HeLa cell proliferation, lncRNA H19 was silenced by RNA interface in HeLa cells. It was found that the silencing of lncRNA H19 declined the viability of HeLa cells in a time dependently. These results are in accordance with previous studies which have reported that several of the lncRNAs inhibit cancer cell proliferation [14]. The mortality of cancer cells is an important factor for their metastasis of the cancers. Hence, inhibiting the cell migration and invasion are considered very imperative for development of treatment strategies for different types of cancers [15]. Therefore, impact of lncRNA H19 silencing on the metastasis cancer cells were also investigated.

The results of wound healing revealed that silencing of lncRNA caused significant halt in HeLa cervical cancer cell migration. Similar results were obtained in Matrigel invasion assay wherein the silencing of lncRNA caused a significant reduction in cell invasion. Similar results have been reported previously wherein the inhibition of lncRNAs halted the invasion of HeLa cells [16]. Furthermore, the immunoblotting revealed that suppression of metastasis was concomitant with suppression of MMP-2 and MMP which are considered important for metastasis of the cancer cells [17]. To sum up, these results indicate that lncRNA H19 may prove to beneficial for treating cervical cancer.

CONCLUSION

The results obtained in the study indicate that lncRNA H19 is highly enhanced in cervical cancer cells. Silencing of lncRNA causes decline in cervical cancer cell proliferation and metastasis. Thus, lncRNA H19 may prove to be a potent target for drugs in cervical cancer therapy.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Huawei Xin and Mingzhe Li performed most experiments and contributed to this work equally under supervision of Xiaoliu Liu and Yan Zhang. Xiaoling Cheng, Tao Wang helped.

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