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LncRNA MIAT Promotes Proliferation of Cervical Cancer Cells and Acts as an Anti-apoptotic Factor

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Abstract

There are a sub-population of cells in tumor tissues known as cancer stem cells (CSCs) which have similar features with stem cells, including self-renewal and differentiation capacity. Recently, it was established that not only stem cells factors such as Oct4, but also ES-associated lncRNAs are contributing to various regulatory aspects of CSCs. Myocardial infarction associated transcript (MIAT) is a lncRNA which expressed in embryonic stem cells (ESCs) and its expression was regulated by ES-associated transcription factors, Oct4 and Nanog. Here, our results revealed that suppression of MIAT reduces cell proliferation and causes G1 arrest in HeLa cancer cells through downregulation of cell cycle progression factors including, CyclinD1 and PCNA. Furthermore, our data showed that downregulation of MIAT represses migration of HeLa cells which implicated that MIAT may be contributed to the migration of HeLa cells. We further detected MIAT silencing promotes apoptosis of Hela cancer cells and influences the expression of known apoptotic factors, Bcl2 and Bax. Based on our findings, LncRNA MIAT might be involved in the cervical cancer malignant progression and it could be the candidate as a potential therapeutic target to combat cancer.

Keywords: LncRNA MIAT; Cervical cancer; G1 arrest; Apoptosis

Introduction

In tumor tissues, there are a sub-population of cells known as cancer stem cells (CSCs) which shares two defining characteristics with stem cells, including self-renewal and differentiation capacity. Based on cancer stem cell hypothesis, these cells are responsible to cancer initiation, progression and metastasis (Maugeri-Saccà *et al*., 2014; Vinogradov and Wei, 2012). Stemness factors such as Oct4, Nanog and Sox2 have been found to contribute in various biological functions of CSCs. Recent advances in high throughput technology for analysis of human genome revealed that most of the transcriptome is constituted by noncoding RNAs (ncRNAs). These transcripts divide in two major groups, small ncRNAs, and long ncRNAs (lncRNAs) (Atkinson *et al*., 2012; Kashi *et al*., 2016). Recently, it was demonstrated that not only stem cells factors such as Oct4, Nucleostemin, Sox2 and Klf4, but also ES-associated lncRNAs are involving in various regulatory features of CSCs. Myocardial infarction associated transcript (MIAT) is an lncRNA which expressed in embryonic stem cells (ESCs) and its expression was regulated by ES-associated transcription factors, Oct4 and Nanog (Sheik Mohamed *et al*., 2010). Inhibition of MIAT leads to disruption of its regulatory loop with Oct4 which was found to induce ES cell differentiation. Recently, MIAT has been detected to be upregulated in non-small-cell lung cancer (NSCLC) and aggressive form of chronic lymphocytic leukemia (Li *et al*., 2016). In this study, we investigate the potential role of LncRNA MIAT in human cervical cancer cells by knocking-down LncRNA MIAT expression in human adenocarcinoma cell line, HeLa, using RNAi technology.

Materials and Methods

Cell line

The human cervical adenocarcinoma cell line (HeLa) was cultured in RPMI-1640 medium (Gibco Life Technologies, Germany), enriched with 10 % fetal bovine serum, 100 U/ml penicillin and 10 µg/ml streptomycin in a humidified atmosphere of 5% CO2 incubator.

MIAT knock down by means of siRNA

To suppress MIAT, smart pool of siRNAs against MIAT was purchased from Dharmacon. Scramble siRNA with no known target in the cells was also purchased from Dharmacon being used for distinguishing sequence-specific silencing from non-specific effects of siRNA. Hela cells were seeded into 12 well plates in growth medium without antibiotics. One day after seeding, the MIAT and scramble siRNAs were introduced into cells by using lipofectamine 2000 (Invitrogen, USA). Two days after transfection, the cells were harvested for genes expression analysis to determine the efficiency of MIAT suppression.

RNA extraction, cDNA synthesis and real-time PCR

Total RNA from cultured cell was isolated with Trizol solution (Invitrogen, USA) according to the manufacturer's instructions. The quality of the extracted RNA was measured by UV spectrophotometry (260/280-nm ratio) and its integrity was detected by visual observation of samples on 1% agarose gel electrophoresis. The first strand of cDNA was synthesized by using 1 µg RNA, 200 U/µl MMLV reverse transcriptase (Fermentase, Lithuania), 20U RNase inhibitor, dNTP mix (final concentration of 1 mM) with random hexamer priming in a 20 µl reaction.
Primers were designed by using Gene Runner software, version 4.0, and their confidence of unity attachment to the targets was checked by BLAST (Basic Local Alignment Search Tool) (Table 1). Quantitative PCR was done using SYBR Premix Ex TaqTM II (Takara, Japan) on Rotor-Gene 6000 instrument (Corbett Life Science, Australia).

The identity of PCR products were verified by visual observation on a 1.5% agarose gels and sequencing with an Applied Biosystems 3730XL sequencer (Macrogen, Seoul, South Korea).

Cell cycle analysis

Cell cycle analysis was performed according to our previous study (Asadi *et al*., 2011). In brief, the cells were harvested after Trypsin-EDTA treatment and washed in PBS. Then the cells were stained with 50 µg/ml propidium iodide solution containing 0.1 % Triton X-100 and 0.1 % sodium citrate. The single cell suspensions were then used for flow cytometric (Partec, Germany) analysis. Cell cycle profiles were analyzed using flowjo 7.6.1 software.

Cell proliferation assay

The cells were plated into 96-well plate and they were stained with 100 µl sterile MTT dye (0.5 mg/ml, Sigma) at specified time points for 4 hours at 37°C. Then the cultured medium was discarded from the cells and 150 µl of dimethyl sulfoxide (DMSO, Sigma) was added to the cells. The absorbance was measured at 570 nm.

Migration assay

To determine the influence of MIAT inhibition on HeLa cell migration, we performed wound healing assay. Briefly, the cells were seeded on 12-well plates and were starved in the medium containing 1% FBS when the cells was confluent. Then we made a straight scratch into a confluent monolayer cells by using a sterile white pipette tip. We further measured and photographed the speed of cell migration when they moved fill the void space at different time points. The analysis of migration was down by Image J software (Tafrihi *et al*., 2014).

Apoptosis assay

Forty-eight hours after siRNA transfection, the cells were harvested in the appropriate manner and were washed with PBS. Cells were then suspended in binding buffer and were stained with Annexin V and PI (Apoptosis Detection Kit, Biolegend) in the dark at room temperature, according to the manufacturer's

recommendations. The stained cells were examined by flow cytometer equipped with flow max software (Partec, Germany). The cells were categorized into apoptotic and viable cells. All of the samples assayed were in triplicates.

Table 1. The primers were used for Real-Time PCR

Gene	Primers	Amplicon size (bp)
MIAT	F: 5′-CAAAGAGCCCTCTGCACTAG-3′ R: 5′-ACCTTGGTTACCCCTGTGATG-3′	128
β-actin	F:5′-ACCACCTTCAACTCCATCATG-3′ R:5′-CTCCTTCTGCATCCTGTCG-3′	120
Oct4 (POU5F1)	F:5′-AGTGAGAGGCAACCTGGAGA-3′ R:5′-TTACAGAACCACACTCGGACC-3′	140
Nanog	F:5′-TAACCTTGGCTGCCGTCTCT-3′ R:5′-AAGCAAAGCCTCCCAATCC-3′	154
Bax	F:5′-GGACGAACTGGACAGTAACATGG-3′ R:5′-GCAAAGTAGAAAAGGGCGACAAC-3′	150
Bcl2	F:5′-CTGCACCTGACGCCCTTCACC-3′ R:5′-CACATGACCCCACCGAACTCAAAGA-3′	119
Cyclin D1	F:5′-ACAAACAGATCATCCGCAAACAC-3′ R:5′-TGTTGGGGCTCCTCAGGTTC-3′	144
PCNA	F:5′-AGGTGGAGAACTTGGAAATGG-3′ R:5′-CGTTGAAGAGAGTGGAGTGG-3′	160
P21	F:5′-ACCATGTGCACCTGTCACTG-3′ R:5′-TTCCAGGACTGCAGGCTTC-3′	200

Statistical analysis

Experiments were replicated two or three times, the $2^{-\Delta\Delta Ct}$ method was utilized to quantify the relative levels of gene expression. The significant difference between groups was determined by independent sample T-test which was performed by the SPSS 22.0 software. $P<0.05$ was considered as significant.

Results

Suppression of MIAT reduces cell proliferation and causes G1 arrest in HeLa cells

To investigate the role of LncRNA MIAT in cervical cancer, we first knocked down MIAT in HeLa cells using smart pool MIAT siRNA and then we assessed the influence of this suppression on cell cycle progression and cell proliferation.

We first found the expression level of MIAT was intensely decreased in MIAT knockdown cells compared with the cells transfected with scramble siRNA (Fig. 1A). MTT assay showed that restricted MIAT expression leads to suppression of cancer cell proliferation (Fig. 1B). In line with MTT data the qPCR data showed that the expression of well-known cell proliferation genes including Cyclin D1 and PCNA, dramatically downregulated following MIAT suppression (Fig. 1C). The cell cycle distribution of HeLa cells showed that a significant increase in the proportion of cells in G1 phase and a decreased in the proportion of cells in S and G2/M phase in MIAT knock down cells compared to cells were transfected with scramble siRNA. (Fig. 1D). Altogether, the results exhibited that MIAT may be have critical role in tumorigenesis through cell cycle regulation.

Downregulation of MIAT represses migration of HeLa cells

To determine the function of MIAT in cancer cell migration, we monitored and photographed the scratch-wound gap of HeLa cells before and after MIAT silencing. The results indicated that downregulation of MIAT restricted cancer cells migration (Fig. 2A, 2B). In addition, we analyzed the expression level of stemness factors including Oct4 and Nanog, following MIAT suppression (Fig. 2C). Our data revealed that the expression of Oct4 and Nanog was significantly decreased when MIAT was silenced. The results implicated that MIAT may be contributed in migration of HeLa cells.

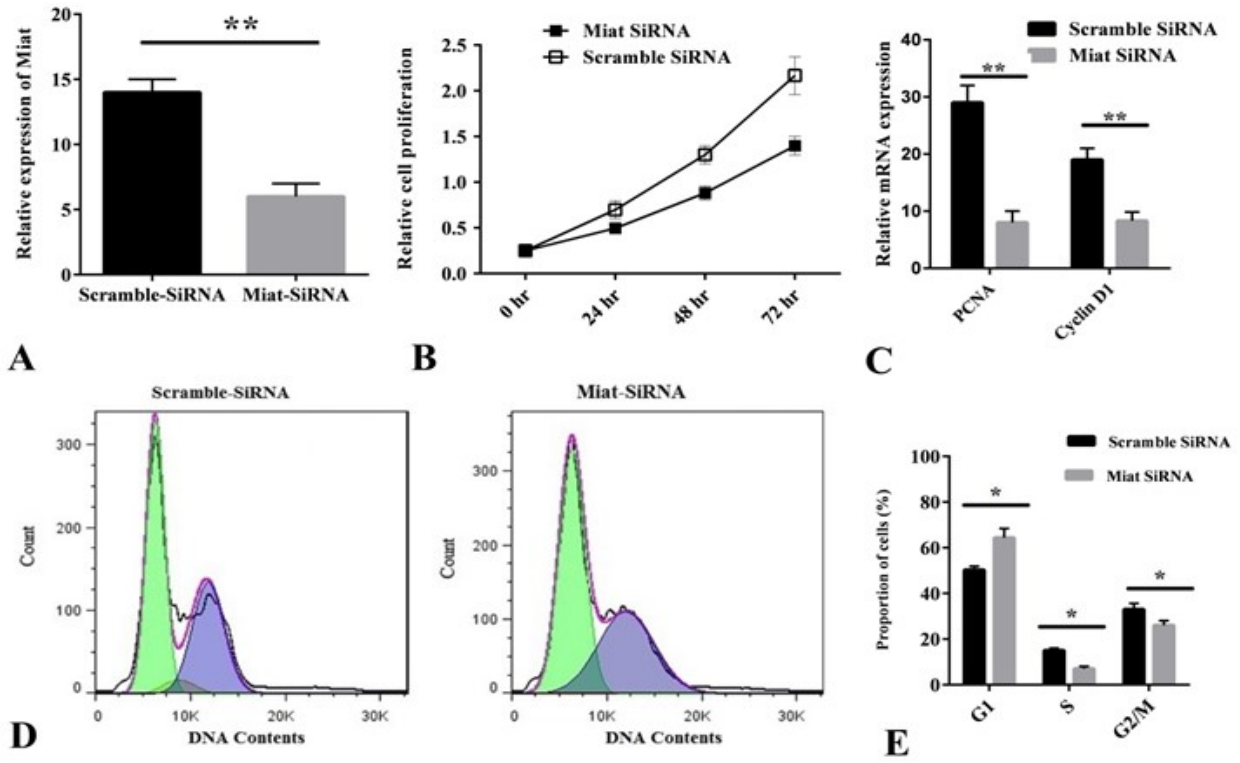

Fig.1. MIAT down regulation induces G1 arrest in HeLa cell line: (A) The expression level of MIAT significantly decreased in MIAT-siRNA transfected cells; (B) Cell proliferation assay showed that MIAT inhibition restricts cancer cell proliferation; (C) The expression level of two main genes involved in cell cycle regulation including CyclinD1 and PCNA were significantly decreased following MIAT silencing; (D) Cell cycle analysis by flow cytometry in HeLa cells transfected by scramble and MIAT siRNAs; (E) Barcharts show the percentage of cells in different phases of cell cycle. Values shown represent the mean ± SE. * p value <0.05, ** p value <0.01.

Knock down of MIAT induces cellular apoptosis of HeLa cells

Induction of apoptosis is a considerable mechanism to prevent tumor growth, thus we evaluated the impact of MIAT suppression on apoptosis. Thus we examined Hela cells with or without MIAT inhibition by staining with Annexin-V and propidium iodide. Our data showed that downregulation of MIAT induced apoptosis in Hela cells (Fig. 3B). In line with the latter finding, we found that siRNA-mediated depletion of MIAT in Hela cells influenced the expression of known apoptotic factors, Bcl2 and Bax (Fig. 3C). These results revealed that LncRNA MIAT may be act as anti-apoptotic factor in HeLa cancer cells.

Discussion

Increasing evidence implicates that the previous assumed “junk DNAs”; actively transcripts to non-coding RNAs and this non–protein-coding portion of the genome is not spurious transcriptional noise. There are two major classes of ncRNAs based on their transcript size: small ncRNAs (<200 bp) and long ncRNAs (lncRNAs) (>200 bp) (Mercer *et al*., 2009). LncRNAs were found to be interacting with ncRNAs, mRNAs, proteins and genomic DNA and act as tethers, guides, decoys, and scaffolds. They are have crucial roles in different cellular processes including; gene regulation, epigenetic, transcription, post-transcription, and translation (Rinn and Chang, 2012). Recent studies established that differential expression pattern of lncRNAs in many human malignancies and propose that this lncRNAs dysregulation could contribute to fundamental aspects of tumor biology including tumor initiation, progression and invasion (Lalevée and Feil, 2015).

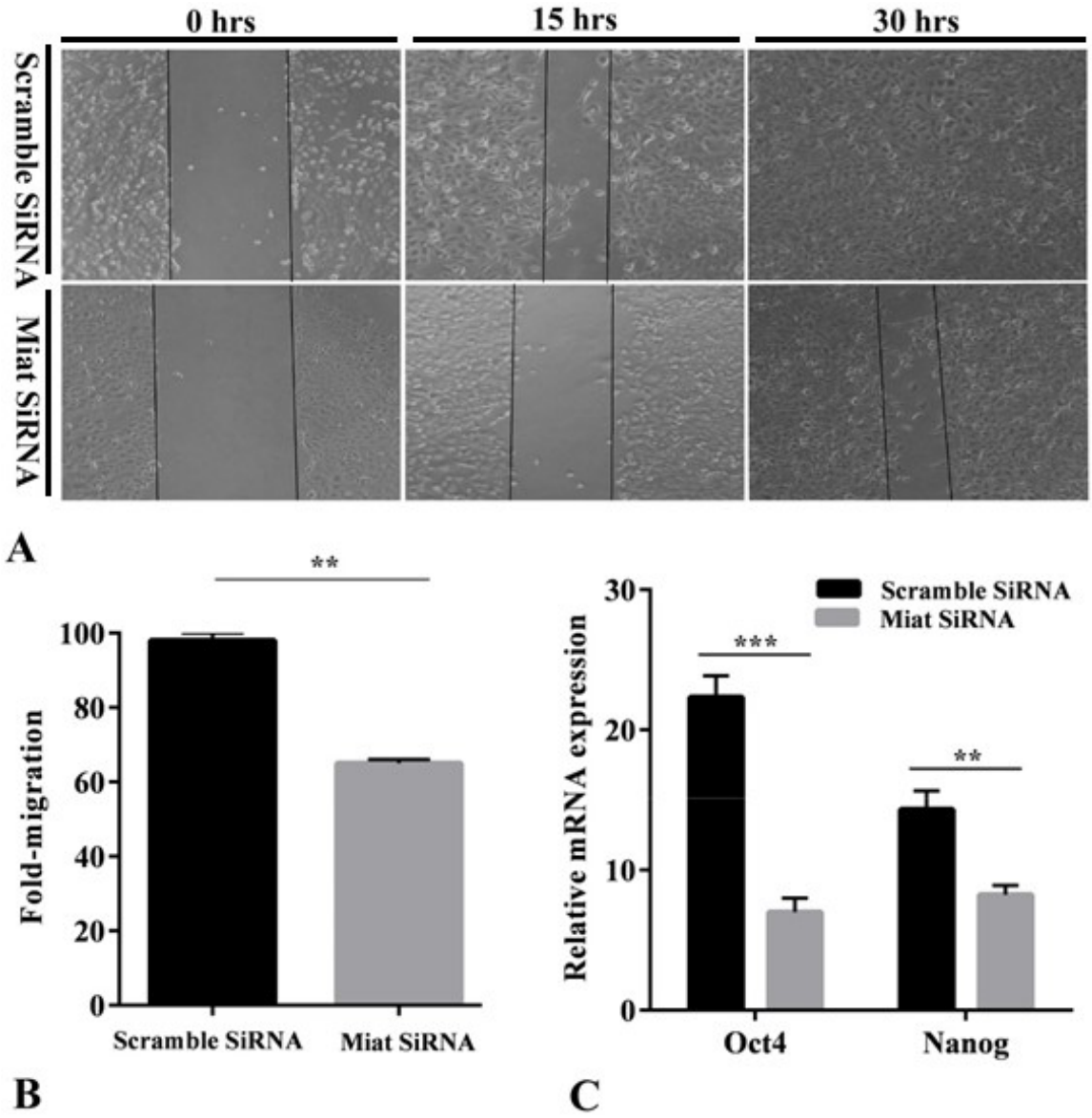

Fig. 2. MIAT knockdown inhibits migration of HeLa cell line: **(**A and B) Wound healing assay was done and MIAT suppression leads to inhibition of cell migration of HeLa cells; (C) Bar charts show that the expression level of Oct4 and Nanog was significantly downregulated when MIAT was suppressed. ** P value <0.01, *** p value <0.001.

MIAT is an lncRNA which was demonstrated to be contributing in several disorders including cardiac fibrosis (Qu *et al*., 2017), and paranoid schizophrenia (Rao *et al*., 2015) but its function in cancer cells is mostly unknown. Recent study showed that MIAT is transcriptionally regulated by stem cells-associated transcription factors Oct4 and Nanog. Consistent with these studies, our data revealed that silencing of MIAT downregulates the Oct4 and Nanog (Mohamed *et al*., 2010). Last findings established that LncRNA MIAT has crucial role in invasiveness of chronic lymphocytic leukemia and non-small lung cancer (Lai *et al*., 2017; Zhang *et al*., 2017). Consistent with latter findings, were determined knock down of MIAT restricted the migration of Hela cancer cells. We further determined that MIAT inhibition leads to repression of cancer cell proliferation and G1 arrest through downregulation of cell cycle progression factors, CyclinD1 and PCNA.

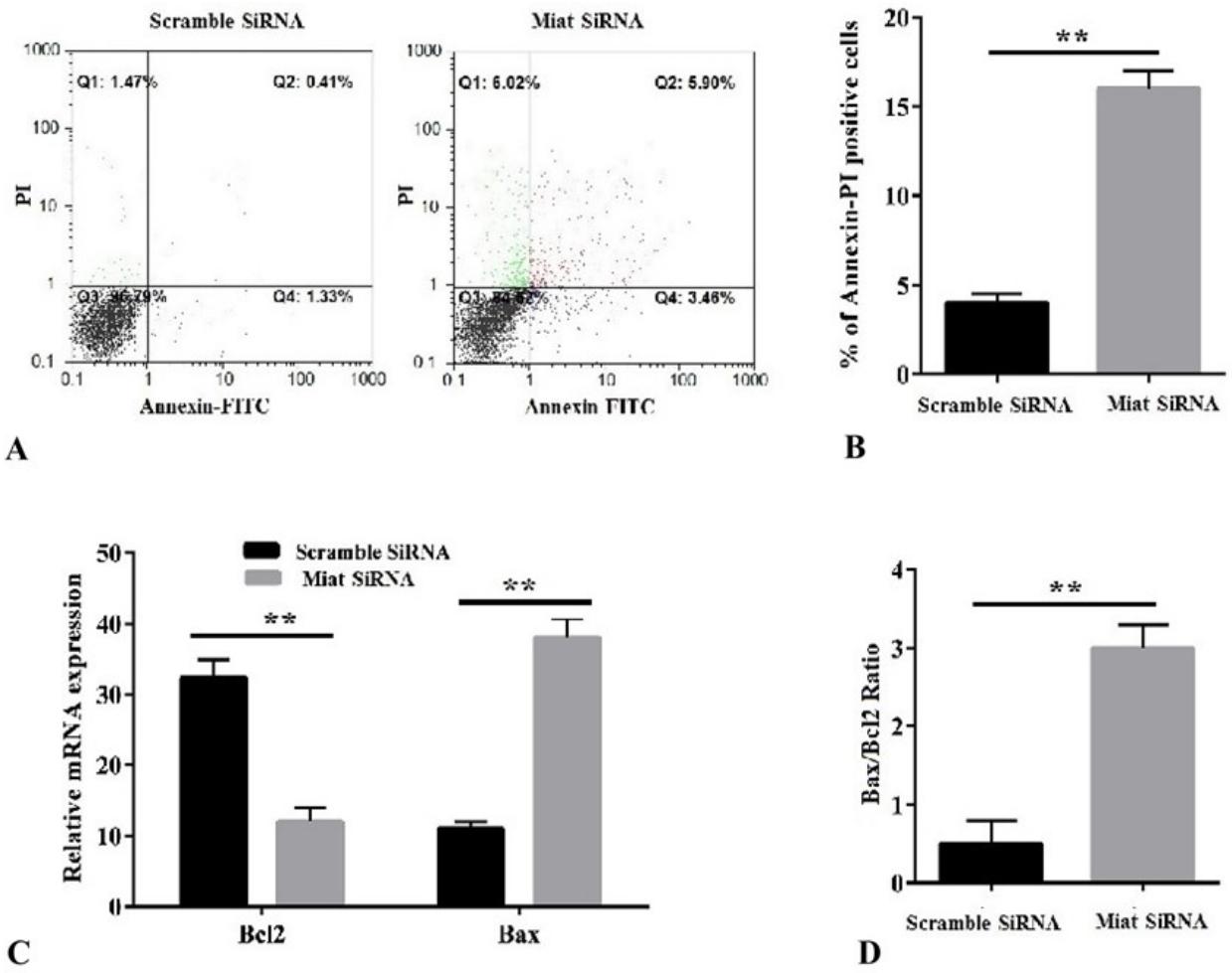

Fig. 3. MIAT knock down promotes apoptosis in HeLa cell line: (A) Annexin V-PI staining was done by flow cytometer and our data showed that apoptotic cells were significantly increased in HeLa cell after MIAT inhibition; (B) Bar charts show the percentage of apoptotic cells in scramble and MIAT siRNA transfected cells; (C) The qPCR analysis of Bcl2 and Bax showed that the expression of Bcl2 and Bax was significantly altered following MIAT downregulation; (D) The Bax/Bcl2 ratio was significantly increased after MIAT-siRNA transfection. ** P value <0.01.

Recent report exhibits that MIAT can act as pro-apoptotic factor in lung cancer and its silencing promotes the cellular apoptosis (Zhang *et al*., 2017). Consistent the data of the report, we detected MIAT inhibition promotes apoptosis of Hela cancer cells. Our data suggests that LncRNA MIAT might be involved in malignant progression of cervical cancer and it could be candidate as potential tumor marker and therapeutic target to combat cancer.

Conclusion

For the first time, in this study we reported that inhibition of LncRNA MIAT can disrupt the proliferation of HeLa cells by inducing G1 arrest and promoting cellular apoptosis. Altogether, the results of this study propose that MIAT can be considering as a potential biomarker in cervical cancer progression and treatment.

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