

Long non-coding RNA HOTAIR regulates proliferation, migration and invasion of human cervical cancer cells by modulating expression of MAPK1

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Abstract

Introduction: Accumulating evidence suggests that long non-coding RNAs (lncRNAs) are dysregulated in cancer cells and may be responsible for the development and progression of this disease. Herein, the role and therapeutic potential of aberrantly expressed lncRNA HOTAIR were investigated in cervical cancer.

Material and methods: The expression profile of the lncRNA HOTAIR was determined by quantitative RT-PCR. CCK-8 and colony formation assays were used for determination of cell viability. DAPI and annexin V/PI assays were used for detection of apoptosis. Wound healing and transwell assays were used to monitor cell migration and invasion.

Results: The results showed that the expression of lncRNA HOTAIR was significantly ($p < 0.01$) upregulated (up to 4.1-fold) in cervical cancer cell lines. Silencing of lncRNA HOTAIR expression resulted in inhibition of the proliferation of the DoTc2 cervical cancer cells via induction of apoptotic cell death. HOTAIR silencing also resulted in decrease of the migration and the invasive properties of the cervical cancer cells. HOTAIR has been reported to interact with MAPK1 in cancer cells, and in this study MAPK1 was found to be overexpressed (up to 3.7-fold) in all the cervical cancer cells and silencing of HOTAIR inhibited the expression of MAPK1 in DoTc2 cervical cancer cells. Silencing of MAPK1 in DoTc2 cells also inhibited their proliferation and metastasis via induction of apoptosis. Co-transfection experiments showed that silencing of MAPK1 and lncRNA HOTAIR causes inhibition of DoTc2 cell growth synergistically.

Conclusions: These results indicate that lncRNA HOTAIR may prove to be an important therapeutic target for management of cervical cancer.

Key words: cervical cancer, HOTAIR, MAPK1, apoptosis, cell invasion, cell viability.

Introduction

Cervical cancer, being one of the common types of cancers in women, is ranked as the second most prevalent cancer worldwide [1]. Although cervical cancer is more frequent in underdeveloped countries, it still ac-

counts for 10% of all cancers in women [2]. Approximately 0.37 million new cervical cancer cases are detected throughout the globe annually [3]. The treatment for cervical cancer involves radical hysterectomy, chemotherapy and/or radiotherapy [4]. However, the clinical outcomes are still unsatisfactory, owing to the adverse effects of chemotherapeutic agents and the dearth of potent therapeutic targets. Hence, the identification of novel, effective and safer anticancer agents and efficient therapeutic targets may help to curb the incidence of cervical cancer and enhance the overall survival rate. Long non-coding RNAs (lncRNAs) have gained enormous attention as therapeutic targets for the management of diseases such as cancer [5]. Although the human genome is very large, a very small part of it codes for proteins. The major part of the human genome is inactive or may be responsible for the transcription of non-coding RNAs [6]. Generally, the non-coding RNAs are either classified as long chain or short non-coding RNAs depending on the size of transcripts [7]. In the last few decades, a lot of research has been dedicated to exploration of the functional roles of microRNAs owing to their diverse functions [8]. However, with progress in the field of science, lncRNAs, which are generally more than 200 nucleotides in length, have been reported to be involved in diverse processes, many of which are cancer related [9]. The lncRNAs have been shown to be aberrantly expressed under disease conditions and are now believed to exhibit the potential to act as therapeutic targets for drugs [10]. HOTAIR (HOX anti-sense intergenic RNA) is one of the important lncRNAs and a lot of research has been aimed at exploring its therapeutic potential in different types of cancers [11]. In breast and pancreatic cancer, HOTAIR has been shown to be aberrantly overexpressed and regulates cell invasion [12, 13]. Moreover, HOTAIR has been shown to play a role in metastasis of oral and pancreatic cancer [14, 15]. In yet another study knockdown of HOTAIR in breast cancer has been shown to be accompanied by suppression of cell migration [16]. With this background the present study explored the therapeutic potential of HOTAIR in cervical cancer.

Although the therapeutic potential of HOTAIR has also been explored in cervical cancer [17], the present study for the first time reports that HOTAIR regulates the growth and invasion of cervical cancer cells by interacting with MAPK1.

Material and methods

Cell lines and culture conditions

The normal cervical cell line (HCvEpC) and cervical cancer cell lines (CaSki, DoTc2, SiHA, C-33A) were procured from American Type Culture Col-

lection (Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in a CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C with 98% humidity and 5% CO₂. All transfections were carried out with the help of Lipofectamine 2000 as per the manufacturer's protocol.

qRT-PCR analysis

The total RNA from the cervical cancer cell lines was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. The cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and amplified with Platinum SYBR Green qPCR Super Mix-UDG reagents (Invitrogen) using the CFX96 sequence detection system (Bio-Rad, Hercules, CA, USA). The cycling conditions for qRT-PCR were as follows: 95°C for 25 s, 40 cycles of 95°C for 20 s, and 55°C for 1 min. Actin was employed as an internal control. The quantitative variation between the samples was determined by the 2^{-ΔΔC_q} method.

Cell viability assay

The CCK-8 assay was used for the determination of cell viability of the cervical cancer cells. In brief, the transfected DoTc2 cells were seeded in 96-well plates and incubated at 37°C for 24 h and subjected to treatment with 10 microliters of CCK-8 solution. The cells were then again subjected to incubation for 2 h at 37°C in a humidifier (5% CO₂, 95% O₂). OD₄₅₀ was taken at different time intervals (0, 12, 24, 48 and 96 h) with the help of a microplate reader.

Apoptosis assays

The transfected cervical cancer DoTc2 cells (0.6 × 10⁶) were seeded in 6-well plates and subjected to incubation for 12 h. Following incubation, the DoTc2 cells were subjected to incubation for 24 h at 37°C. As the cells sloughed off, 10 µl cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were covered with cover slips and examined with a fluorescent microscope. Annexin V/PI staining of the DoTc2 cells was performed to determine the percentage of apoptotic cells. In brief, the cells were washed and subjected to incubation at room temperature for 25 min in the dark in 100 µl of 1X binding buffer containing 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI). Afterward, apoptosis was analyzed by a FACScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA).

Transwell and wound healing assay

The invasion abilities of the transfected DoTc2 cells were examined by transwell chamber assay. In brief, 1×10^4 DoTc2 cells were seeded in the upper chamber of the transwell (8 μ m pore size polycarbonate filters). Then the chambers were placed into 24-well plates and these were subjected to incubation at 37°C for 48 h. The inserts were coated with extracellular matrix gel (50 μ l) (ECM, Sigma, USA). Swabbing was performed to remove the non-invaded cells from the upper surface. However, the invaded cells on the lower surface were subjected to fixation with methanol for about 35 min, followed by staining with crystal violet (0.5%) for about 50 min, subjected to washing with PBS and finally counted under a light microscope (5 fields). Wound healing assay was employed to investigate the migration of the transfected DoTc2 cells. In brief, after 24 h of transfection into DoTc2 cells, the medium was removed and cells were subjected to PBS washing. A sterile pipette tip was employed to scratch a wound in each well and cells were subjected to washing again and a photograph was taken. The plates were subjected to culturing at 24 h and a photograph was taken again under an inverted microscope.

Western blotting

The DoTc2 cells were subjected to washing with ice-cold PBS and then suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 μ g of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with TBS and then exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualized by an enhanced chemiluminescence reagent.

Statistical analysis

Data are shown as mean \pm SD. Statistical analysis was done using Student's *t*-test with GraphPad prism 7 software. Values of $p < 0.01$ were taken as significant.

Results

LncRNA HOTAIR is upregulated in cervical cancer cell lines

The expression of lncRNA HOTAIR was examined in one normal and four cervical cancer cell lines. The results showed that the expression of lncRNA HOTAIR was significantly ($p < 0.01$) but

aberrantly upregulated in all the cervical cancer cell lines. The expression of HOTAIR was 2.9- to 4.1-fold higher in cervical cancer cell lines relative to the normal cells (Figure 1 A). Moreover, the highest expression of lncRNA HOTAIR was found in the case of DoTc2.

Silencing of HOTAIR inhibits proliferation of DoTc2 cells via apoptosis induction

To infer the role of lncRNA HOTAIR in DoTc2 cervical cancer cells, the expression of lncRNA HOTAIR was silenced (Figure 1 B). The results showed that silencing of the lncRNA HOTAIR expression in DoTc2 cells resulted in a significant ($p < 0.01$) decline in the cell viability (Figure 1 C). The silencing of HOTAIR in the DoTc2 cells also reduced their potential to form colonies (Figure 1 D). Investigation of the underlying mechanisms by DAPI staining revealed that silencing of lncRNA HOTAIR caused apoptotic cell death of the DoTc2 cells (Figure 1 E). Annexin V/PI staining also showed that the apoptotic DoTc2 cell percentage increased significantly upon lncRNA HOTAIR silencing (Figure 1 F). The percentage of apoptotic DoTc2 cells was 4.21% in NC and 42.66% in the Si-HOTAIR transfected DoTc2 cells. Additionally, lncRNA HOTAIR silencing also caused upregulation of Bax and caspase-3, which was also accompanied by a decline in the expression of Bcl-2 (Figures 1 G, 2).

Silencing of HOTAIR suppresses migration and invasion of DoTc2 cells

The effects of HOTAIR cells on the migration and invasion of the DoTc2 was also investigated by transwell and wound healing assays respectively. The wound healing assay results showed that HOTAIR silencing inhibited the migration of the DoTc2 cells as evident from the high cell free migration area in the case of Si-HOTAIR transfected DoTc2 cells relative to NC transfected DoTc2 cells (Figure 3 A). The transwell assay showed that the invasion of the DoTc2 cells was significantly inhibited upon HOTAIR silencing (Figure 3 B).

LncRNA HOTAIR exerts its effects via suppression of MAPK1

Previous studies have indicated an interaction between lncRNA HOTAIR and MAPK1 in ovarian cancer. Therefore, the expression of MAPK1 was examined in all the four cervical and one normal cell line, and it was observed that MAPK1 was upregulated in all the cervical cancer cells by 3.1- to 3.7-fold relative to the normal cells (Figure 3 A). However, silencing of HOTAIR in DoTc2 cells resulted in suppression of MAPK1, indicative of direct interaction between MAPK1 and HOTAIR in DoTc2

cells (Figure 3 B). Next the expression of MAPK1 was silenced in the DoTc2 cells (Figure 3 C) and the results showed that silencing of MAPK1 resulted in inhibition of the cell viability and colony formation potential of the DoTc2 cells (Figures 3 D and E). These inhibitory effects triggered by MAPK1 silencing were mainly due to the induction of apoptosis as evident from the DAPI and Annexin V/PI staining (Figures 3 F and G). The apoptosis induced by MAPK1 silencing was also accompanied by upregulation of Bax and downregulation of Bcl-2 (Figure 3 H).

MAPK1 silencing inhibits migration and invasion of DoTc2 cells

The wound healing assay results showed that MAPK1 silencing decreased the migration of the

DoTc2 cells, as is clear from the high cell free migration area in the case of Si-MAPK1 transfected DoTc2 cells relative to NC transfected DoTc2 cells (Figure 4 A). The transwell assay showed that the invasion of the DoTc2 cells was significantly inhibited upon MAPK1 silencing (Figure 4 B). These results are similar to those of HOTAIR silencing in DoTc2 cells.

LncRNA HOTAIR and MAPK1 act synergistically in DoTc2 cells

To examine whether silencing of both MAPK1 and lncRNA HOTAIR exhibits synergistic effects on the viability of the DoTc2 cells, the DoTc2 cells were co-transfected with Si-HOTAIR and Si-MAPK1 constructs. The results of the CCK-8 assay showed that the growth inhibitory effects of Si-HOTAIR

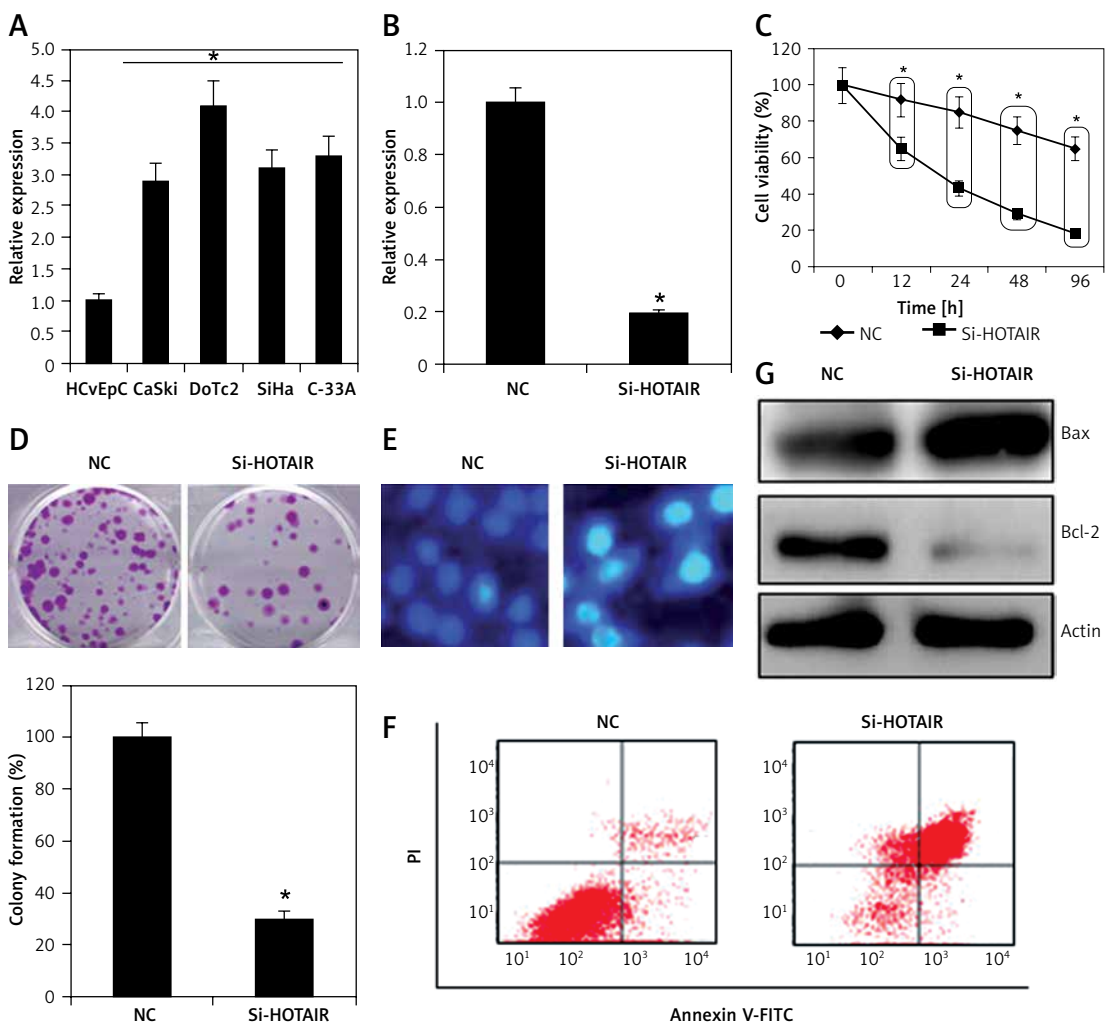


Figure 1. LncRNA HOTAIR regulates proliferation of cervical cancer cells. **A** – Expression of lncRNA HOTAIR in normal HCvEpC and cervical cancer cell lines. **B** – Expression of lncRNA HOTAIR in NC or Si-HOTAIR transfected DoTc2 cells. **C** – Cell viability of the NC or Si-HOTAIR transfected DoTc2 cells. **D** – Colony formation of the NC or Si-HOTAIR transfected DoTc2 cells. **E** – DAPI staining of NC or Si-HOTAIR transfected DoTc2 cells. **F** – Annexin V/PI staining of NC or Si-HOTAIR transfected DoTc2 cells. **G** – Expression of Bax and Bcl-2 in NC or Si-HOTAIR transfected DoTc2 cells. The experiments were performed in triplicate and results are expressed as mean \pm SD (* p < 0.01 for cancerous vs. non-cancerous cells and NC vs. Si-HOTAIR)

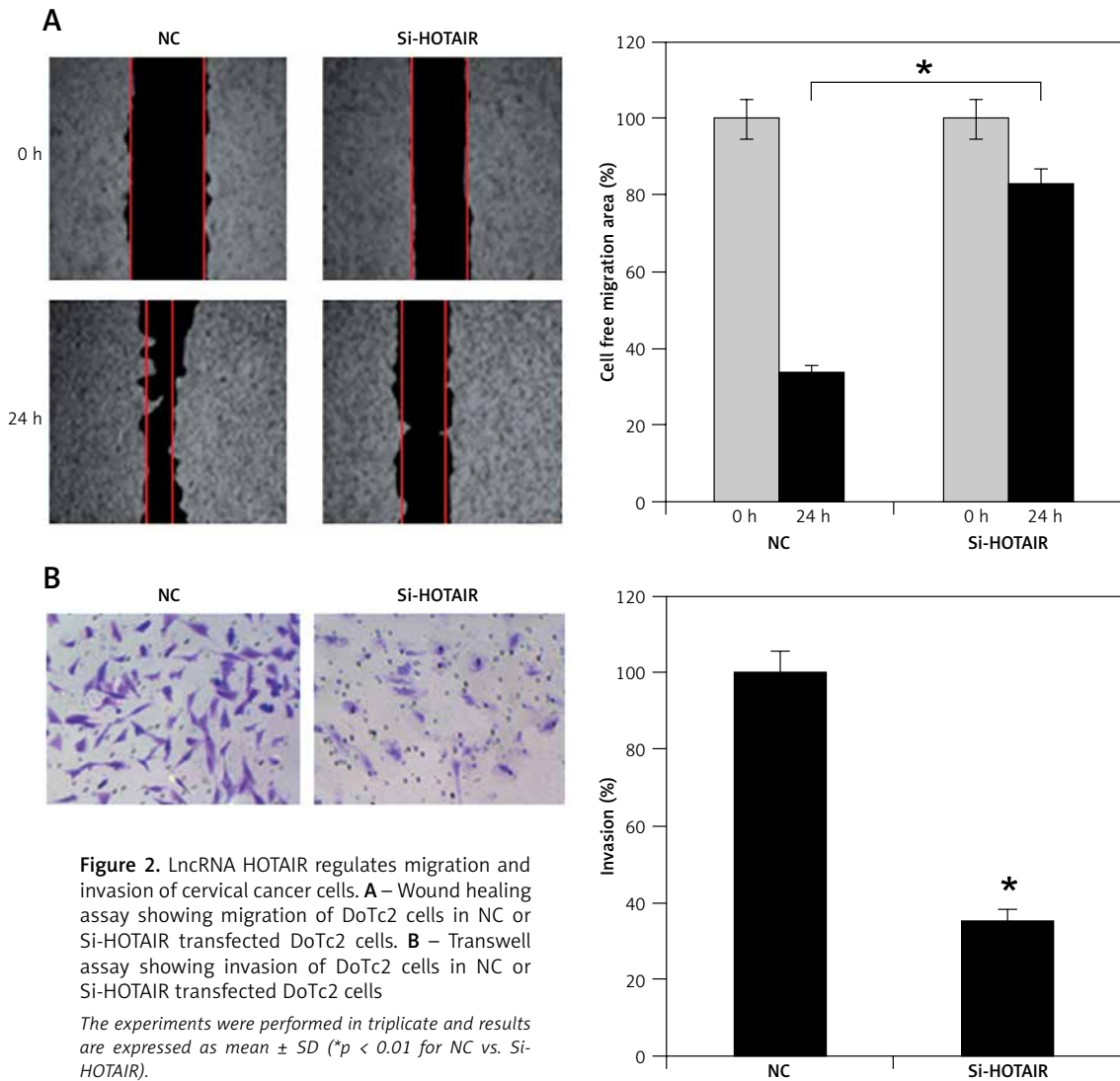


Figure 2. LncRNA HOTAIR regulates migration and invasion of cervical cancer cells. **A** – Wound healing assay showing migration of DoTc2 cells in NC or Si-HOTAIR transfected DoTc2 cells. **B** – Transwell assay showing invasion of DoTc2 cells in NC or Si-HOTAIR transfected DoTc2 cells
*The experiments were performed in triplicate and results are expressed as mean ± SD (*p < 0.01 for NC vs. Si-HOTAIR).*

and Si-MAPK1 were more profound together than individually, indicating synergistic effects of HOTAIR and MAPK1 silencing on the proliferation of the DoTc2 cervical cancer cells (Figure 5).

Discussion

Cervical cancer is considered as the second most common type of cancer in women across the world [18]. Since the clinical outcomes are far from satisfactory due to the adverse effects of the existing drugs and lack of efficient therapeutic targets, the identification of potent anticancerous molecules and therapeutic targets is required [3, 4]. Long non-coding RNAs have attained importance because of their diverse functions in biological pathways [12]. LncRNA HOTAIR has been reported to be involved in the development and progression of several types of cancers [19]. In the present study, we for the first time explored the therapeutic potential of lncRNA HOTAIR via its interaction with MAPK1.

The results revealed that lncRNA HOTAIR is aberrantly overexpressed in human cervical cancer and silencing of HOTAIR caused a significant ($p < 0.01$) decrease in the cell viability and colony formation via induction of apoptosis. Furthermore, HOTAIR silencing also caused a decrease in the migration and invasiveness of DoTc2 cervical cancer cells. Several previous studies have shown the effects of HOTAIR silencing on the growth and metastasis of cancer cells [20, 21]. In lung adenocarcinoma, suppression of HOTAIR caused a decline in cell growth by arresting the lung adenocarcinoma cells at the G_0/G_1 check point of the cell cycle [20]. In another study, HOTAIR silencing resulted in a decrease in the invasiveness of gastric cancer cells via modulation of HER2 expression [21]. A previously carried out study provided a clue about the interaction between HOTAIR and MAPK1 in ovarian cancer [22]. Therefore, we sought to determine whether HOTAIR regulates the growth and metastasis of

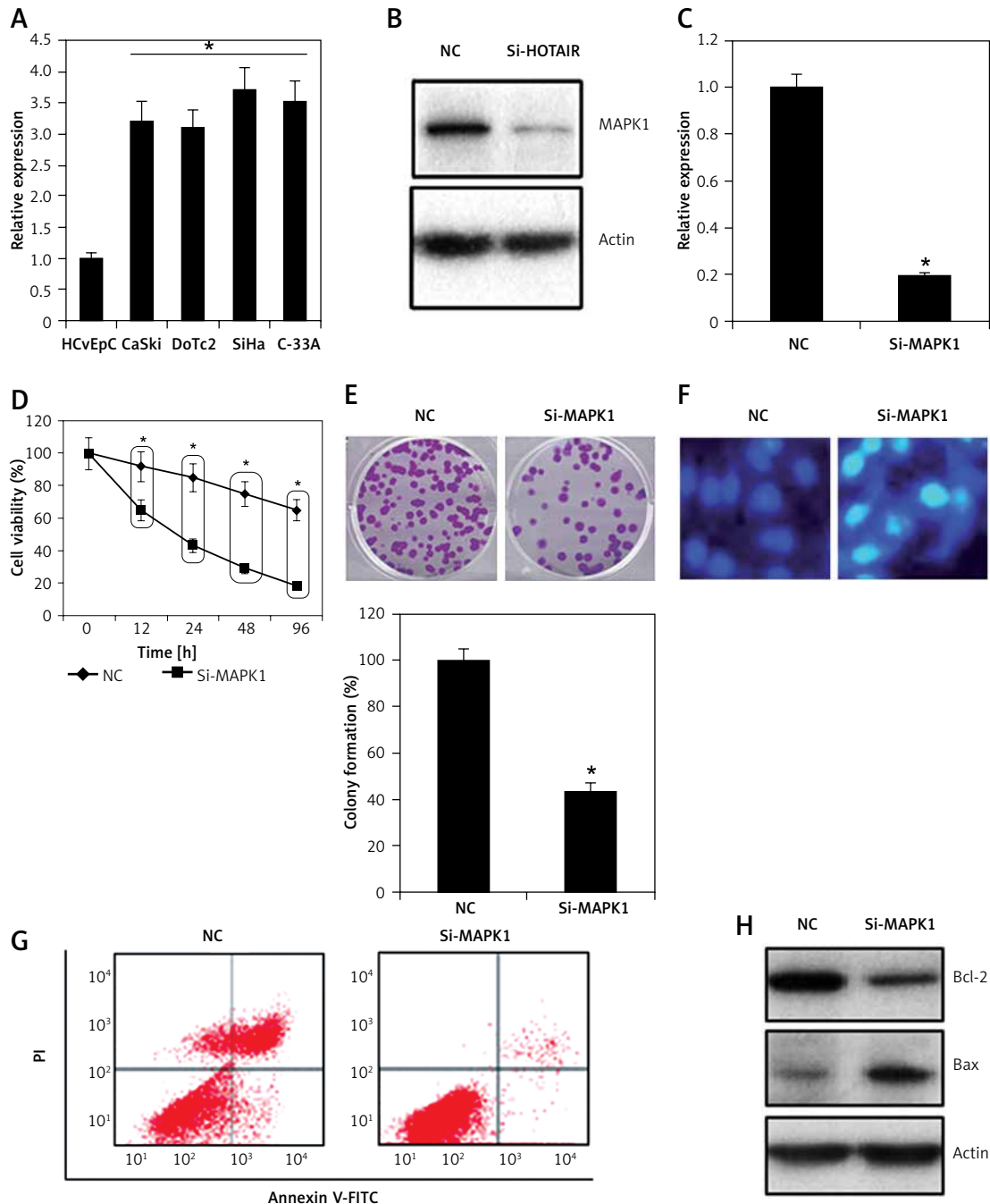
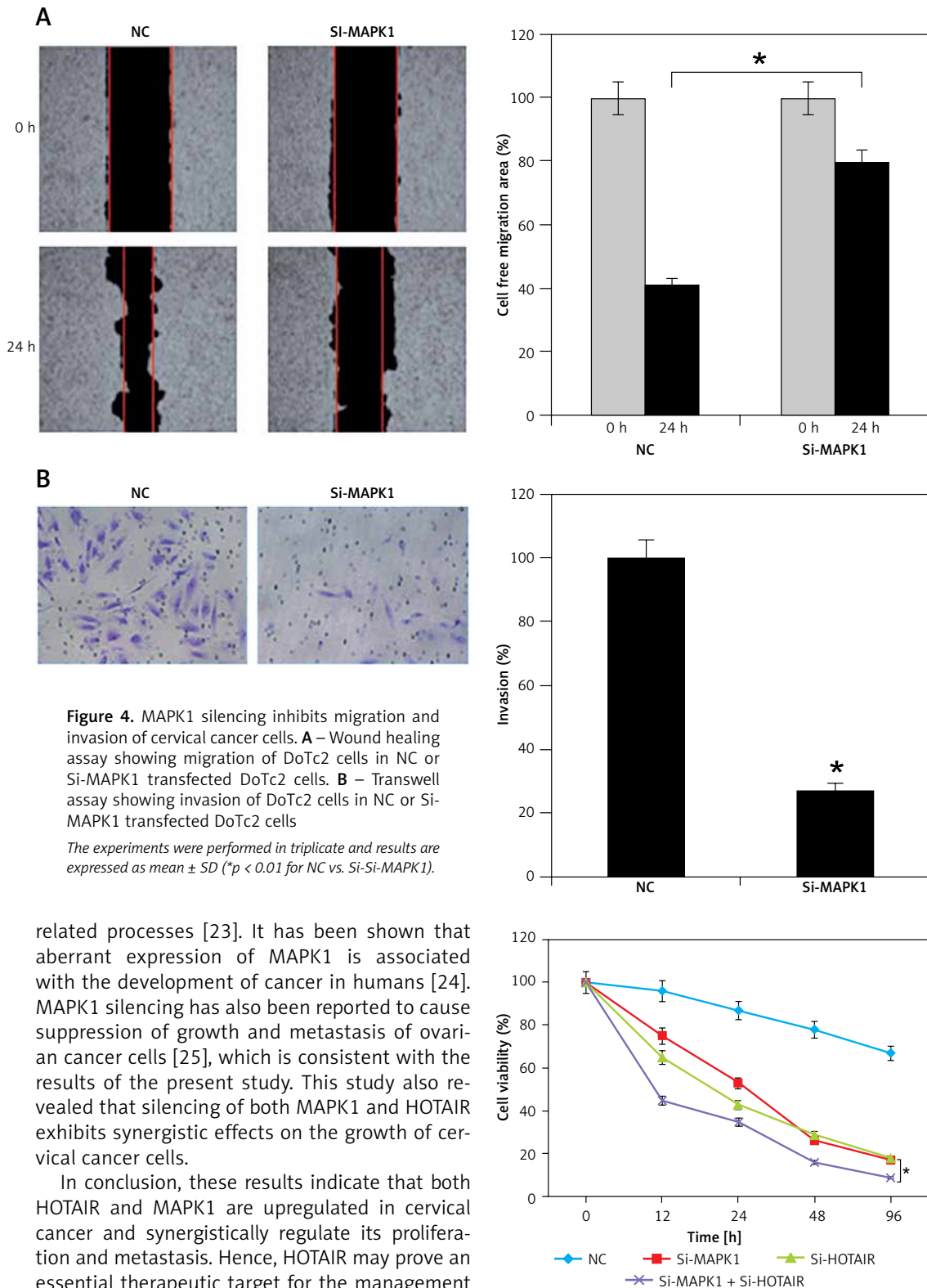


Figure 3. LncRNA HOTAIR regulates proliferation of cervical cancer cells via MAPK1. **A** – Expression of MAPK1 in normal HcVepC and cervical cancer cell lines. **B** – Expression of MAPK1 in NC or Si-HOTAIR transfected DoTc2 cells as depicted by western blot analysis. **C** – Expression of MAPK1 in NC or Si-MAPK1 transfected DoTc2 cells. **D** – Cell viability of the NC or Si-MAPK1 transfected DoTc2 cells. **E** – Colony formation of the NC or Si-MAPK1 transfected DoTc2 cells. **F** – DAPI staining of NC or Si-MAPK1 transfected DoTc2 cells. **G** – Annexin V/PI staining of NC or Si-MAPK1 transfected DoTc2 cells. **H** – Expression of Bax and Bcl-2 in NC or Si-MAPK1 transfected DoTc2 cells. The experiments were performed in triplicate and results are expressed as mean \pm SD (* $p < 0.01$ for NC vs. Si-Si-MAPK1).

cervical cancer cells by interacting with MAPK1. Consequently, the expression of MAPK1 was determined in all the cervical cancer cell lines and MAPK1 was found to be overexpressed in all the cervical cancer cell lines. Nonetheless, silencing of HOTAIR resulted in suppression of MAPK1 expression in the DoTc2 cervical cancer cells,

suggestive of cross talk between MAPK1 and HOTAIR. Moreover, like HOTAIR, the silencing of MAPK1 also inhibited the growth, migration and invasion of cervical cancer cells via activation of apoptosis. The MAPK1 pathway has been shown to play an imperative role in the development of cancer via regulation of growth and metastasis



related processes [23]. It has been shown that aberrant expression of MAPK1 is associated with the development of cancer in humans [24]. MAPK1 silencing has also been reported to cause suppression of growth and metastasis of ovarian cancer cells [25], which is consistent with the results of the present study. This study also revealed that silencing of both MAPK1 and HOTAIR exhibits synergistic effects on the growth of cervical cancer cells.

In conclusion, these results indicate that both HOTAIR and MAPK1 are upregulated in cervical cancer and synergistically regulate its proliferation and metastasis. Hence, HOTAIR may prove an essential therapeutic target for the management of cervical cancer and drugs can be developed to target the expression of both of these genes.

Conflict of interest

The authors declare no conflict of interest.

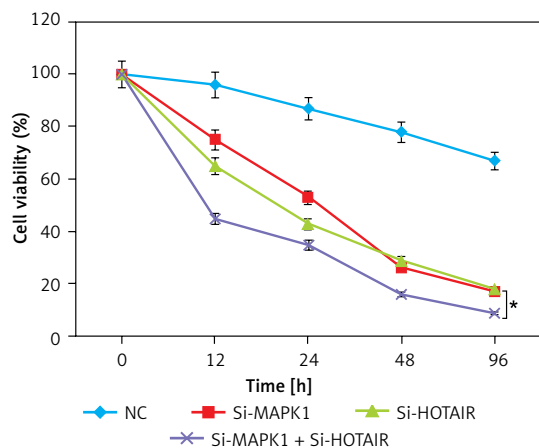
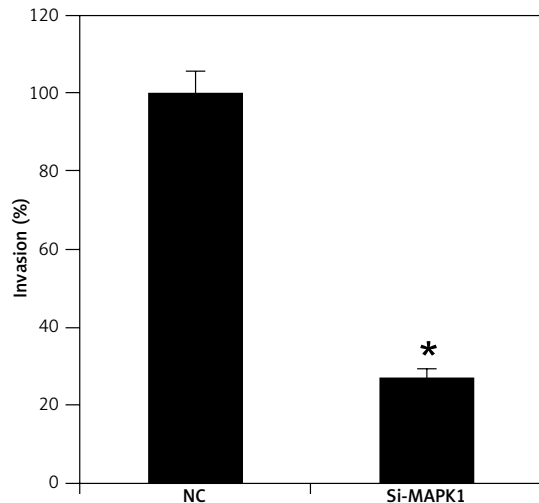
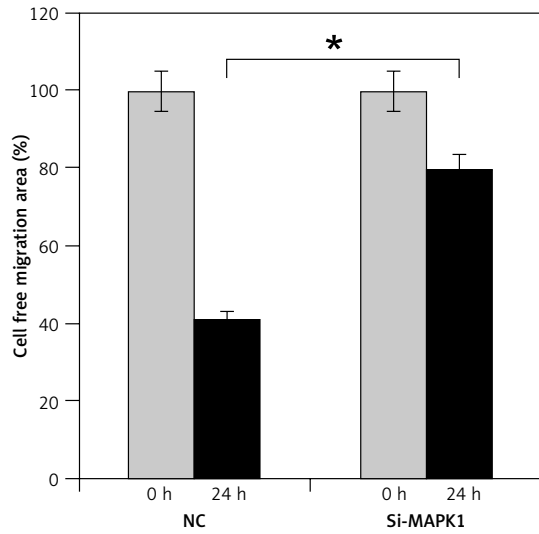


Figure 5. MAPK1 and lncRNA HOTAIR silencing exert synergistic inhibitory effects on proliferation of DoTc2 cells

*The experiments were performed in triplicate and results are expressed as mean ± SD (*p < 0.01 for Si-HOTAIR and Si-MAPK1 vs. Si-HOTAIR + Si-MAPK1)*

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