Immunol Genet J, Vol. 8, No. 1, 2025, pp.111-123 DOI: https://doi.org/10.18502/igj.v8i1.17996

Original Article

Breast Cancer-Associated Fibroblasts Could Induce the PI3K/Akt/mTOR Signaling Pathway Through Downstream Long Non-Coding RNA HOTAIR

Mona Sadeghalvad[1], Hamid-Reza Mohammadi-Motlagh[2], Kamran Mansouri[2], Ali Mostafaie[2], Farshid Noorbakhsh[1], Sadaf Alipour[3,4], Foroozan Ghalkhani[5], Ramesh Omranipour[3], Seid-Shahram Miraghaee[2], Sarah Kiani[2], Nima Rezaei[1,6,7*]

[1] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
[3] Breast Disease Research Center, Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Surgery, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran
[5]Imam Reza Hospital, AJA University of Medical Sciences, Tehran, Iran
[6] Research Center for Immunodeficiencies, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
[7] Network of Immunity in Infection, Malignancy and Autoimmunity (NIIMA), Universal Scientific Education and Research Network (USERN), Tehran, Iran

Received: 06 November 2024; Accepted: 11 February 2025

Abstract

Background: Cancer-associated fibroblasts (CAFs) play an important role in the initiation and progression of tumor cells. These cells can trigger signaling pathways involved in tumor progression. HOTAIR, an increased long non-coding RNA (lncRNA) in breast cancer, has a vital role in the tumorigenesis and development of breast cancer cells.

Methods: In this study, a fibroblast cell culture medium was used to investigate its possible role in inducing the HOTAIR expression and PI3K/Akt/mTOR pathway in breast cancer cells. CAFs and normal fibroblasts (NFs) were isolated from tumors of 6 patients with breast cancer and subjects with healthy breasts, respectively. The MCF-7 cells were cultured in a medium obtained from CAFs (CAF-CM) or NFs (NF-CM), and then the expression of HOTAIR and PI3K/Akt/mTOR in MCF-7 cells was assessed using Real-Time PCR. HOTAIR was silenced in MCF-7 cells using siRNAs and then cultured in CAF-CM or NF-CM. Subsequently, the phosphorylation status of PI3K/Akt/mTOR proteins was analyzed by western blotting.

Results: Fibroblast culture medium enhanced the expression of HOTAIR and activation of the PI3K/Akt/mTOR pathway in breast cancer cells. By HOTAIR silencing, reduced activity of the PI3K/Akt/mTOR pathway, as well as the lower effect of fibroblast culture medium in the induction of PI3K/Akt/mTOR pathway, was seen.

Conclusion: HOTAIR can play a role as a mediator in inducing the PI3K/Akt/mTOR pathway in breast cancer cells by the effect of cancer-associated fibroblast cells.

Keywords: Breast Cancer; HOTAIR; PI3K/Akt/mTOR Pathway; Tumor-Associated Fibroblasts; Tumor Microenvironment

***Corresponding Author:** Nima Rezaei, MD, PhD
Research Center for Immunodeficiencies, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
E-mail: rezaei_nima@tums.ac.ir

How to cite this article
Sadeghalvad M, Mohammadi-Motlagh HR, Mansouri K, Mostafaie A, Noorbakhsh F, Alipour S, et al. Breast Cancer-Associated Fibroblasts Could Induce the PI3K/Akt/mTOR Signaling Pathway Through Downstream Long Non-Coding RNA HOTAIR. Immunol Genet J, 2025; 8(1): 111-123. DOI: https://doi.org/10.18502/igj.v8i1.17996

Introduction

Breast cancer is one of the most common human cancers, accounting for 25% of all cancers in women and 12% of all cancers in the world (1). Despite the many studies conducted on the characteristics of cancer cells, the mechanisms involved in the ability of tumor cells to invade and survive remain largely unknown (2).

Cancer-associated fibroblasts (CAFs) are among the largest populations of cells in the tumor microenvironment (TME) involved in the initiation, growth, progression, metastasis, angiogenesis, and inhibition of tumor cell apoptosis (3-5). CAFs mediate their effects mainly by secreting growth factors such as tumor growth factor β (TGF-β), vascular endothelial growth factor (VEGF). (6, 7). These mediators promote tumor cell growth and metastasis by activating several signaling pathways in tumor cells including PI3K/Akt/mTOR (8-12).

The PI3K/Akt/mTOR signaling pathway regulates many important cellular processes in cell growth through increased protein synthesis and glucose and lipid metabolism (12, 13). This signaling pathway is involved in regulating processes including apoptosis, cell cycle and metastasis in many types of cancers and can be regulated by several mediators including non-coding RNAs (12). Recently, the role of long non-coding RNAs (lncRNAs), a major class of non-coding transcripts, has been given great importance in the pathogenesis of several cancers, including breast cancer (14-18). These molecules can regulate a variety of cellular processes by influencing or interacting with different elements, including proteins, DNA, RNA molecules, and transcription factors (19- 29). HOTAIR (HOX transcript antisense intergenic RNA) is an lncRNA involved in the proliferation, progression, survival, and metastasis of various types of tumors including breast, liver, pancreas, and lung cancer (16, 30-33). Increased expression of HOTAIR is associated with the migration and invasion of breast tumor cells, and might worsen the prognosis and severity of the disease (34-36). Recent studies have shown a relationship between the activation of the PI3K/Akt/mTOR pathway and HOTAIR lncRNA in cancers such as melanoma and osteosarcoma. It has been demonstrated that the increase in HOTAIR expression leads to an activation of mTOR pathway and, as a result, an increase in metastasis and tumor progression (37, 38). In the current study, to investigate the paracrine effects of fibroblasts on breast cancer cells, the effect of fibroblast culture medium on the induction of the PI3K/Akt/mTOR signaling pathway in breast cancer cells was studied in vitro. Moreover, the expression of HOTAIR was investigated as a probable mediator in the activation of the PI3K/Akt/mTOR pathway by fibroblasts.

Materials and Methods

Study Design and Subjects

The study was in accordance with the Declaration of Helsinki and conducted with approval from the Tehran University of Medical Sciences (TUMS) Ethics Committee with the number of IR.TUMS.MEDICINE.REC.1398.386. Two groups including patients with breast cancer and healthy people, were studied after obtaining a consent form. Fresh human mammary tumor samples (0.7 -1cm in diameter) were isolated from six cases of invasive ductal breast carcinomas (IDC, Luminal A) at the time of surgical excision. The patients with newly diagnosed IDC without any background of receiving chemotherapy or anti-cancer therapies were included, and the patients with benign tumors and non-invasive cancers were excluded from the study. Normal breast tissue was obtained from six healthy age-matched subjects without any background in breast cancer or receiving hormone therapies who were under reduction mammoplasty. After pathological examination, the tissues were classified as cancerous and healthy.

Isolation of Human Breast Fibroblasts

Fresh tissues obtained from the patients with cancer or the healthy individuals were transported to the laboratory in sterile normal saline on ice. Tissues were washed twice with sterile normal saline and then minced in a sterile glass petri dish using sterile scalpels and scissors and then transferred to a sterile tube. Dulbecco's modified Eagle's medium (DMEM) containing collagenase I (1 mg/mL) (Sigma), fetal bovine serum 10% (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island,

NY) was filtered through a 0.2 µm filter and added to the minced tissue. The tubes incubated for 5 hours at 37 °C in a shaker at 230 rpm (approximate angle position of 45°). Then, the tubes were centrifuged at 2000 xg for 3 min. The supernatant was discarded, and the obtained pellet was washed with DMEM. The pellet was cultured in 25 cm2 tissue culture flasks containing FBS 15%, 100 U/ml penicillin, and 100 µg/ml streptomycin, and maintained in a humidified incubator at 37 °C with 5% CO2. The medium was changed after 6 days when fibroblasts sprouted from the tissue to remove debris. Finally, fibroblast cells were confirmed using light microscopy and immunocytochemistry (ICC).

Cell Culture

Fibroblast cells were cultured in the complete medium (supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin/100 µg/ml streptomycin). After 48 hours, the medium was replaced with a serum-free medium and cultured for the subsequent 48 hours. Thereafter, the culture medium (CM) was collected, centrifuged at 2500 xg for 10 minutes, filtered through a 0.2 µm filter, and kept at -80°C until use. The breast cancer cell line MCF-7 was purchased from the National Cell Bank, Pasteur Institute (Tehran, Iran). Cells were cultured in complete DMEM and were maintained in a humidified incubator at 37 °C with 5% CO2.

Cytokine Assay

The level of IL-6 and IL-10 cytokines in the fibroblast cells' culture medium was measured by the standard enzyme-linked immunosorbent assay method (R & D).

Immunocytochemistry (ICC)

Both NFs and CAFs were plated in 6-well plates. After confluency of about 70%, cells were washed with PBS, twice for 5 minutes each time and analyzed for α-smooth muscle cell actin (α-SMA), vimentin, cytokeratin, CD31, and CD34 (DAKO). Briefly, cell fixation was performed using paraformaldehyde 4% in PBS for 20 min at room temperature (RT). After washing, peroxidase blocking solution was added and incubated for 10 minutes RT in a dark place. A permeabilizing solution, PBS containing Triton X-100 0.1%, was added for the detection of α-SMA and cytokeratin (intracellular markers). Cells were washed using PBS containing Tween 0.05%. Blocking was performed using BSA 1% in PBS for 1 hour at RT. After washing, primary antibodies were added, and the cells were incubated for 2 hours at RT. HRP-Conjugated secondary antibody (Razi Biotech, Iran) was added and incubated for 1 hour at RT. Then 3, 3'-Diaminobenzidine (DAB, Razi Biotech, Iran) was added as a substrate and the plate was incubated for 5 minutes. Hematoxylin was added after washing, and the cells were analyzed using light microscopy.

RNA Interference and Transfection

Smart pool siRNA-HOTAIR and scramble sequences (negative control siRNA) were purchased from Dharmacon. Smart pool siRNA-HOTAIR consisted of a mixture of 4 siRNAs targeting HOTAIR. Target sequences for siRNA-HOTAIR were as follows: 5'AGACGAAGGUGAAAGCGAA-3', 5'-CAAUAUAUCUGUUGGGCGU-3', 5'G GACUGGGAGGCGCUAAU-3', 5'CAGU GAAUGGAACGGAUU-3'.

MCF-7 cells were cultured in separate groups for transfection with siRNA-HOTAIR (siR-HOTAIR) or siRNA-scramble (siR-NC), an un-transfected cells. MCF-7 cells were transfected with siRNA-HOTAIR or scrambled sequences using Attractene transfection reagent (Qiagen) according to the manufacturer's instructions. Briefly, 3×104 MCF-7 cells/well were seeded into 24-well plates. The transfection medium was prepared by adding 1.5 µl of Attractene transfection reagent to 100 µl of serum-free DMEM medium containing siR-HOTAIR or siR-NC at a final concentration of 100 nM. The tube was incubated at room temperature for 20 minutes. The mixture was then added drop wise to cells maintained in 100 µl serum free media in 24-well plates. After 5 hours, the medium in each group of MCF-7 cells was replaced with DMEM (control group), CAF-CM, or NF-CM. Cells were maintained in a humidified incubator at 37°C with 5% CO2 and were evaluated 72h after transfection.

RNA Extraction and cDNA Synthesis

RNA was extracted from MCF-7 cells transfected with siR-HOTAIR, siR-NC, and untransfected cells according to the manufacturer's instructions

(RNX plus isolation kit, Sinaclon, Iran). The RNA purity was assessed by 1% agarose gel electrophoresis, and its concentration was measured using a NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Scientific, USA). The extracted RNA was kept at −70°C until use. The cDNA synthesis was performed using the Prime Script RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Briefly, 50–100 ng of total RNA in a 10-µl sample volume was reverse transcribed using both oligo-dT/random hexamer primers. cDNA was kept at −20°C until analysis of gene expression.

Quantitative Real-time Polymerase Chain Reaction

Real-time reverse transcription PCR was performed in order to investigate the efficiency of HOTAIR downregulation by siRNAs and also the expression of PI3K, Akt, and mTOR mRNAs. The reaction was performed in a total volume of 20 µl including 1 µl of cDNA, 0.5 µM of each forward and reverse primer, 10 µl of SYBR® Premix Ex Taq™ II (Takara Bio, Inc., Otsu, Japan), and 8 µl of H2O. The qPCR reactions were performed on the Rotor-Gene 6000 machine (Corbett Research, Australia) using the universal thermal cycling parameters, including an initial denaturation at 95°C for 30 sec, and 40 cycles, including a denaturation at 95°C for 5 sec, an annealing at 60°C (for HOTAIR, PI3K, and mTOR), and 57°C for 20 sec (for AKT), and an extension at 72°C for 30 sec. Finally, a melting curve analysis was performed at 60-95°C. The primer sequences were indicated in **Table 1**. The relative expression levels were normalized to the endogenous control β-actin and were expressed as 2-ΔΔCt (39). Data were expressed as fold changes in the amount of mRNA.

Immunoblotting Assay

The immunoblotting assay was performed according to a previously described method (40). To analyze the expression of PI3K, phospho-PI3K (p-PI3K, p85 alpha + gamma (Tyr467 + Tyr199), Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), and β-actin. The MCF-7 cells (transfected with siR-HOTAIR, siR-NC, and non-transfected) were treated with the NF-CM, CAF-CM, and DMEM for 72 hours. Cells were washed twice with phosphate buffer saline (PBS) and the proteins were extracted using 0.5 ml of lysis buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl), containing a protease inhibitor cocktail (Roche). Cell lysates were collected by centrifugation (14,000 xg for 15 min at 4 °C) and kept in -20 °C until use. The Bradford assay was used to determine the protein concentration. The cell lysate was mixed in SDS-PAGE sample buffer containing 10% SDS and then incubated for 10 minutes and resolved in 10% polyacrylamide separating gels at 150 V. The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using tank blotting. The membranes were rinsed 3 times in TBS containing 0.05% (v/v) Tween 20 (TBS-Tween 20), and then blocked in PBS-BSA 2% (w/v) for 2 h. The membranes were incubated at 4˚C overnight in primary anti-human PI3K (Biolegend), phospho-PI3K (p-PI3K, p85 alpha + gamma (Tyr467 + Tyr199) (Bioss Inc)), Akt (Biolegend), p-Akt (Ser473, R&D), mTOR (Biolegend), p-mTOR (Ser2448, Biolegend), and β-actin (Biolegend). After washing three times in TBS-Tween 20, the membranes were incubated for 90 minutes in corresponding secondary antibodies (human anti-mouse IgG, or human anti-rabbit IgG HRP conjugated, Razi Biotech, Iran). Finally, the target protein bands were detected by chemiluminescence assay.

Cell Proliferation Assay

Cell growth was evaluated by 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) assay 72h post transfection. Briefly, 2 × 103 cells/well were seeded in 96-well plates. MTT solution with a concentration of 5 mg/ml in PBS was added to each well, and plates were incubated at 37°C for 3h. Thereafter, the supernatants were discarded, and the formazan crystals were solubilized by dimethyl sulfoxide (DMSO). The absorbance was measured using a microplate reader (Awareness Stat Fax 2100) at 570 nm.

Statistical Analysis

One-way ANOVA and independent sample t-test were used to detect significant differences between groups. Data analysis was performed by SPSS software version 21 (SPSS, Chicago, IL, USA) and GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA). $P < 0.05$ was

considered statistically significant.

Results

Fibroblast Isolation and Characterization

Isolated fibroblasts had spindle-shaped or stellate-like morphology and progressively formed the structure of a network. Both CAFs and NFs were positive for α-SMA and vimentin using immunocytochemistry **(Figure 1)**. Whereas CD31 and CD34 are endothelial cell markers and cytokeratin is an epithelial marker, they were negative for both CAFs and NFs (Figure 1). IL-6 and IL-10 cytokines were higher in the CAFs-CM compared to NFs-CM The results of evaluating the level of cytokines IL-6 and IL-10 in fibroblasts' culture medium showed that the levels of both IL-6 and IL-10 in CAF-CM were significantly higher than NF-CM (IL-6: p <0.01, IL-10: p <0.05). In addition, the level of IL-6 in both CAF-CM and NF-CM was significantly higher than the level of IL-10 in these mediums (p <0.01) **(Figure 2)**. The fibroblasts' culture medium upregulates the expression of HOTAIR and PI3K/Akt/mTOR signaling pathway in MCF-7 cells. The expression of HOTAIR, PI3K, Akt, and mTOR mRNAs was assessed in MCF-7 cells treated with CAF-CM, NF-CM, and DMEM (control group). Results showed that the expression of HOTAIR was increased in MCF-7 cells treated with CAF-CM and NF-CM compared to the control group (p<0.01). The effect of CAF-CM on HOTAIR expression was significantly higher than the effect of NF-CM (p<0.01) **(Figure 3)**. Treatment with CAF-CM and NF-CM compared to the control group led to a significant increase in expression of HOTAIR (p<0.01), PI3K, Akt, and mTOR (NF-CM: p<0.05, CAF-CM: p<0.01). Also, treatment with CAF-CM led to a significant increase in PI3K, Akt, and mTOR expression compared to treatment with NF-CM (p<0.01) (Figure 3). The fibroblasts' culture medium enhanced the phosphorylation of PI3K, Akt, and mTOR in MCF-7 cells. To investigate the effect of fibroblasts' culture medium on the activation of the PI3K/Akt/mTOR signaling pathway in MCF-7 breast cancer cells, the ratio of phosphorylated to total form (p/t) of PI3K, Akt, and m-TOR proteins was analyzed in untreated and treated MCF-7 cells **(Figure 4)**. The results showed that both NF-CM and CAF-CM treatment led to an increase in the p/t ratio of PI3K, Akt, and mTOR proteins compared to the control group treated with DMEM. On the other hand, the p/t ratio of these proteins was significantly higher in MCF-7 cells treated with CAF-CM compared to the cells treated with NF-CM and the control group (Figure 4). These results suggest the effect of fibroblasts' culture medium on the activation of the PI3K/Akt/mTOR signaling pathway in breast cancer cells.

HOTAIR Downregulation

To analyze the role of lncRNA HOTAIR on the activation of the PI3K/Akt/mTOR signaling pathway, HOTAIR was downregulated in MCF-7 cells using siRNAs. The efficiency of HOTAIR downregulation was investigated by Real-Time PCR. The expression of HOTAIR showed a significant decrease (p<0.01) after transfection with specific siRNAs (siR-HOTAIR) compared to the group of cells transfected with siRNA-Scramble (siR-NC) and non-transfected cells **(Figure 5A)**. The MTT assay result showed that HOTAIR silencing resulted in a significant decrease in the transfected MCF-7 cells compared with the cells transfected with scrambled sequences or cells without transfection (p < 0.01) **(Figure 5B)**. This finding may confirm that the knockdown of lncRNA-HOTAIR could result in the suppression of the proliferation of MCF-7 cells.HOTAIR downregulation resulted in less activation of the PI3K/Akt/mTOR signaling pathway in MCF-7 cells. The p/t ratio of PI3K, Akt, and mTOR proteins after HOTAIR downregulation was assessed by western blotting. The results indicated that with HOTAIR downregulation, the p/t ratio of PI3K, Akt, and mTOR proteins was significantly decreased in MCF-7 cells compared to the untransfected control (mock) or siR-NC group **(Figure 6)**. This result could indicate a possible role of HOTAIR in the activation of the PI3K/Akt/mTOR signaling pathway in MCF-7 breast cancer cells. In addition, the p/t ratio of PI3K, Akt, and mTOR proteins was significantly decreased in the cells whose HOTAIR was knocked down and then were treated with CAF-CM or NF-CM, compared to the mock or siR-NC groups (Figure 6). These results show that HOTAIR probably plays a role as a mediator in the induction of the PI3K/Akt/mTOR signaling pathway by fibroblasts.

Table 1. The primer sequences used in the gene expression analysis.

Gene name	Forward (5'>3')	Reverse (5'>3')
HOTAIR	GGTAGAAAAAGCAACCACGAAGC	**ACATAAACCTCTGTCTGTGAGTGCC**
PI3K	GAACGAGTGGTTGGGCAATG	**CCTCGCAACAGGTTTTCAGC**
AKT	ACAGGTGGAAGAACAGCTCG	**ACAGGTGGAAGAACAGCTCG**
mTOR	GCTTGATTTGGTTCCCAGGACAGT	**GTGCTGAGTTTGCTGTACCCATGT**
β-actin	**TGGCACCCAGCACAATGAA**	**CTAAGTCATAGTCCGCCTAGAAGCA**

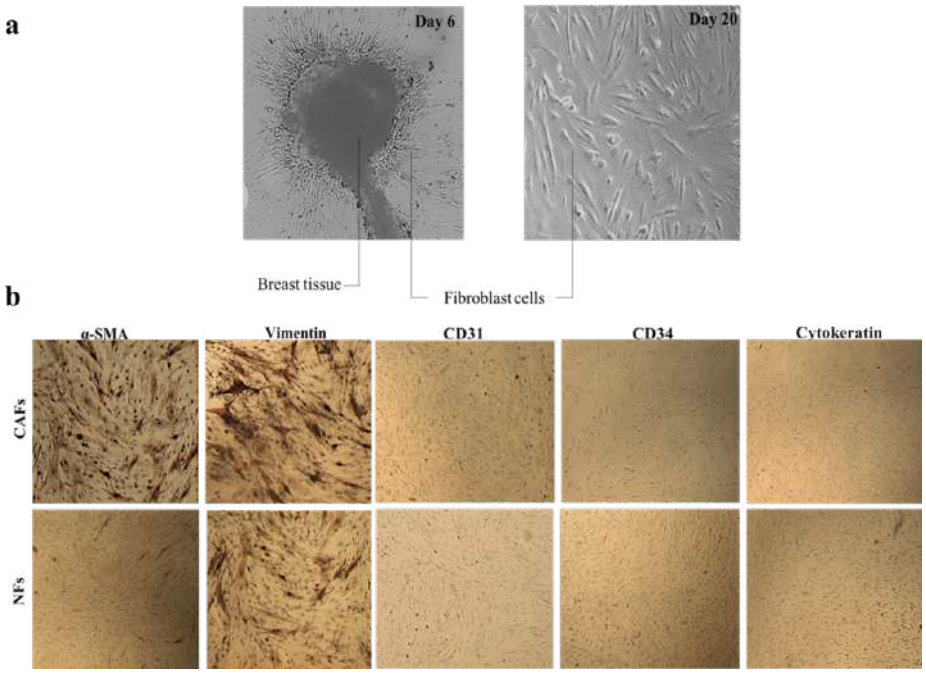

Figure 1. Characterization of isolated fibroblasts with light microscopy. The expression of α-SMA, vimentin, CD31, CD34, and cytokeratin in the fibroblasts isolated from patients with invasive ductal breast carcinoma (CAFs) and healthy individuals (NFs).

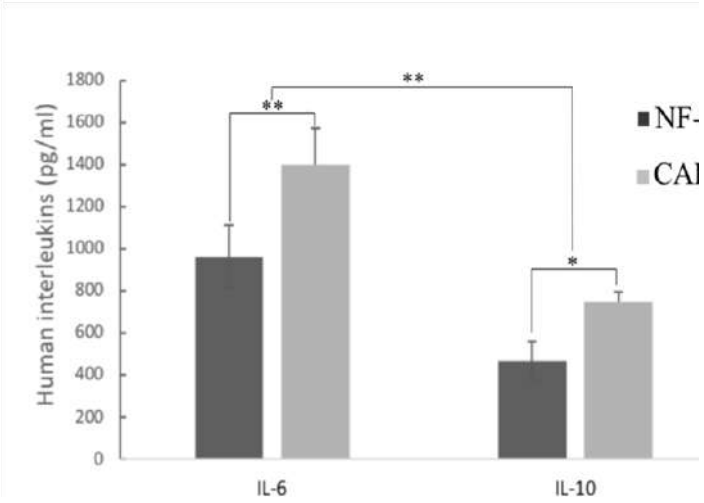

Figure 2. The level of IL-6 and IL-10 in cancer-associated fibroblasts' culture medium (CAF-CM) and normal fibroblasts' culture medium (NF-CM). N=6 in each group, *$p < .05$, **$p < .01$.

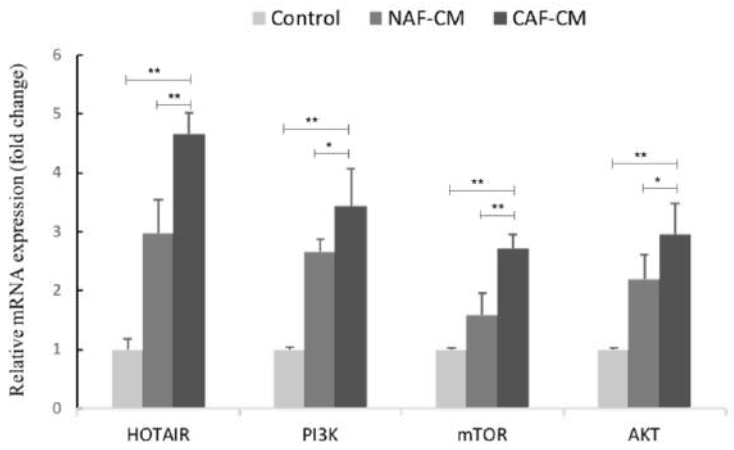

Figure 3. The expression level of HOTAIR, mTOR, PI3K, and AKT in MCF-7 cells after treatment with cancer-associated fibroblasts' culture medium (CAF-CM) and normal fibroblasts' culture medium (NF-CM). Data presented as relative gene expression changes in the treated cells compared to the controls (*$p < 0.05$, **$p < 0.01$).
Abbreviations: HOX transcript antisense RNA; mTOR, Mammalian target of rapamycin; PI3K, Phosphoinositide 3-kinases; AKT, protein kinase AKT.

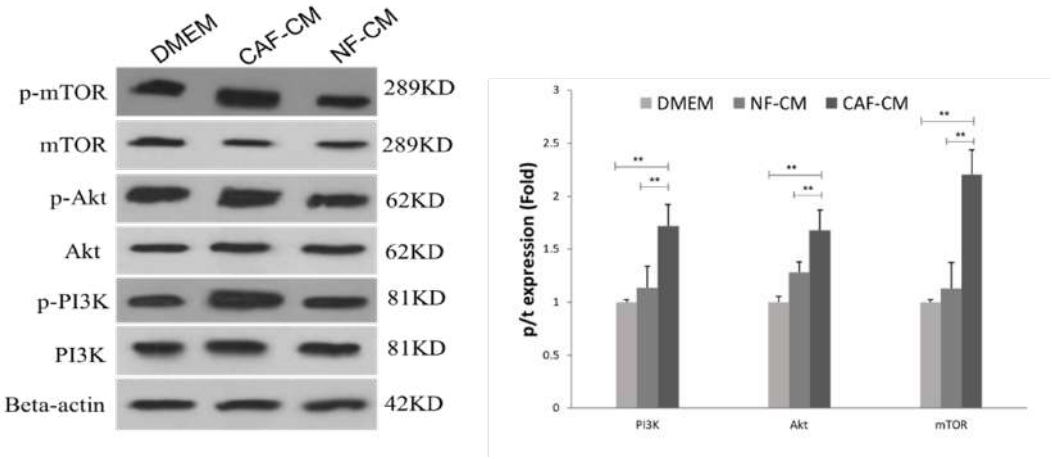

Figure 4. The phosphorylated/total (p/t) ratio of PI3K, Akt, and mTOR proteins in MCF7 cells treated with DMEM (negative control), cancer-associated fibroblast culture medium (CAF-CM), and normal fibroblast culture medium (NF-CM). Data presented as changes in the treated cells compared to the controls (*$p < 0.05$, ** $p < 0.01$). Density determination of bands was carried out by Image J software.

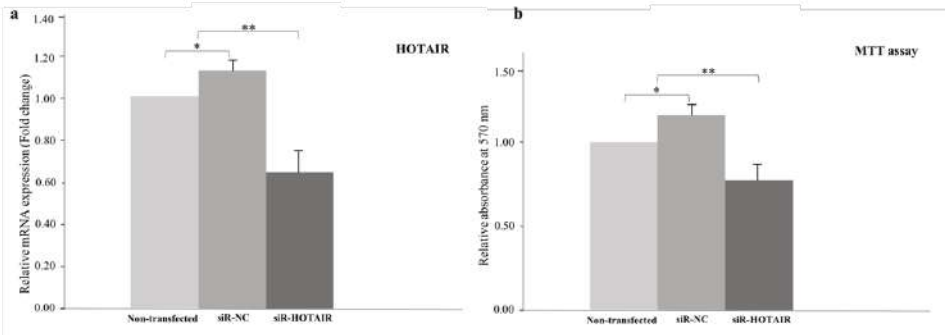

Figure 5. (A) The expression level of HOTAIR and (B) MTT assay in MCF-7 cells without transfection (non-transfected), and MCF-7 cells transfected with siRNA-scramble (siR-NC) or siRNA-HOTAIR (siR-HOTAIR). Data in Figure 5A are presented as relative gene expression changes in the treated cells compared to the controls (*$p < 0.05$, ** $p < 0.01$). Values are expressed as the mean ± standard deviation (n=3). HOTAIR, HOX transcript antisense RNA.

Discussion

In this study, a fibroblast cell culture medium was used to investigate its possible role in inducing the PI3K/Akt/mTOR pathway in breast tumor cells by downstream lncRNA HOTAIR. The result of this study showed that breast tumor-associated fibroblasts, as a part of the TME, can induce the activation of the PI3K/Akt/mTOR signaling pathway and also the expression of lncRNA HOTAIR in MCF-7 breast tumor cells. The role of TME in increasing the progression and invasion of tumor cells has been studied for years (41, 42). Fibroblast cells are an important part of the TME, and many studies have shown their importance in the progression, invasion, and survival of cancer cells (3, 5-7). Studies have shown that HOTAIR is one of the increased lncRNAs in breast cancer that plays a vital role in the proliferation, invasion, and metastasis of breast cancer cells. Besides, increased expression of HOTAIR is associated with poor prognosis in patients with breast cancer (43-45).

The results of this study showed that the expression of HOTAIR increased about 4.7 and 2.6 times in the MCF-7 group treated with CAF-CM or NF-CM compared to the control group. In line with the present study, Ren Y *et al*. found that treatment of breast cancer cells with CAF-CM led to the up-regulation of HOTAIR and increased epithelial-mesenchymal transition (EMT) in cancer cells. They showed that TGF-β1 produced by CAFs is an important mediator to induce HOTAIR in breast cancer cells (46). On the other hand, the downregulation of HOTAIR inhibits tumor growth caused by CAFs and reduces lung metastasis in the MDA-MB-231 orthotopic animal model (46). Regarding the role of CAF in inducing the HOTAIR in other types of cancers, Sun and colleagues have recently shown that isolated CAFs from NSCLC patients secrete high levels of CCL5, which has been implicated in the induction of HOTAIR expression, which subsequently leads to the reduction of cisplatin (DDP)-induced apoptosis in tumor cells (47). Therefore, the results of this study and previous studies further indicate that CAFs may play a role in the growth and progression of cancer cells by inducing HOTAIR expression. In the present study, downregulation of HOTAIR using siRNAs led to a significant decrease in the ratio of phosphorylated to total (p/t) form of PI3K, Akt, and mTOR proteins in MCF-7 cells, suggesting a lower activation of this pathway following HOTAIR silencing. In line with the present study, previous studies have also reported the relationship between HOTAIR and the PI3K/Akt/mTOR signaling pathway in different types of cancers, including osteosarcoma, melanoma, gastric, and breast cancers (37, 38, 48, 49). Of course, the exact mechanism of this connection is still unknown. Recently, we showed that HOTAIR downregulation could decrease the expression of PI3K, Akt, and mTOR in MCF-7 cells (50). Another study showed that HOTAIR knockdown significantly reduced the phosphorylation of PI3K, Akt and mTOR in doxorubicin-resistant MCF-7 cells and inhibited this signaling pathway, which resulted in a decrease in the resistance of breast cancer cells to doxorubicin. In addition, the reduction of HOTAIR expression led to decreased cell proliferation and increased apoptosis in doxorubicin-resistant MCF-7 cells (48). The role of HOTAIR in inducing the PI3K/Akt/mTOR signaling pathway is also shown in other types of cancers that are summarized in **Table 2**. Therefore, the results of the present study and previous studies show that HOTAIR can be considered as a mediator in inducing the PI3K/Akt/mTOR signaling pathway in cancer cells. Although the exact mechanism and relationship of this lncRNA with this signaling pathway are not fully known, it can be postulated that HOTAIR or other lncRNAs can exert their regulatory activities at the level of transcription, post-transcription, and also at the post-translational level (19, 51). **Figure 7** summarizes the possible mechanisms through which fibroblasts may be involved in the induction of lncRNA HOTAIR and, subsequently, in the induction of the PI3K/Akt/mTOR signaling pathway. Among the mechanisms that justify the effect of HOTAIR knockdown on PI3K/Akt/mTOR pathway activity, HOTAIR's role in inhibiting the *HOXD* gene can be mentioned. By interacting with two protein complexes named PRC2 and LSD1, HOTAIR leads to H3K27me3 methylation of the target regions in the *HOXD* gene and, as a result, decreases the expression of its target molecules (43, 52). It has been shown that increased expression of HOTAIR can lead to increased invasion of breast cancer cells by decreasing HOXD10 expression (53).

On the other hand, it has been shown that the

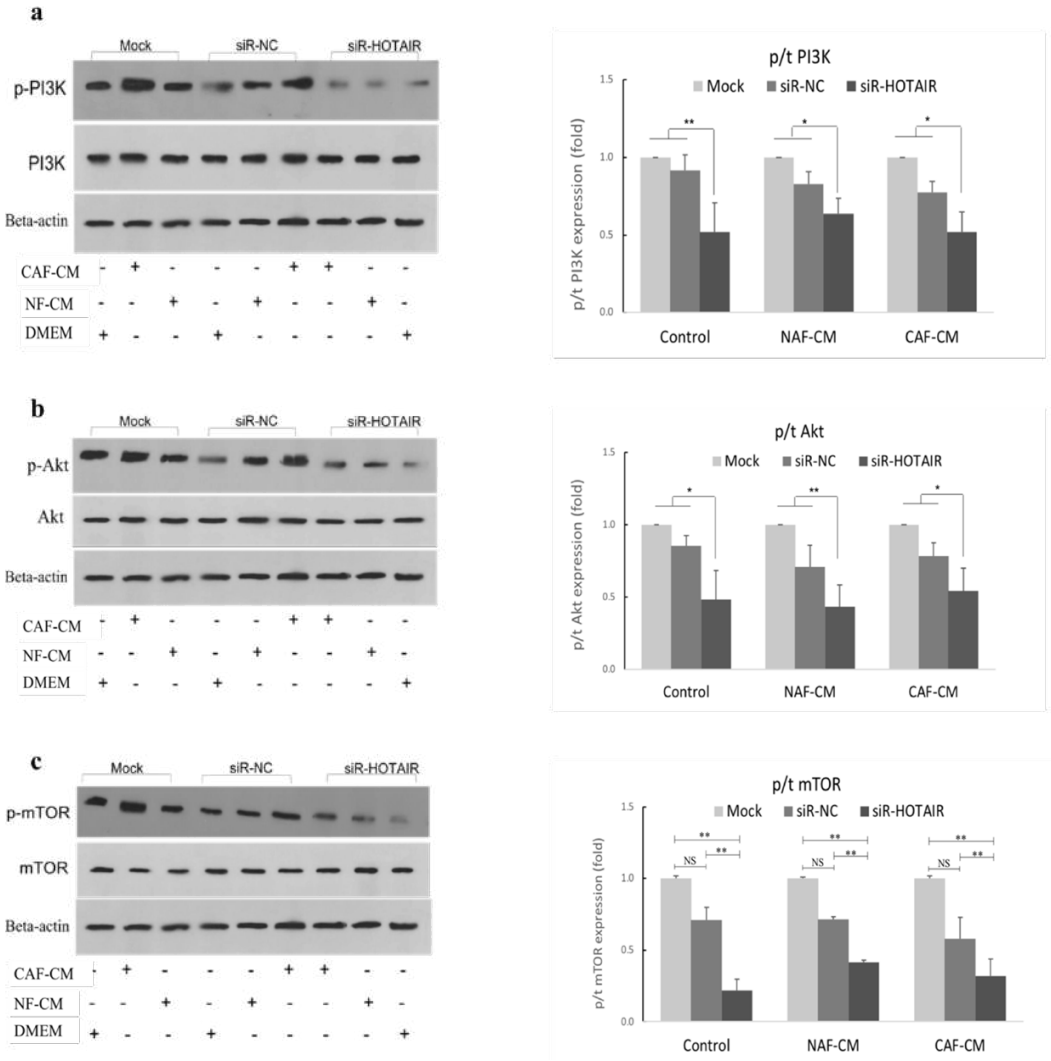

Figure 6. The ratio of phosphorylated to total expression (p/t) of (A) PI3K, (B) Akt, (C) mTOR protein in non-transfected (mock), transfected with siRNA-Scramble (siR-NC), and transfected with siRNA-HOTAIR (siR-HOTAIR) groups. CAF-CM: treated with supernatant culture of cancer-associated fibroblasts. NF-CM: treated with culture medium of normal fibroblasts. Control: treatment with DMEM medium. The number of samples in each group is n=5, data are displayed as Mean±SEM (NS: not significant, * p<0.01, ** p<0.05). (Density determination of bands with Image J software).

Table 2. The role of HOTAIR in inducing the PI3K/AKT/mTOR signaling pathway in some cancers.

Cancer type	Role	Ref
Gastric cancer	HOTAIR plays a role in the resistance of gastric cancer cells to cisplatin, and this resistance is reduced by inhibiting HOTAIR. This effect of HOTAIR is mediated by the PI3K/Akt axis and mir- 34a, and therefore, inhibition of HOTAIR can result in decreased levels of Akt phosphorylation and inhibition of PI3K/Akt pathway	**(49)**
Melanoma cancer	Reduced expression of HOTAIR was associated with the reduction of mTOR protein expression. An increase in HOTAIR expression was related to an increase in the mTOR protein phosphorylation	**(37)**
Osteosarcoma	HOTAIR has led to the proliferation and metastasis of osteosarcoma cancer cells through the induction of the PI3K/Akt/ mTOR pathway so that the inhibition of HOTAIR with siRNAs led to a decrease in the phosphorylation of PI3K, Akt and mTOR proteins	**(37)**

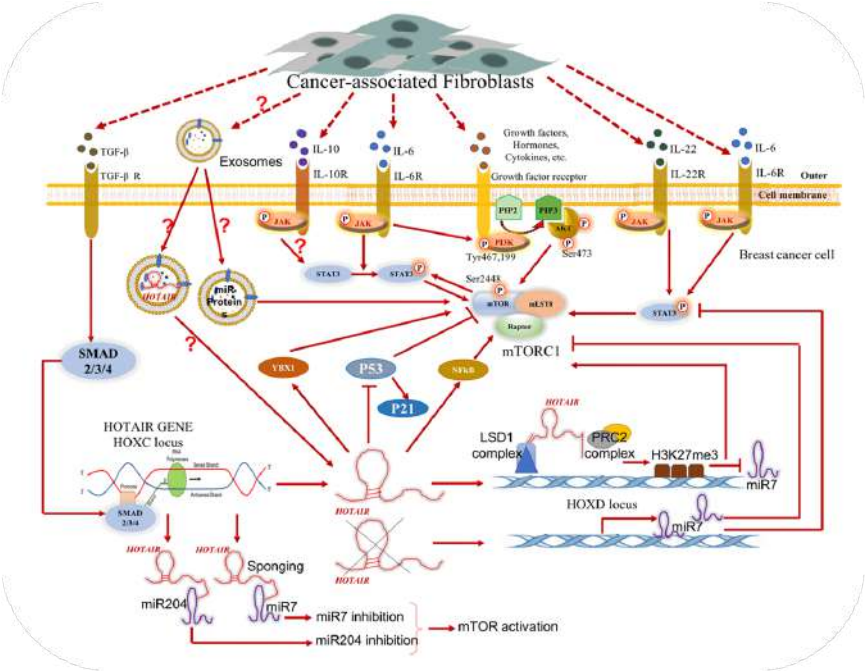

Figure 7. The proposed pathway for the role of fibroblast cells in the tumor microenvironment in the induction of lncRNA HOTAIR and subsequently in the induction of the PI3K/Akt/mTOR signaling pathway. HOTAIR has the ability to create a sponge with miR7 and can inhibit its function in breast cancer cells. miR7 is a tumor suppressor and is itself an inhibitor of the mTOR pathway (55). Therefore, by forming a sponge with miR7 and inhibiting it in breast cancer cells, HOTAIR can lead to an increase in the expression of the PI3K/Akt/mTOR pathway and an increase in tumor invasion. In addition, the reduction of HOXD10 expression under the effect of HOTAIR has led to the reduction of miR7 expression and increased invasion and induction of EMT in cancer cells (55).
Abbreviations: HOTAIR, HOX antisense intergenic RNA; mTOR, Mammalian target of rapamycin; PI3K, Phosphoinositide 3-kinases; PIP2, Phosphatidylinositol 4,5-bisphosphate; PIP3, Phosphatidylinositol-3,4,5-trisphosphate; Ser, Serine amino acid; Tyr, Tyrosine amino acid; IL-10R, interleukin 10 receptor; IL-6 R, interleukin-6 receptor; miR, micro RNA; TGF-β, Tumor growth factor beta; LSD1, lysine-specific demethylase 1A; PRC2, Polycomb repressive complex 2; STAT, Signal transducer and activator of transcription; JAK, Janus kinase.

reduction of HOXD10 expression under the effect of HOTAIR leads to the induction of the PI3K/Akt/mTOR pathway and increases the invasion and metastasis of tumor cells (54). Therefore, it may be possible that HOTAIR, by methylation of the HOXD10 region and reducing its expression, indirectly plays a role in inducing the expression of the PI3K/Akt/mTOR pathway so that the downregulation of HOTAIR in this study leads to a decrease in the expression and activity of this pathway. The results of this study show that by reducing the expression of HOTAIR using siRNAs, the CAF-CM and NF-CM's effects on the activation of PI3K/Akt/mTOR pathway is significantly reduced compared to the time that the expression of HOTAIR is not manipulated. This result can indicate the role of HOTAIR as a mediator in inducing this signaling pathway under the effect of fibroblast cells. This is the first study regarding the role of CAFs in the induction of the PI3K/Akt/mTOR pathway by HOTAIR in breast cancer cells. The precise relationship between CAF cells and the HOTAIR-mediated induction of PI3K/Akt/mTOR pathway in cancer cells is still not fully understood. A study by Li and colleagues in 2019 showed that the level of the cytokine IL-22 was greatly increased in the lung CAF-CM compared to normal fibroblasts. Treatment with lung CAF-CM significantly increased the proliferation, migration, and invasion of lung cancer cells and also reduced the apoptosis of cancer cells through the activation of PI3K/Akt/mTOR (55). Considering the role of fibroblasts in the induction of HOTAIR and PI3K/Akt/mTOR signaling pathway in this study, the molecular mechanism of this relationship and finding the mediators that have a role in this pathway need more investigation.

Conclusion

It has been found that many invasive behaviors

of tumor cells are strongly influenced by the environment around the tumor. Fibroblast cells as an important part of the TME that have an important role in the progression, invasion, and survival of cancer cells by producing several mediators. As shown in this study, fibroblasts are able to induce HOTAIR expression in MCF-7 breast tumor cells. By downregulation of HOTAIR, we found that the activity of the PI3K/Akt/mTOR signaling pathway was reduced, which could indicate the possible role of HOTAIR in the induction of this signaling pathway. On the other hand, with HOTAIR downregulation, the effect of fibroblasts in inducing the activity of PI3K/Akt/mTOR was reduced. These results can indicate the role of HOTAIR as a mediator in inducing the PI3K/Akt/mTOR pathway by the effect of fibroblast cells.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

This study was supported by a research grant received from the Tehran University of Medical Sciences, Tehran, Iran (grant number 98-01-30-41086)

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359–86.
2. Wang Y, Zhou BP. Epithelial-mesenchymal transition in breast cancer progression and metastasis. Chin J Cancer. 2011;30:603.
3. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335–48.
4. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Cunha G, Hein P, et al. Carcinoma-associated fibroblasts stimulate tumor progression of initiated human epithelium. Breast Cancer Res. 2000;2:1.
5. Busch S, Acar A, Magnusson Y, Gregersson P, Rydén L, Landberg G. TGF-beta receptor type-2 expression in cancer-associated fibroblasts regulates breast cancer cell growth and survival and is a prognostic marker in pre-menopausal breast cancer. Oncogene. 2015;34:27–38.
6. Gout S, Huot J. Role of cancer microenvironment in metastasis: focus on colon cancer. Cancer Microenviron. 2008;1:69–83.
7. Hlatky L, Tsionou C, Hahnfeldt P, Coleman CN. Mammary fibroblasts may influence breast tumor angiogenesis via hypoxia-induced vascular endothelial growth factor up-regulation and protein expression. Cancer Res. 1994;54:6083–6.
8. Nagaraj NS, Datta PK. Targeting the transforming growth factor-β signaling pathway in human cancer. Expert Opin Investig Drugs. 2010;19:77–91.
9. Heldin CH, Landström M, Moustakas A. Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial–mesenchymal transition. Curr Opin Cell Biol. 2009;21:166–76.
10. Nye MD, Almada LL, Fernandez-Barrena MG, Marks DL, Elsawa SF, Vrabel A, et al. The transcription factor GLI1 interacts with SMAD proteins to modulate transforming growth factor β-induced gene expression in a p300/CREB-binding protein-associated factor (PCAF)-dependent manner. J Biol Chem. 2014;289:15495–506.
11. Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A. TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol Biol Cell. 2005;16:1987–2002.
12. LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. Drug Resist Updat. 2008;11:32–50.
13. Dowling RJ, Topisirovic I, Fonseca BD, Sonenberg N. Dissecting the role of mTOR: lessons from mTOR inhibitors. Biochim Biophys Acta Proteins Proteom. 2010;1804:433–9.
14. Sun X, Du P, Yuan W, Du Z, Yu M, Yu X, et al. Long non-coding RNA HOTAIR regulates cyclin J via inhibition of microRNA-205 expression in bladder cancer. Cell Death Dis. 2015;6:e1907.
15. Okugawa Y, Toiyama Y, Hur K, Toden S, Saigusa S, Tanaka K, et al. Metastasis-associated long non-coding RNA drives gastric cancer development and promotes peritoneal metastasis. Carcinogenesis. 2014;35:2731–9.
16. Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, et al. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene. 2013;32:1616–25.
17. Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, et al. Long noncoding RNA HO-

TAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Cancer Res. 2011;71:6320–6.
18. Xu C, Yang M, Tian J, Wang X, Li Z. MALAT-1: a long non-coding RNA and its important 3'end functional motif in colorectal cancer metastasis. Int J Oncol. 2011;39:169–75.
19. Fitzgerald KA, Caffrey DR. Long noncoding RNAs in innate and adaptive immunity. Curr Opin Immunol. 2014;26:140–6.
20. Hung T, Wang Y, Lin MF, Koegel AK, Kotake Y, Grant GD, et al. Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nat Genet. 2011;43:621–9.
21. Gomez JA, Wapinski OL, Yang YW, Bureau JF, Gopinath S, Monack DM, et al. The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-γ locus. Cell. 2013;152:743-54.
22. Huang Z, Luo Q, Yao F, Qing C, Ye J, Deng Y, et al. Identification of differentially expressed long non-coding RNAs in polarized macrophages. Sci Rep. 2016;6:1-10.
23. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011;147:358-69.
24. Sumazin P, Yang X, Chiu HS, Chung WJ, Iyer A, Llobet-Navas D, et al. An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. Cell. 2011;147:370-81.
25. Rubio-Somoza I, Weigel D, Franco-Zorilla JM, García JA, Paz-Ares J. ceRNAs: miRNA target mimic mimics. Cell. 2011;147:1431-2.
26. Gong C, Maquat LE. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. Nature. 2011;470:284-8.
27. Liu X, Li D, Zhang W, Guo M, Zhan Q. Long non-coding RNA gadd7 interacts with TDP-43 and regulates Cdk6 mRNA decay. EMBO J. 2012;31:4415-27.
28. Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N. Requirement for Xist in X chromosome inactivation. Nature. 1996;379:131-7.
29. Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature. 2012;482:339-46.
30. Pádua Alves C, Fonseca AS, Muys BR, Bueno RBL, Bürger MC, Souza JE, et al. The lincRNA Hotair is required for epithelial-to-mesenchymal transition and stemness maintenance of cancer cell lines. Stem Cells. 2013;31:2827-32.
31. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature. 2010;464:1071-6.
32. Yang Z, Zhou L, Wu LM, Lai MC, Xie HY, Zhang F, et al. Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann Surg Oncol. 2011;18:1243-50.
33. Nakagawa T, Endo H, Yokoyama M, Abe J, Tamai K, Tanaka N, et al. Large noncoding RNA HOTAIR enhances aggressive biological behavior and is associated with short disease-free survival in human non-small cell lung cancer. Biochem Biophys Res Commun. 2013;436:319-24.
34. Teschendorff AE, Lee SH, Jones A, Fiegl H, Kalwa M, Wagner W, et al. HOTAIR and its surrogate DNA methylation signature indicate carboplatin resistance in ovarian cancer. Genome Med. 2015;7:1-12.
35. Xue X, Yang YA, Zhang A, Fong K, Kim J, Song B, et al. LncRNA HOTAIR enhances ER signaling and confers tamoxifen resistance in breast cancer. Oncogene. 2016;35:2746-55.
36. Malek E, Jagannathan S, Driscoll JJ. Correlation of long non-coding RNA expression with metastasis, drug resistance and clinical outcome in cancer. Oncotarget. 2014;5:8027.
37. Li E, Zhao Z, Ma B, Zhang J. Long noncoding RNA HOTAIR promotes the proliferation and metastasis of osteosarcoma cells through the AKT/mTOR signaling pathway. Exp Ther Med. 2017;14:5321-8.
38. Shang Z, Feng H, Cui L, Wang W, Fu H. Propofol promotes apoptosis and suppresses the HOTAIR-mediated mTOR/p70S6K signaling pathway in melanoma cells. Oncol Lett. 2018;15:630-4.
39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 2001;25:402-8.
40. Sanadgol N, Mostafaie A, Mansouri K, Bahrami G. Effect of palmitic acid and linoleic acid on expression of ICAM-1 and VCAM-1 in human bone marrow endothelial cells (HBMECs). Arch Med Sci. 2012;8:192-8.
41. Sadeghalvad M, Mohammadi-Motlagh HR, Rezaei N. Immune microenvironment in different molecular subtypes of ductal breast carcinoma. Breast Cancer Res Treat. 2021;185:261-79.
42. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumor microenvironment in tumorigenesis. J Cancer. 2017;8:761.
43. Mozdarani H, Ezzatizadeh V, Parvaneh RR. The emerging role of the long non-coding RNA HO-

TAIR in breast cancer development and treatment. J Transl Med. 2020;18:1-15.
44. Ma MZ, Li CX, Zhang Y, Weng MZ, Zhang MD, Qin YY, et al. Long non-coding RNA HOTAIR, a c-Myc activated driver of malignancy, negatively regulates miRNA-130a in gallbladder cancer. Mol Cancer. 2014;13:1-14.
45. Sørensen KP, Thomassen M, Tan Q, Bak M, Cold S, Burton M, et al. Long non-coding RNA HOTAIR is an independent prognostic marker of metastasis in estrogen receptor-positive primary breast cancer. Breast Cancer Res Treat. 2013;142:529-36.
46. Ren Y, Jia HH, Xu YQ, Zhou X, Zhao XH, Wang YF, et al. Paracrine and epigenetic control of CAF-induced metastasis: the role of HOTAIR stimulated by TGF-β1 secretion. Mol Cancer. 2018;17:1-14.
47. Sun X, Chen Z. Cancer-associated fibroblast-derived CCL5 contributes to cisplatin resistance in A549 NSCLC cells partially through upregulation of lncRNA HOTAIR expression. Oncol Lett. 2021;22:1-11.
48. Li Z, Qian J, Li J, Zhu C. Knockdown of lncRNA-HOTAIR downregulates the drug-resistance of breast cancer cells to doxorubicin via the PI3K/AKT/mTOR signaling pathway. Exp Ther Med. 2019;18:435-42.
49. Cheng C, Qin Y, Zhi Q, Wang J, Qin C. Knockdown of long non-coding RNA HOTAIR inhibits cisplatin resistance of gastric cancer cells. Int J Biol Macromol. 2018;107:2620-9.
50. Sadeghalvad M, Mansouri K, Mohammadi-Motlagh HR, Noorbakhsh F, Mostafaie A, Alipour S, et al. Long non-coding RNA HOTAIR induces the PI3K/AKT/mTOR signaling pathway in breast cancer cells. Rev Assoc Med Bras. 2022;68:456-62.
51. Zhang X, Wang W, Zhu W, Dong J, Cheng Y, Yin Z, et al. Mechanisms and functions of long non-coding RNAs at multiple regulatory levels. Int J Mol Sci. 2019;20(22):5573.
52. Hajjari M, Salavaty A. HOTAIR: an oncogenic long non-coding RNA in different cancers. Cancer Biol Med. 2015;12(1):1-9.
53. Yang H, Zhou J, Mi J, Ma K, Fan Y, Ning J, et al. HOXD10 acts as a tumor-suppressive factor via inhibition of the RHOC/AKT/MAPK pathway in human cholangiocellular carcinoma. Oncol Rep. 2015;34(4):1681-91.
54. Zhang J, Liu S, Zhang D, Ma Z, Sun L. Homeobox D10, a tumor suppressor, inhibits the proliferation and migration of esophageal squamous cell carcinoma. J Cell Biochem. 2019;120(8):13717-25.
55. Ping W, Liu XM, Lei D, Zhang XJ. mTOR signaling-related MicroRNAs and Cancer involvement. J Cancer. 2018;9(4):667-73.