








Long non-coding RNA HOTAIR induces the PI3K/AKT/mTOR signaling pathway in breast cancer cells

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SUMMARY

OBJECTIVE: The phosphoinositide 3-kinase/protein kinase AKT/mammalian target of rapamycin signaling pathway is essential for proper cellular metabolism and cell growth. However, aberrant activation of this pathway has been linked to the progression and metastasis of breast cancer. Recently, the role of long non-coding RNAs in interfering with the cell signaling pathways involved in cell growth and metabolism has been identified. HOX antisense intergenic RNA is a long non-coding RNA whose abnormal expression has been associated with development, therapy resistance, and metastasis of breast cancer. The purpose of this study was to investigate whether the long non-coding RNA HOX antisense intergenic RNA is linked to the phosphoinositide 3-kinase/protein kinase AKT/mammalian target of rapamycin signaling pathway in breast cancer cells.

METHODS: HOX antisense intergenic RNA was silenced in the breast cancer cell line MCF-7 using siRNAs. Subsequently, the gene expression level of HOX antisense intergenic RNA, PI3K, AKT, and mTOR was assessed using real-time RT-PCR. Also, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay was used to analyze cell proliferation.

RESULTS: The results revealed that HOX antisense intergenic RNA knockdown can downregulate the expression of PI3K, AKT, and mTOR RNAs compared to negative control in MCF-7 cells. In addition, the proliferation of breast cancer cells was significantly reduced following the HOX antisense intergenic RNA silencing.

CONCLUSION: This study may introduce HOX antisense intergenic RNA as a molecule involved in the upregulation of the phosphoinositide 3-kinase/protein kinase AKT/mammalian target of rapamycin signaling pathway in breast cancer cells that may contribute to breast cancer cell proliferation.

KEYWORDS: MCF-7 cells. HOTAIR long non-coding RNA. RNA. Long non-coding. Gene expression.

INTRODUCTION

Breast cancer (BC), the most prevalent malignancy in women, is classified as a heterogeneous group of disorders with extremely varied clinical outcomes^{1,2}. Although the specified process and related cellular mechanisms driving BC progression are not fully understood, new technologies and molecular research have significantly increased the understanding of cancer biology and discovered potential anticancer treatment targets.

The significance of non-coding RNAs in the development of various malignancies has recently been addressed in several studies^{3,4}. Long non-coding RNA (lncRNA) is a kind of RNA

molecule that has a length of more than 200 nucleotides and does not code for proteins⁵. HOX antisense intergenic RNA (HOTAIR) is an lncRNA whose aberrant expression is shown to be linked to BC progression, treatment resistance, and cancer cell metastasis⁶. It has been indicated that HOTAIR can alter the expression of many of the essential genes related to cell signaling pathways, including TGF- β , JAK/STAT, and PTEN pathways, which ultimately leads to increased cancer cell invasion as well as metastasis⁷.

The phosphoinositide 3-kinase/protein kinase AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway

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plays a critical role in controlling normal cellular metabolism and cell proliferation⁸. However, abnormal activation of this pathway is shown to be associated with growth, metabolism, and survival of cancer cells in a variety of human cancers, including BC⁹. The activated mTOR signaling pathway has been shown to be associated with decreased patient survival and correlated with a worse prognosis in BC¹⁰. Understanding the mechanisms involved in regulating this pathway could be a step toward finding effective therapeutic agents to inhibit cancer progression. In this study, we aimed to evaluate whether lncRNA HOTAIR is associated with the PI3K/AKT/mTOR signaling pathway in BC cell line MCF-7.

METHODS

Cell culture

The BC cell line MCF-7 was purchased from the National Cell Bank, Pasteur Institute (Tehran, Iran). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY, USA). Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

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 four siRNAs targeting HOTAIR. Target sequences for siRNA-HOTAIR were as follows: 5'-AGACGAAGGUGAAAGCGAA-3', 5'-CAAUAUAUCUGUUGGGCGU-3', 5'-GGGACUGGGAGGCGCAAU-3', and 5'-CAGUGGAAUGGAACGGAU-3'.

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 containing siRNA-HOTAIR or scrambled sequences at a final concentration of 100 nM. The tube was incubated at room temperature for 20 min. The mixture was then added drop-wise to cells maintained in serum-free media in 24-well plates. The medium was replaced with DMEM 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin after 5 h. Cells were maintained in a humidified incubator at 37°C with 5% CO₂ and were evaluated 48 h after transfection.

RNA extraction and cDNA synthesis

RNA was extracted from MCF-7 cells transfected with siRNA-HOTAIR and control groups, including MCF-7 cells without transfection or cells transfected with siRNA-scramble according to the manufacturer's instruction (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 instruction. 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.

Measurement of mRNA expression

Real-time RT-PCR analysis 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.), 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 s, and 40 cycles including a denaturation at 95°C for 5 s, an annealing at 60°C (for HOTAIR, PI3K, and mTOR) and 57°C (for AKT) for 20 s, and an extension at 72°C for 30 s. Finally, a melting curve analysis was performed at 60–95°C.

The primer sequences are mentioned in Table 1. The relative expression levels were normalized to the endogenous control β-actin and were expressed as 2^{-ΔΔCt11}. Data were expressed as fold changes in the amount of mRNA.

Cell proliferation assay

Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay 48 h 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 3 h. 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

For statistical analysis, the Prism software version 6.07 and Excel version 16.0 were used, and all data were expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the statistical significance of

Table 1. Primer sequences.

Gene name	Forward (5'→3')	Reverse (5'→3')
HOTAIR	GGTAGAAAAAGCAACCACGAAGC	ACATAAACCTCTGTCTGTGAGTGCC
PI3K	GAACGAGTGGTTGGGCAATG	CCTCGCAACAGGTTTTCAGC
AKT	ACAGGTGGAAGAACAGCTCG	ACAGGTGGAAGAACAGCTCG
mTOR	GCTTGATTGGTTCCAGGACAGT	GTGCTGAGTTTGTGTACCCATGT
β-Actin	TGGCACCCAGCACAAATGAA	CTAAGTCATAGCCGCCTAGAAGCA

the results. A $p < 0.05$ was considered to show significant differences between groups.

RESULTS

Transfection and gene-silencing efficiency

To find the optimal time for HOTAIR silencing, RNA isolation and analysis of the HOTAIR expression were performed at different time points, including 12, 24, 48, and 72 h (data not shown). Then, RNAs were extracted from both transfected and control groups 48 h post transfection as the optimal time. The interference efficiency of siRNA-HOTAIR was determined by real-time RT-PCR through measurement of the expression of HOTAIR in the transfected cells group compared to the negative control group. Our findings showed that the expression of HOTAIR was significantly decreased in cells transfected with siRNA-HOTAIR compared to cells transfected with siRNA-scramble or cells without transfection ($p < 0.05$) (Figure 1A).

HOX antisense intergenic RNA silencing could downregulate phosphoinositide 3-kinase/protein kinase AKT/mammalian target of rapamycin signaling pathway in MCF-7 cells

To investigate the effect of HOTAIR silencing on the PI3K/AKT/mTOR signaling pathway in MCF-7 cells, the expression of *PI3K*, *AKT*, and *mTOR* genes was assessed in both transfected and control groups using real-time RT-PCR. As shown in Figure 1, the mRNA levels of *PI3K*, *AKT*, and *mTOR* in MCF-7 cells were significantly decreased in cells transfected with siRNA-HOTAIR compared to cells transfected with siRNA-scramble or cells without transfection ($p < 0.05$ for *PI3K* and *mTOR* and $p < 0.01$ for *AKT*).

HOX antisense intergenic RNA silencing decreased the proliferation of MCF-7 cells

To determine the effect of the HOTAIR knockdown on cell growth, the MTT assay was performed. The optical density (OD)

values were used to assess the proliferation rate. As shown in Figure 2, HOTAIR silencing resulted in a significant decrease in the percent of MCF-7 cells compared with the cells transfected with scrambled sequences or cells without transfection ($p < 0.05$). This finding may confirm that knockdown of lncRNA-HOTAIR could result in suppression of the proliferation of MCF-7 cells.

DISCUSSION

In recent years, significant efforts have been made to enhance the diagnosis and treatment of BC. Nevertheless, the pathogenic mechanism involved in BC progression remains largely unknown. Identifying the molecular mechanisms underlying cancer development and progression may be important in clinical prognosis, patients' survival, and application of efficient therapies. Recently, the role of lncRNAs, a major class of newly identified non-coding transcripts, in the pathogenesis of several malignancies, including BC, has been shown to have great importance³. lncRNAs exert their regulatory effects at both transcriptional and post-transcriptional levels. They can regulate a variety of cellular processes by influencing or interacting with different elements, including protein, DNA, and RNA molecules¹². In this study, we aimed to evaluate the potential role of lncRNA HOTAIR in regulating the PI3K/AKT/mTOR signaling pathway in BC cells.

HOTAIR was shown to be one of the most upregulated cancer-associated lncRNAs in BC. It has been demonstrated that HOTAIR interacts with the Polycomb Repressive Complex 2 (PRC2) to alter chromatin state and promote cancer metastasis^{7,13}. Accumulating evidence suggests that high level of HOTAIR is correlated with a worse prognosis of patients with BC^{14,15}. A study by Yu et al.¹⁶ showed that HOTAIR silencing could significantly inhibit MCF-7 cell proliferation and increase apoptosis of MCF-7 cells through regulating the P53/AKT/JNK signaling pathway. Accordingly, the present study indicated a decreased proliferation of MCF-7 cells following knockdown of HOTAIR. However, HOTAIR silencing in other cancer cells, including oral squamous cell carcinoma¹⁷,

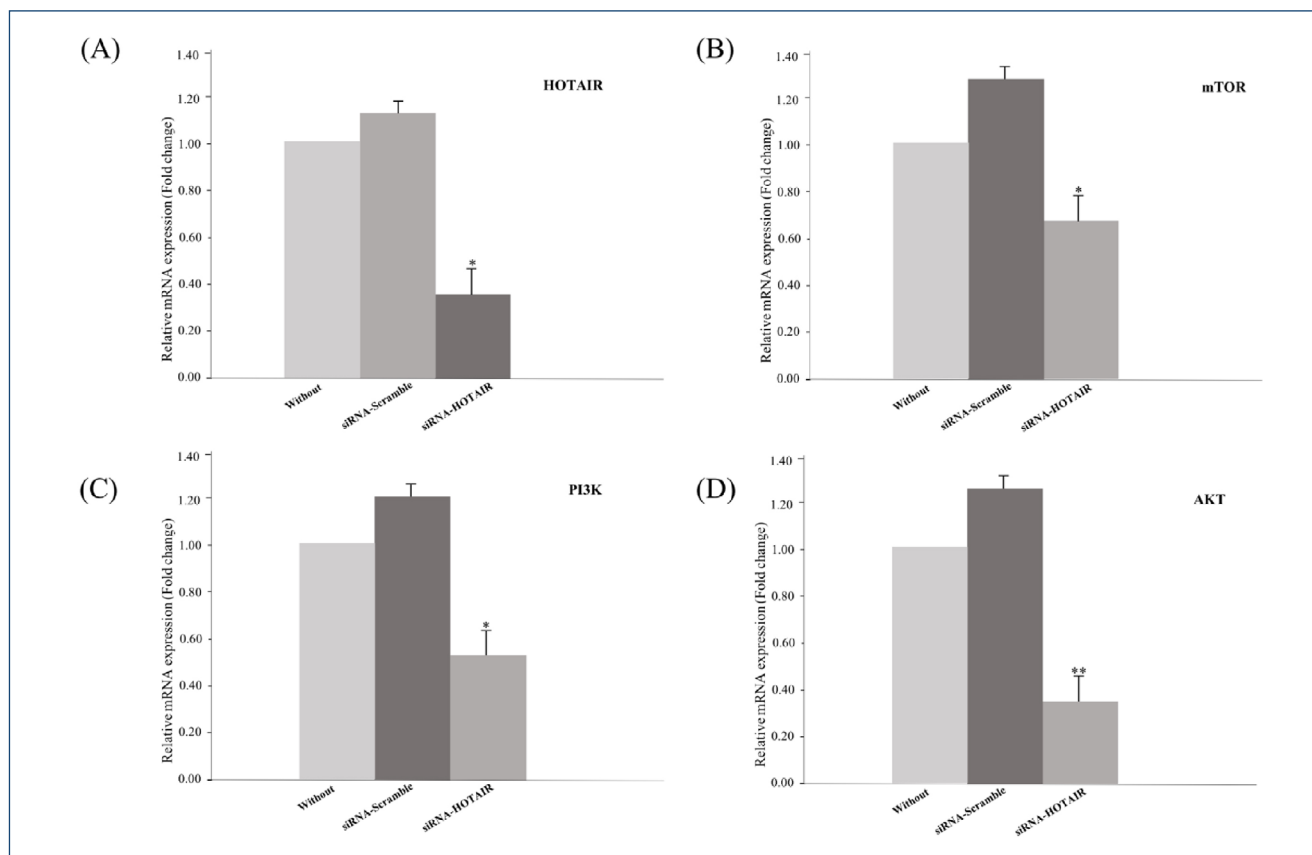


Figure 1. The expression level of HOTAIR, mTOR, PI3K, and AKT in MCF-7 cells without transfection and in MCF-7 cells transfected with siRNA-scramble or siRNA-HOTAIR. Silencing of HOTAIR by siRNAs downregulated the expression of *HOTAIR* (A), *mTOR* (B), *PI3K* (C), and *AKT* (D) genes in MCF-7 cells. Data presented as relative gene expression changes in the treated cells compared to the controls (* $p < 0.05$, ** $p < 0.01$). HOTAIR, HOX antisense intergenic RNA; lncRNA, long non-coding RNA; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinases; AKT, protein kinase AKT.

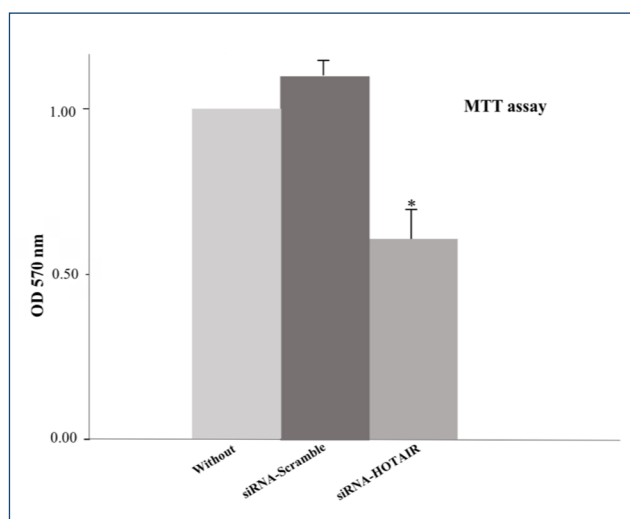


Figure 2. HOTAIR silencing decreased the proliferation of MCF-7 breast cancer cells. The MTT assay was performed in MCF-7 cells without transfection and in MCF-7 cells transfected with siRNA-scramble or siRNA-HOTAIR. Values are expressed as the mean \pm standard deviation ($n = 3$). * $p < 0.05$ compared to the control group. HOTAIR, HOX antisense intergenic RNA; lncRNA, long non-coding RNA; OD, optical density.

gastric cancer¹⁸, and lung cancer¹⁹, has reduced the invasion and metastasis of cancer cells. These data support the hypothesis that HOTAIR could be considered an important mediator in cancer cell proliferation, survival, and metastasis.

Among the tumor-associated signaling pathways, the PI3K/AKT/mTOR signaling pathway plays a central role in regulating the expression of a variety of signaling molecules involved in cell proliferation, survival, apoptosis, and metastasis^{20,21}. On the other hand, this pathway itself can be regulated by many mediators in the tumor microenvironment as well as some hormones in BC. Interestingly, prolactin-mediated activation of the PI3K pathway may be involved in proliferation and cytoskeletal dynamics, leading to the progression of mammary tumors²². Therefore, identifying the regulators of this pathway may be an important step toward inhibiting cancer progression through targeted therapies.

Since the significant role of HOTAIR has been shown in increasing the proliferation of several types of cancer cells, in this study, we hypothesized whether this lncRNA could serve a role

in inducing the PI3K/AKT/mTOR signaling pathway. In this study, we suggested the lncRNA HOTAIR as an upstream molecule involved in the upregulation of the PI3K/AKT/mTOR signaling pathway (Figure 3). HOTAIR silencing resulted in a significantly reduced expression level of PI3K, AKT, and mTOR molecules. In accordance with the present study, previous studies have also shown the important role of HOTAIR in inducing the mTOR signaling pathway in several types of cancers. Li et al. revealed that HOTAIR could enhance osteosarcoma

cell growth through activation of the AKT/mTOR signaling pathway. In their study, silencing of HOTAIR using siRNAs reduced the phosphorylation of AKT/mTOR signaling pathway proteins²³. Furthermore, a previous study demonstrated that the knockdown of HOTAIR decreases doxorubicin resistance in BC cells via the PI3K/AKT/mTOR signaling pathway, demonstrating that HOTAIR could be considered a therapeutic target for BC therapy²⁴. Furthermore, a study by Hui et al. showed the association between the upregulated HOTAIR and

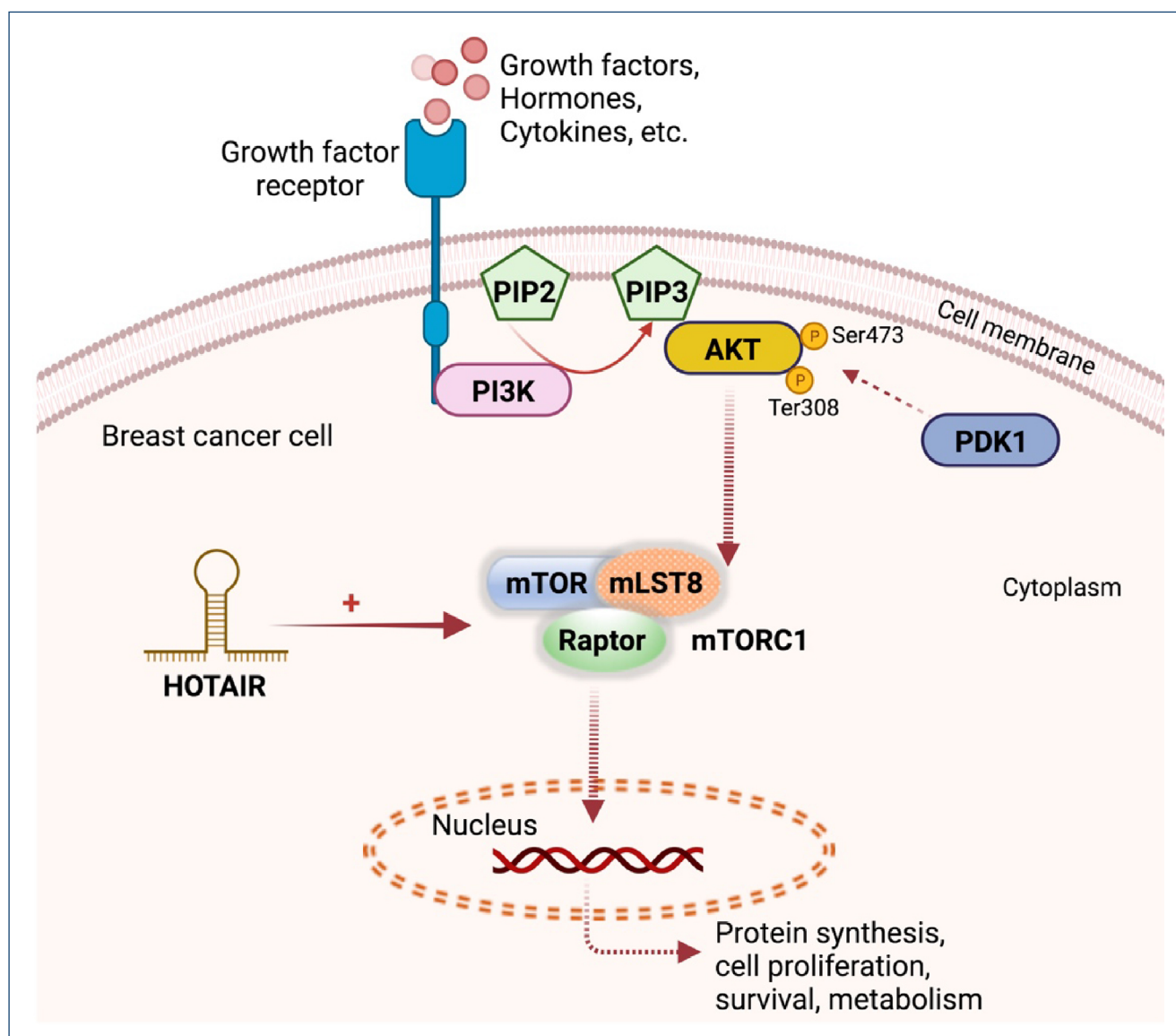


Figure 3. Role of lncRNAs HOTAIR in inducing the mTOR signaling pathway. The mTOR pathway, consisting three principal molecular components, namely, PI3K, AKT, and mTOR, plays an important role in regulating several pivotal cellular processes such as cell proliferation, survival, apoptosis, metabolism, and metastasis. Thus, the lncRNAs HOTAIR silencing using siRNAs, as a therapeutic approach, may result in decreasing cancer progression. HOTAIR may act as a molecule upstream of the PI3K/AKT/mTOR signaling pathway in BC cells, which may ultimately be involved in inducing tumor growth. 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; PDK1, phosphoinositide-dependent kinase 1. Image created with BioRender.com.

abnormal activated PI3K/AKT pathway in adenocarcinoma of the esophagogastric junction²⁵.

Consequently, based on the obtained findings, it can be concluded that lncRNA HOTAIR can play a role in BC progression, maybe through upregulation of the PI3K/AKT/mTOR signaling pathway. Although the exact mechanism of HOTAIR in inducing the gene expression of PI3K, Akt, and mTOR molecules is not yet known, it is thought that HOTAIR can directly or indirectly affect the activity of transcription factors and their expression. It is possible that HOTAIR regulates the gene expression of PI3K, AKT, and mTOR by interaction with the regulators of this pathway.

Therefore, further studies are required to investigate the interaction between this lncRNA and regulators of this pathway, as well as the activation state of proteins under the influence of HOTAIR.

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AUTHORS' CONTRIBUTIONS

MS: Conceptualization, Data curation, Formal Analysis, Validation, Experimental work, Writing – original draft. **KM:** Conceptualization, Writing – review & editing, Validation. **HRMM:** Conceptualization, Formal Analysis, Writing – review & editing. **FN:** Conceptualization, Formal Analysis, Writing – review & editing. **AM:** Conceptualization, Formal Analysis, Writing – review & editing. **SA:** Conceptualization, Formal Analysis, Writing – review & editing. **NR:** Conceptualization, Data curation, Formal Analysis, Validation, Writing – review & editing, Project Administration.

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