LncRNA HOTAIR influences cell growth, migration, invasion, and apoptosis via the miR-20a-5p/HMGA2 axis in breast cancer

Wenyan Zhao¹, Donghua Geng¹, Shuqiang Li¹, Zhaofu Chen² & Ming Sun²

¹Department of General Surgery, Shengjing Hospital Affiliated China Medical University, Shenyang 110004, Liaoning, China
²Department of Urology, Shengjing Hospital Affiliated China Medical University, Shenyang 110004, Liaoning, China

Abstract

To study the regulatory effect of lncRNA HOTAIR/miR-20a-5p/HMGA2 axis on breast cancer (BC) cell growth, cell mobility, invasiveness, and apoptosis. The microarray data of lncRNAs and mRNAs with differential expression in BC tissues were analyzed in the Cancer Genome Atlas (TCGA) database. LncRNA HOX transcript antisense RNA (lncRNA HOTAIR) expression in BC was assessed by qRT-PCR. Cell viability was confirmed using MTT and colony formation assay. Cell apoptosis was analyzed by TdT-mediated dUTP nick-end labeling (TUNEL) assay. Cell mobility and invasiveness were testified by transwell assay. RNA pull-down and dual luciferase assay were used for analysis of the correlation between lncRNA HOTAIR and miR-20a-5p, as well as relationship of miR-20a-5p with high mobility group AT-hook 2 (HMGA2). Tumor xenograft study was applied to confirm the correlation of lncRNA HOTAIR/miR-20a-5p/HMGA2 axis on BC development in vivo. The expression levels of the lncRNA HOTAIR were upregulated in BC tissues and cells. Knockdown lncRNA HOTAIR inhibited cell propagation and metastasis and facilitated cell apoptosis. MiR-20a-5p overexpression in BC suppressed cell growth, mobility, and invasiveness and facilitated apoptosis. HMGA2 was a target of miR-20a-5p, which significantly induced carcinogenesis of BC. BC cells progression was mediated by lncRNA HOTAIR via affecting miR-20a-5p/HMGA2 axis in vivo. LncRNA HOTAIR affected cell growth, metastasis, and apoptosis via the miR-20a-5p/HMGA2 axis in breast cancer.

Introduction

Breast cancer (BC) is considered the leading cause of cancer-related death in women worldwide [1–4]. At present, gene therapy against neoplasms has been a novel research focus in cancer treatment [5, 6]. Using gene therapy against BC, it is an urgent need to elucidate novel mechanisms correlated with BC development.

Noncoding RNAs (ncRNAs) have become the focus of “next generation” biology [7], which consist of long noncoding RNAs (lncRNAs) and microRNAs (miRNAs). Until now, a few lncRNAs have been demonstrated to be dysregulated in breast cancer, which is closely related to breast cancer diagnosis and prognosis [8, 9]. HOX transcript antisense intergenic RNA (HOTAIR) located in chromosome 12 and owned 2.2 kb in length approximately, which is transcribed from the HOXC locus and epigenetically acts as a repressor of HOXD [10]. In addition, lncRNA HOTAIR has been found to be closely associated with cell metastasis in multiple cancers, such as colorectal [11], hepatocellular [12], pancreatic [13], gastrointestinal stromal [14], lung [15], and breast [16] carcinomas. Notably,
HOTAIR expression is increased in breast cancer, which provides a powerful biomarker of tumor metastases and patient death [16, 17]. However, the molecular mechanism of HOTAIR in BC remains unknown.

MiRNAs are short (20-22 nt), noncoding, and highly stable RNAs that are involved in post-transcriptional regulation of gene expression [18]. Dysregulation of miRNAs is a major culprit of tumorigenesis in breast cancers because their loss leads to the increased expression of targeted genes, including oncogenes. For example, Browne et al. [19] showed that miR-378-mediated suppression of Runx1 alleviated the aggressive phenotype of triple-negative MDA-MB-231 human breast cancer cells. Playing the role of cancer inhibitor, miR-20a-5p has been found to be downregulated in the majority of cancer cells. For example, miR-20a-5p was confirmed to repress MICA and MICB expression by binding to the mRNA 3′-UTRs in human cancer cells (mainly HeLa, 293T, DU145 cells) [20]. Meanwhile, miR-20a-5p was also experimentally verified as new pharmacogenomic biomarkers for metformin in MCF-7 or MDA-MB-231 cell lines [21]. Therefore, it is suggested that miR-20a-5p may hold great promise as an accessible biomarker for BC. However, the role of miR-20a-5p in breast cancer needs to be further illuminated.

High mobility group AT-hook 2 (HMGA2) binds to AT-rich regions in DNA, altering chromatin architecture [22] to promote the action of transcriptional enhancers. HMGA2 is highly expressed in most malignant epithelial tumors, including breast [23], pancreas [24], and nonsmall cell lung cancer [25], suggesting that HMGA2 could promote tumor progression in breast cancer.

In this study, we explored the role of lncRNA HOTAIR/miR-20a-5p/HMGA2 axis in the development of BC. LncRNA HOTAIR functioned as the sponge of miR-20a-5p to upregulate HMGA2 expression. Therefore, decrease in lncRNA HOTAIR may serve as prognostic as well as predication marker for BC patients and used as a novel therapeutic target.

Materials and Methods

Clinical samples

A total of 20 BC patients who underwent a mastectomy at Shengjing Hospital Affiliated China Medical University were recruited to the study. All specimens were pathologically confirmed as breast cancer and did not receive radiotherapy or chemotherapy prior to surgery. After resection, the tumor and adjacent tissues were frozen by liquid nitrogen, and the specimens were immediately stored at −80°C. The Ethics Committee of Shengjing Hospital Affiliated China Medical University approved this study, and written informed consents were acquired from all enrolled patients.

Bioinformatics analysis

LncRNAs and mRNAs with differential expressions in BC tissues were analyzed in the Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). Differentially expressed lncRNA and mRNA were identified using a t-test (P < 0.05) combined with fold change (FC) (log2(FC)>2 for upregulated lncRNAs and log2(FC)<2 for downregulated lncRNAs). The Kaplan-Meier curve was used to test lncRNA association with time to progression. The binding sites between lncRNA HOTAIR and miR-20a-5p, and the target genes of miR-20a-5p were predicted using miRcode (http://www.mircode.org/) and TargetScan 7.1 database (www.targetscan.org).

Cell culture

Three BC carcinoma cell lines MCF7, SKBR3, MDA-MB-231 and human breast epithelial cell lines MCF-10A were all purchased from BeNa Culture Collection Biological Technology Co., Ltd. (Beijing, China). Breast cancer cell lines MCF7 and MDA-MB-231 were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS and 100 U/mL Penicillin/Streptomycin in a 5% CO2 incubator. SKBR3 were cultured in McCoy’s 5A Media (modified with tricine) and MCF-10A was cultured in RPMI-1640. Cells were collected at 90% confluence, and the medium was changed every 48–72 h.

Cell transfection

MDA-MB-231 cells at exponential stage were used for transfection. Before transfection, 1 × 106 BC cells were cultured in 6-well plates with 2 mL complete medium for 24 h until they were 90% confluent. Si-HOTAIR, hsa-miR-20a-5p mimics, hsa-miR-20a-5p inhibitor, si-HMGA2, pCDNA-HMGA2, and negative control (NC) were purchased from Shanghai GenePharma Inc. (Shanghai, China). The vectors and microRNAs were transfected, respectively, into MDA-MB-231 cell line by Lipofectamine 3000 reagents and cultured with Opti-MEM serum-free medium following the instructions. Cells were grouped into (1) NC group; (2) si-HOTAIR group; (3) miR-20a-5p-mimics group; (4) miR-20a-5p-inhibitor group; (5) si-HOTAIR+miR-20a-5p-inhibitor group; (6) HMGA2
group; (7) si-HMGA2 group; (8) HMGA2 + miR-20a-5p-mimics group.

**qRT-PCR**

The total RNA from BC tissues and cells was extracted by TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), and 200 ng extracted RNA was reverse transcribed into cDNA by ReverTra Ace qPCR RT Kit (Toyobo, Japan) before qRT-PCR. Quantitative PCR was carried out using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan) and a LightCycler 480 Real-Time PCR system (Roche, Shanghai, China). The GAPDH and U6 gene was used as an endogenous control gene for normalizing the expression of target genes. Each sample was analyzed in triplicate. The thermocycling program consisted of holding at 94°C for 2 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 56°C, and 60 sec at 72°C. Melting curve data were then collected to verify PCR specificity and the absence of primer dimers. Primer sequences are exhibited in Table 1.

**MTT assay**

Cell viability was monitored using MTT cell viability assay kit (Sigma). The transfected cells were seeded in 96-well plates (200 μL, 3 × 10³ cells/well). 10 μL MTT (5 mg/mL) was added to each well and continued incubating for 4 h, followed by the precipitate dissolving in dimethyl

---

**Table 1. Primer sequences.**

| Primer sequences (5'–3')     |
|-----------------------------|
| MiR-20a-5p forward          |
| UAAAGUGCUUAUAGUGCAGGUGAG    |
| MiR-20a-5p reverse          |
| CUACCUCACUAUAAGCACUUUA      |
| U6 forward                  |
| CTCGTCTCCGACAGCATATACT      |
| U6 reverse                  |
| CGCTTACGAAATTGGCGTGT        |
| HOTAIR forward              |
| CAGTGGGGAACCTGACTCG         |
| HOTAIR reverse              |
| GTGCCCTCTCTCTCTTACC         |
| HMGA2 forward               |
| GGGGCGCGACATCAAT            |
| HMGA2 reverse               |
| ACTGCAGTGTCTTCCCTCCATGA     |
| GAPDH forward               |
| TCAAGGCTGAGAACGGGAGAG       |
| GAPDH reverse               |
| TGGACTCCACGACGTACTCA        |

---

**Figure 1.** HOTAIR was overexpressed in BC tissues and cells. (A) Volcano plot: HOTAIR was analyzed by lncRNA microarray analysis and selected as a promising lncRNA involved breast cancer (BC) tumorgenesis. (B) Heatmap: LncRNA HOTAIR was overexpressed in BC tumor tissues compared with adjacent tissues. (C) Kaplan-Meier analysis showed that high expression of HOTAIR obtaining an adverse overall survival. (D–E) QRT-PCR was used to detect expression levels of HOTAIR in BC tumor tissues and cells including normal MCF-10A cells and breast cancer cell lines SKBR3, MCF7, MDA-MB-231, demonstrating that LncRNA HOTAIR was overexpressed. * Compared with the control group, \( P < 0.05 \). ** Compared with the control group, \( P < 0.01 \).
Figure 2. HOTAIR affected cells proliferation, migration, invasiveness, and apoptosis in BC. (A) Transfection efficiency of si-HOTAIR was confirmed by qRT-PCR methods. (B) MTT assay: HOTAIR knockdown in MDA-MB-231 cells significantly inhibited cell proliferation. (C) Colony formation assay: colony formation of BC cells was decreased by knockdown of HOTAIR. (D) Transwell migration assay: HOTAIR knockdown in MDA-MB-231 cells reduced migration of BC cells. Bar: 20 μm. (E) Transwell invasive assay: HOTAIR knockdown significantly suppressed cell invasive capacity in BC cells. (F) Detection of apoptosis was using the TUNEL assay (100×). MDA-MB-231 cells were transfected with control siRNA, si-HOTAIR. Apoptosis rate of BC cell was significantly increased after si-HOTAIR treatment. Bar: 20 μm. **Compared with NC group, P < 0.01.
sulfoxide (DMSO, 100 μL). The absorbance was measured at 490 nm under a microplate spectrophotometer.

**Colony formation assay**

Cells (1 × 10^3 cells per well) were seeded in a 6-well plate and incubated for 1 week at 37°C. Then, cells were washed twice in PBS, fixed with 4% formaldehyde for 15 min and stained for 10–30 min with GIMSA. The colonies (a diameter ≥ 100 μm) were counted in triplicate assays.

**Transwell assay**

For the transwell migration assay, the breast cancer cells were trypsinized and placed in the upper chamber of each insert (Corning, Cambridge, USA) containing the noncoated membrane. Lower chambers were supplemented with 1% fetal bovine
IncRNA HOTAIR/HMGA2/miR-20a-5p Axis in BC

W. Zhao et al.
serum (600 μL). After 24 h incubation at 37°C, the upper surface of the membrane was removed with a cotton tip, while the cells on the lower surface were stained for 30 min with 0.1% crystal violet. For the invasion assay, matrigel chambers (BD Biosciences, San Jose, CA, USA) were carried out conforming to manufacturer’s instructions. Briefly, transfected MDA-MB-231 cells (200 μL, 5000 cells per well) were collected, resuspended in medium without serum, and then shifted to the hydrated matrigel chambers (50 μL). The bottom chambers were incubated overnight in 500 μL DMEM culture medium with 10% FBS. The cells on the upper surface were scraped, whereas the invasive cells on the lower surface were fixed and colored with 0.1% crystal violet for half an hour.

**TUNEL staining**

Cells were transfected with si-HOTAIR or miR-20a-5p mimic or miR-20a-5p inhibitor and then cultured...
overnight. After rising twice with PBS, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized in 0.25% Triton-X 100 for 20 min. TUNEL assays were carried out conforming to the manufacturer’s instructions (Roche). Briefly, the cells were first incubated in terminal deoxynucleotidyl transferase (TdT) reaction cocktail for 45 min at 37°C, followed by treatment with Click-IT reaction cocktail. The nucleus was stained with hematoxylin or methyl green.

**RNA pull-down assay**

RNA pull-down assay was conducted through Flag-MS2 bp-MS2bs-based pull-down assay. Specifically, pcDNA3-FlagMS2 bp and pcDNA3-HOTAIR-MS2bs were co-transfected to MDA-MB-231 cells, and the cells were collected after 2 days. About $1 \times 10^7$ cells were dissolved in the soft lysis buffer plus 80 U/mL RNasin (Promega Madison, WI, USA). Fifty microliters of ANTI-FLAG M-280 Magnetic Beads (Invitrogen) was supplemented to each binding reaction tube and incubated for 4 h. Beads were washed six times in the lysis buffer. The retrieved supernatant was detected by qRT-PCR.

**Luciferase reporter assay**

Cells ($1 \times 10^5$ per milliliter) were transfected with 100 ng plasmids and 200 nmol/L miR-20a-5p mimics, miR-20a-5p inhibitors or their negative control. After 2 days, the cells were lysed with 80 μL 1× Passive Lysis Buffer and tested through a dual luciferase assay (Promega). For HOTAIR and HMGA2 promoter analysis, the HOTAIR and HMGA2 promoter was amplified and cloned into a psiCHECK TM-2 vector (Promega). Luciferase activity was evaluated through the dual luciferase assay system (Promega).

**Tumor xenograft in vivo**

A total of 30 BALB/c nude mice were chosen and assigned to five groups: (1) NC group (injected with MDA-MB-231 cells), (2) si-HOTAIR (injected with MDA-MB-231 cells with HOTAIR knockdown), (3) miR-20a-5p inhibitor group (injected with MDA-MB-231 cells with miR-20a-5p knockdown), (4) si-HMGA2 group (injected with MDA-MB-231 cells with HMGA2 knockdown), (5) si-HOTAIR+miR-20a-5p inhibitor group (injected with MDA-MB-231 cells with both HOTAIR and miR-20a-5p knockdown). 0.2 mL of above cell suspension that contained $2 \times 10^3$ or $2 \times 10^4$ or $2 \times 10^5$ cells was injected into the left or right back of each mice. Tumor sizes were assessed once per week by a digital caliper. The tumor volumes were determined by measuring their length ($l$) and width ($w$) and calculating the volume ($V$) as follows: $V = lw^2/2$. After 28 days, the mice were euthanized and tumor tissues were weighted. The animal studies were approved by the Institutional Animal Care and Use Committee of Shengjing Hospital Affiliated China Medical University, and were performed according to institutional guidelines.

### Statistical analysis

Statistical data were analyzed using GraphPad Prism 6.0 (GraphPad Software) and were presented as mean ± standard deviation. Student's t-test was employed to evaluate difference between individual groups. The criterion of statistical significance was $P < 0.05$.

### Results

**lncRNA HOTAIR was overexpressed in BC tissues and cells**

Microarray analysis was used to identify differential expressed lncRNA in BC tissues and its adjacent tissues. Among them, lncRNA HOTAIR had been reported to act as tumor-promoting molecular in multiple tumors, and it was significantly upregulated in BC tissues, predicting its expression and biological function in BC tumorigenesis (Fig. 1A and B). The prognosis analysis of lncRNA HOTAIR and the results showed that the high expression of lncRNA HOTAIR brought out an adverse role in survival depending on Kaplan-Meier analysis (Fig. 1C). In addition, lncRNA HOTAIR expression in the BC tissues was upregulated by about 2.27-fold in comparison with the adjacent tissues ($P < 0.01$, Fig. 1D). To determine its role in BC development, we explored the expression of lncRNA HOTAIR in BC cells (MDA-MB-231, SKBR3, MCF-7) and normal cells (MCF-10A), and found that the expression of lncRNA HOTAIR was considerably increased in the BC cell lines compared to MCF-10A cells ($P < 0.05$, Fig. 1E). Besides, lncRNA HOTAIR expression in MDA-MB-231 was higher than other cell lines, thereafter selected as conducting the following experiments. These findings suggested that lncRNA HOTAIR might participate in the development of BC.

**lncRNA HOTAIR enhanced the progression of BC cells**

To investigate the biological functions of lncRNA HOTAIR in BC cells, we decreased expression of lncRNA HOTAIR in MDA-MB-231 cells by transfection of small interfering RNA (siRNA). QRT-PCR showed that lncRNA HOTAIR...
expression remarkably downregulated in si-HOTAIR transfected MDA-MB-231 cells compared with control groups ($P < 0.01$, Fig. 2A). MTT and colony formation assay showed that lncRNA HOTAIR suppression remarkably reduced cellular viability of MDA-MB-231 cells compared to control groups ($P < 0.01$, Fig. 2B and C). Furthermore, we explored whether lncRNA HOTAIR was involved in cell metastasis in MDA-MB-231 cells. Transwell migration assay showed that lncRNA HOTAIR inhibition remarkably decreased migration ability in MDA-MB-231 cells ($P < 0.01$, Fig. 2D). By using transwell invasion assay, we observed that the number of invaded cells was obviously decreased in the lncRNA HOTAIR knockdown cells compared with control groups ($P < 0.01$, Fig. 2E). As well, TUNEL assay showed that lncRNA HOTAIR knockdown in MDA-MB-231 cells significantly promoted cell apoptosis ($P < 0.01$, Fig. 2F). Taken together, these findings indicated that lncRNA HOTAIR enhanced carcinogenesis of BC cells in vitro.

**LncRNA HOTAIR targeted miR-20a-5p and repressed its expression**

Bioinformatics prediction showed that lncRNA HOTAIR could potentially bind to miR-20a-5p, miR-107, miR-137, miR-133b as well as miR-200b. QRT-PCR assay showed that miR-20a-5p and miR-107 expressions were significantly higher than control group after lncRNA HOTAIR knockdown and miR-20a-5p was the highest increase ($P < 0.01$, Fig. 3A). MiR-137 and miR-133B were slightly upregulated ($P < 0.05$, Fig. 3A), whereas miR-200b was observed no significance ($P > 0.05$, Fig. 3A). Thus, we then explored the relationship of lncRNA HOTAIR and miR-20a-5p. We employed biotinylated miR-20a-5p probe to pull down the lncRNA HOTAIR. Data indicated endogenous lncRNA HOTAIR was enriched specifically in miR-20a-5p probe detection compared with control group, suggesting that miR-20a-5p is a direct inhibitory target of lncRNA HOTAIR ($P < 0.01$, Fig. 3B). In addition, relying on the miRCode (http://www.mircode.org/), the targeting relationship between lncRNA HOTAIR and miR-20a-5p was displayed in Fig. 3C. Meanwhile, there was a negative correlation between lncRNA HOTAIR expression and miR-20a-5p expression ($R^2 = 0.9008$, $P < 0.01$, Fig. 3D). The above results demonstrated that lncRNA HOTAIR targeted and regulated miR-20a-5p expression.

**MiR-20a-5p inhibited the progression of BC cells**

QRT-PCR revealed that miR-20a-5p expression level was significantly upregulated after transfected with miR-20a-5p-mimics, but downregulated on miR-20a-5p-inhibitor addition ($P < 0.01$, Fig. 3E). MTT and colony formation assay showed that miR-20a-5p overexpression reduced cell viability, while cell transfected with miR-20a-5p inhibitors exhibited a significantly increased cell proliferation. Moreover, lncRNA HOTAIR knockdown neutralized the activator effects of miR-20a-5p inhibitor ($P < 0.05$, Fig. 4A–C). Meanwhile, miR-20a-5p significantly impaired cell migratory and invasive capacity in MDA-MB-231 cells, whereas lncRNA HOTAIR induced BC cells migratory and invasive capacity. Co-transfected with si-HOTAIR and miR-20a-5p inhibitor, both migratory and invasive capacities were not significantly changed compared with negative control ($P < 0.05$, Fig. 4D and E). TUNEL assays revealed that miR-20a-5p suppression inhibited apoptosis of BC cells, indicating that miR-20a-5p promoted BC cells apoptosis, which was restored on lncRNA HOTAIR knockdown as well ($P < 0.01$, Fig. 4F). Overall, lncRNA HOTAIR/miR-20a-5p axis can exert influence on BC development and procession.

**HMGA2 was differentially expressed and analyzed by mRNA array in BC cells**

To understand the underlying mechanism of miR-20a-5p in BC, we searched the differentially expressed mRNA for its potential target genes via TCGA microarray. 158 upregulated mRNA and 175 downregulated mRNA were found in BC tissues. HMGA2 was one of the candidate genes because its expression was upregulated reaching to 2.05-fold (Fig. 5A and B). And qRT-PCR also confirmed...
HMGA2 was observably higher than adjacent tissues, indicating its carcinogenesis role in BC ($P < 0.01$, Fig. 5C).

**HMGA2 was targeted by miR-20a-5p in BC cells**

In this study, bioinformatics prediction software (TargetScan) showed that miR-20a-5p potentially bound to HMGA2 (Fig. 5D). Dual luciferase reporter assay showed that luciferase activity was remarkably decreased in cells co-transfected with WT-HMGA2 and miR-20a-5p mimics ($P < 0.01$, Fig. 5E). It indicated that miR-20a-5p could directly bind to 3’-UTR of HMGA2, and further repressed gene expression. To investigate their relationship thoroughly, we explored the expression of HMGA2 in MDA-MB-231 cells transfected with miR-20a-5p mimics. The results showed miR-20a-5p mimics significantly reduced the expression of HMGA2 compared with control group ($P < 0.01$, Fig. 5F). On the other hand, overexpression of HMGA2 could not decrease the expression of miR-20a-5p in MDA-MB-231 cells ($P > 0.05$, Fig. 5G). Then, we explored the expression of miR-20a-5p in BC tissues, our findings suggested that lncRNA HOTAIR expression was increased and inverse correlation with miR-20a-5p expression in these 20 clinical BC tissues ($R^2 = 0.8374$, $P < 0.01$, Fig. 5H). Those data demonstrated that miR-20a-5p regulated HMGA2 expression by directly binding to it, but HMGA2 could not induce the degradation of miR-20a-5p in return.

**miR-20a-5p targeted HMGA2 to affect cells proliferation, migration, invasion, and apoptosis**

To explore the potential function of miR-20a-5p/HMGA2 in BC development, MDA-MB-231 cells were classified into NC group, HMGA2 group, si-HMGA2 group, and HMGA2 + miR-20a-5p-mimics group. MTT and colony formation assay were used to analyze cells growth. Results showed that HMGA2 overexpression induced cells growth and increased colonies formation, while transfected with si-HMGA2 significantly suppressed cell proliferation. Moreover, miR-20a-5p overexpression attenuated the activator effects of HMGA2 ($P < 0.05$, Fig. 6A–C). As well, HMGA2 significantly enhanced cell migratory and invasive capacity in MDA-MB-231 cells, whereas HMGA2 knockdown reduced BC cells migratory and invasive capacity. Meanwhile, co-transfected with HMGA2 and miR-20a-5p mimics, both migratory and invasive capacities were not significantly changed compared with NC group ($P < 0.05$, Fig. 6D and E). Moreover, HMGA2 reduced BC cells apoptosis, as evaluated by the TUNEL assay. The simultaneous increase of miR-20a-5p in MDA-MB-231 cells attenuated the role of HMGA2 in BC procession, as shown by the increased cell apoptosis rate ($P < 0.01$, Fig. 6F). These data suggested that HMGA2 played an activated role in BC development mediated by miR-20a-5p.

**lncRNA HOTAIR/miR-20a-5p/HMGA2 axis modulated BC tumor growth in vivo**

To verify the function of lncRNA HOTAIR/miR-20a-5p/HMGA2 axis in BC, we studied on their bio-functional role in tumorogenesis in vivo. Nude mice experiment results indicated knockdown of lncRNA HOTAIR or si-HMGA2 could slowdown mice tumor growth, while miR-20a-5p inhibitor accelerated tumor growth ($P < 0.05$, Fig. 7A–C). Moreover, the tumor volume of mice in si-HOTAIR+miR-20a-5p inhibitor group was significantly smaller than that in miR-20a-5p inhibitor group, while larger than that...
in si-HOTAIR group (P < 0.05). The tumor weight of mice after treatment also displayed similar trend.

Discussion

Herein, lncRNA HOTAIR was found to be overexpressed in breast cancer tissues and cells and mediated miR-20a-5p/HMGA2 in breast cancer. Knockdown of lncRNA HOTAIR could suppress cell viability, further affecting cell propagation, migration, invasiveness, and cell apoptosis capacities in BC. Nevertheless, knockdown of miR-20a-5p in BC cells showed the opposite changes. Our findings indicated that lncRNA HOTAIR and miR-20a-5p could be used as novel markers of BC and were potential therapeutic targets for BC treatment.

LncRNA HOTAIR is one of the important lncRNAs in various tumor carcinogenesis including breast cancer and highly expressed in various cancers, which is closely related to tumor size, advanced and extensive metastasis [13, 26–29]. Overexpression of lncRNA HOTAIR not only influences tumor formation but induces the proliferation, migration, and invasion [16, 27, 30]. Accumulated evidence suggested that lncRNA HOTAIR is an independent biomarker for predicting the risk of metastasis and mortality in breast cancer [16, 31], suggesting its carcinogenic role in BC progression. Our study also disclosed that lncRNA HOTAIR acted as a crucial regulator in BC development and facilitated BC cell propagation and metastasis and promoted tumor growth in vitro and in vivo.

Some previous researches demonstrated that miRNAs can modulate lncRNA HOTAIR expression, including miR-141 [32], miR-148a [7], miR-34a [33]. MiR-20, as a member of the miR-17-92 cluster, has been reported that displayed a higher expression in multiple cancers and involved in carcinogenesis [34]. Recently, miR-17-92 members including miR-20a were also reported to suppress MICA/B protein expression in ovarian tumors [35], glioma [36], and breast tumors [37], contributing to their immune escape. However, on condition of some physiological context and the cell type, miR-20a also plays tumor suppressive function [38–40]. In our study, miR-20a-5p functioned as a tumor suppressor in breast cancer cells. Analysis of miRNA expression profiles revealed a decrease in miR-20a-5p expression in breast carcinoma. Furthermore, we demonstrated that lncRNA HOTAIR functioned as a competing endogenous RNA (ceRNA) to affect miR-20a-5p activity and regulate the miR-20a-5p target genes like HMGA2.

As a transcriptional factor, HMGA2 is increased in many malignant tumors such as lung cancer [41], ovarian cancer [42], and bladder cancer [43], which targets different downstream genes in the process of tumorigenesis [44–46]. Wu et al. [47] proved that HMGA2 expression was positively correlated with tumor histological grade, and it was important with pathogenesis of breast cancer. Therefore, HMGA2 is becoming recognized as a key mediator in numerous mesenchymal and epithelial malignancies. In the current study, we substantiated that HMGA2 was negatively regulated by miR-20a-5p and exerted a certain influence on BC cell growth, cell mobility, invasiveness, and apoptosis.

However, there are some limitations that exist in this study. First, the limited samples might not fully substantiate the accuracy of the results. Second, detailed investigation of genes that comprise the lncRNA HOTAIR/miR-20a-5p/HMGA2 axis should also yield further insight into the mechanism by which lncRNA HOTAIR overexpression induces breast cancer progression. And the relationship between lncRNA HOTAIR and other potential targeting miRNAs needed more attentions and researches. In addition, we just focused on one breast cancer cell line, the MDA-MB-231 cell line, which made our research not strict adequate and the experimental conclusion was not sufficient enough. Moreover, the specific role of lncRNA HOTAIR/miR-20a-5p/HMGA2 in breast cancer remains to be further investigated.

In conclusion, lncRNA HOTAIR expression was elevated in BC tissues and cells. LncRNA HOTAIR could act as a molecular sponge of miR-20a-5p and significantly contributed to BC development and tumorigenesis by activating HMGA2 protein expression. The finding of the lncRNA HOTAIR/miR-20a-5p/HMGA2 network might provide more effective clinical therapeutic strategy for breast cancer patients.

Acknowledgment

This research was supported by Shengjing Hospital Affiliated China Medical University.

Conflict of Interest

None declared.

References

1. Wang, Y., Y. Zhou, Z. Yang, B. Chen, W. Huang, Y. Liu, et al. 2017. Mir-204/zeb2 axis functions as key mediator for malat1-induced epithelial-mesenchymal transition in breast cancer. Tumour Biol. 39:1010428317690998.
2. Torre, L. A., F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal. 2015. Global cancer statistics, 2012. CA Cancer J. Clin. 65:87–108.
3. Jemal, A., F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman. 2011. Global cancer statistics. CA Cancer J. Clin. 61:69–90.
4. Gonzalez-Angulo, A. M., F. Morales-Vasquez, and G. N. Hortobagyi. 2007. Overview of resistance to systemic therapy in patients with breast cancer. Adv. Exp. Med. Biol. 608:1–22.

5. Zhao, X. B., and G. S. Ren. 2016. Lncrna taurine-

6. Shuvalov, O., A. Petukhov, A. Daks, O. Fedorova, A. Ermakov, G. Melino, et al. 2015. Current genome editing tools in gene therapy: new approaches to treat cancer. Curr. Gene Ther. 15:511–529.

7. Tao, S., H. He, and Q. Chen. 2015. Estradiol induces hotair levels via g-meriated mir-148a inhibition in breast cancer. J. Transl. Med. 13:131.

8. Ke, H., L. Zhao, X. Feng, H. Xu, L. Zou, Q. Yang, et al. 2016. Neat1 is required for survival of breast cancer cells through fusch and mir-548. Gene Regul. Syst. Biol. 10:11–17.

9. Xing, Z., P. K. Park, C. Lin, and L. Yang. 2015. Lncrna bcar4 wires up signaling transduction in breast cancer. RNA Biol. 12:681–689.

10. Rinn, J. L., M. Kertesz, J. K. Wang, S. L. Squazzo, X. Xu, S. A. Brugmann, et al. 2007. Functional demarcation of active and silent chromatin domains in human hox loci by noncoding mas. Cell 129:1311–1323.

11. Kogo, R., T. Shimamura, K. Mimori, K. Kawahara, S. Imoto, T. Sudo, et al. 2011. Long noncoding rna hotair regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Can. Res. 71:6320–6326.

12. Geng, Y. J., S. L. Xie, Q. Li, J. Ma, and G. Y. Wang. 2011. Large intervening non-coding rna hotair is associated with hepatocellular carcinoma progression. J. Int. Med. Res. 39:2119–2128.

13. Kim, K., I. Jutooru, G. Chadalapaka, G. Johnson, J. Frank, R. Burghardt, et al. 2013. Hotair is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 32:1616–1625.

14. Niinuma, T., H. Suzuk, M. Nojima, K. Nosho, H. Yamamoto, H. Takamaru, et al. 2012. Upregulation of mir-196a and hotair drive malignant character in gastrointestinal stromal tumors. Can. Res. 72:1126–1136.

15. Nakagawa, T., H. Endo, M. Yokoyama, J. Abe, K. Tamai, N. Tanaka, et al. 2013. 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. 436:319–324.

16. Gupta, R. A., N. Shah, K. C. Wang, J. Kim, H. M. Horlings, D. J. Wong, et al. 2010. Long non-coding rna hotair reprograms chromatin state to promote cancer metastasis. Nature 464:1071–1076.

17. Yang, Z., L. Zhou, L. M. Wu, M. C. Lai, H. Y. Xie, F. Zhang, et al. 2011. Overexpression of long non-coding rna hotair predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann. Surg. Oncol. 18:1243–1250.

18. Bartel, D. P. 2004. Micrornas: genomics, biogenesis, mechanism, and function. Cell 116:281–297.

19. Browne, G., J. A. Dragon, D. Hong, T. L. Messier, J. A. Gordon, N. H. Farina, et al. 2016. Microrna-378-mediated suppression of runx1 alleviates the aggressive phenotype of triple-negative mda-mb-231 human breast cancer cells. Tumour Biol. 37:8825–8839.

20. Stern-Ginossar, N., C. Gur, M. Biton, E. Horwitz, M. Elboim, N. Stanieisky, et al. 2008. Human micrornas regulate stress-induced immune responses mediated by the receptor nkg2d. Nat. Immunol. 9:1065–1073.

21. Li, J., K. Lei, Z. Wu, W. Li, G. Liu, J. Liu, et al. 2016. Network-based identification of micrornas as potential pharmacogenomic biomarkers for anticancer drugs. Oncotarget 7:45584–45596.

22. Fusco, A., and M. Fedele. 2007. Roles of hmga proteins in cancer. Nat. Rev. Cancer 7:899–910.

23. Rogalla, P., K. Drechsler, B. Kazmierczak, V. Rippe, U. Bonk, and J. Bullerdiek. 1997. Expression of hmg1-c, a member of the high mobility group protein family, in a subset of breast cancers: relationship to histologic grade. Mol. Carcinog. 19:153–156.

24. Abe, N., T. Watanabe, Y. Suzuki, N. Matsumoto, T. Masaki, T. Mori, et al. 2003. An increased high-mobility group a2 expression level is associated with malignant phenotype in pancreatic exocrine tissue. Br. J. Cancer 89:2104–2109.

25. Meyer, B., S. Loeschke, A. Schultze, T. Weigel, M. Sandkamp, T. Goldmann, et al. 2007. Hmga2 overexpression in non-small cell lung cancer. Mol. Carcinog. 46:503–511.

26. Sorensen, K. P., M. Thomassen, Q. Tan, M. Bak, S. Cold, M. Burton, et al. 2013. Long non-coding rna hotair is an independent prognostic marker of metastasis in estrogen receptor-positive primary breast cancer. Breast Cancer Res. Treat. 142:529–536.

27. Ge, X. S., H. J. Ma, X. H. Zheng, H. L. Ruan, X. Y. Liao, W. Q. Xue, et al. 2013. Hotair, a prognostic factor in esophageal squamous cell carcinoma, inhibits wif-1 expression and activates wnt pathway. Cancer Sci. 104:1675–1682.

28. Liu, X. H., Z. L. Liu, M. Sun, J. Liu, Z. X. Wang, and W. De. 2013. The long non-coding rna hotair indicates a poor prognosis and promotes metastasis in non-small cell lung cancer. BMC Cancer 13:464.

29. Li, X., Z. Wu, Q. Mei, X. Li, M. Guo, X. Fu, et al. 2013. Long non-coding rna hotair, a driver of malignancy, predicts negative prognosis and exhibits
oncogenic activity in oesophageal squamous cell carcinoma. Br. J. Cancer 109:2266–2278.

30. Ding, C., S. Cheng, Z. Yang, Z. Lv, H. Xiao, C. Du, et al. 2014a. Long non-coding rna hotair promotes cell migration and invasion via down-regulation of rna binding motif protein 38 in hepatocellular carcinoma cells. Int. J. Mol. Sci. 15:4060–4076.

31. Lu, L., G. Zhu, C. Zhang, Q. Deng, D. Katsaros, S. T. Mayne, et al. 2012. Association of large noncoding rna hotair expression and its downstream intergenic cpg island methylation with survival in breast cancer. Breast Cancer Res. Treat. 136:875–883.

32. Chiyomaru, T., S. Fukuhara, S. Saini, S. Majid, G. Deng, V. Shahryari, et al. 2014. Long non-coding rna hotair is targeted and regulated by mir-141 in human cancer cells. J. Biol. Chem. 289:12550–12565.

33. Deng, J., M. Yang, R. Jiang, N. An, X. Wang, and B. Liu. 2017. Long non-coding rna hotair regulates the proliferation, self-renewal capacity, tumor formation and migration of the cancer stem-like cell (csc) subpopulation enriched from breast cancer cells. PLoS ONE 12:e0170860.

34. Jiang, Z., J. Yin, W. Fu, Y. Mo, Y. Pan, L. Dai, et al. 2014. Mirna 17 family regulates cisplatin-resistant and metastasis by targeting tgbetas2 in nsclc. PLoS ONE 9:e94639.

35. Xie, J., M. Liu, Y. Li, Y. Nie, Q. Mi, and S. Zhao. 2014. Ovarian tumor-associated microrna-20a decreases natural killer cell cytotoxicity by downregulating mica/b expression. Cell. Mol. Immunol. 11:495–502.

36. Codo, P., M. Weller, G. Meister, E. Szabo, A. Steinle, M. Wolter, et al. 2014. Microrna-mediated down-regulation of nkg2d ligands contributes to glioma immune escape. Oncotarget 5:7651–7662.

37. Shen, J., J. Pan, C. Du, W. Si, M. Yao, L. Xu, et al. 2017. Silencing nkg2d ligand-targeting mirnas enhances natural killer cell-mediated cytotoxicity in breast cancer. Cell Death Dis. 8:e2740.

38. Yan, H., J. Wu, W. Liu, Y. Zuo, S. Chen, S. Zhang, et al. 2010. Microrna-20a overexpression inhibited proliferation and metastasis of pancreatic carcinoma cells. Hum. Gene Ther. 21:1723–1734.

39. Yu, Z., N. E. Willmarth, J. Zhou, S. Katiyar, M. Wang, Y. Liu, et al. 2010. Microrna 17/20 inhibits cellular invasion and tumor metastasis in breast cancer by heterotypic signaling. Proc. Natl Acad. Sci. USA 107:8231–8236.

40. Yu, Z., C. Wang, M. Wang, Z. Li, M. C. Casimiro, M. Liu, et al. 2008. A cyclin d1/microrna 17/20 regulatory feedback loop in control of breast cancer cell proliferation. J. Cell Biol. 182:509–517.

41. Kumar, M. S., E. Armenteros-Monterroso, P. East, P. Chakravorty, N. Matthews, M. M. Winslow, et al. 2014. Hmg2a functions as a competing endogenous rna to promote lung cancer progression. Nature 505:212–217.

42. Xi, Y. N., X. Y. Xin, and H. M. Ye. 2014. Effects of hmg2a on malignant degree, invasion, metastasis, proliferation and cellular morphology of ovarian cancer cells. Asian Pac. J. Trop. Med. 7:289–292.

43. Ding, X., Y. Wang, X. Ma, H. Guo, X. Yan, Q. Chi, et al. 2014b. Expression of hmg2a in bladder cancer and its association with epithelial-to-mesenchymal transition. Cell Prolif. 47:146–151.

44. Madison, B. B., A. N. Jeganathan, R. Mizuno, M. M. Winslow, A. Castells, M. Cuatrecasas, et al. 2015. Let-7 represses carcinogenesis and a stem cell phenotype in the intestine via regulation of hmg2a. PLoS Genet. 11:e1005408.

45. Yu, K. R., J. H. Shin, J. J. Kim, M. G. Koog, J. Y. Lee, S. W. Choi, et al. 2015. Rapid and efficient direct conversion of human adult somatic cells into neural stem cells by hmg2a/let-7b. Cell Rep. 10:441–452.

46. Parameswaran, S., X. Xia, G. Hegde, and I. Ahmad. 2014. Hmg2a regulates self-renewal of retinal progenitors. Development 141:4087–4097.

47. Wu, J., S. Zhang, J. Shan, Z. Hu, X. Liu, L. Chen, et al. 2016. Elevated hmg2a expression is associated with cancer aggressiveness and predicts poor outcome in breast cancer. Cancer Lett. 376:284–292.