Int J Clin Exp Med 2020;13(4):2675-2683
www.ijcem.com /ISSN:1940-5901/IJCEM0106238

Review Article

Expression of lncRNA-HOTAIR in patients with breast cancer and its effect on biological function

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Received December 11, 2019; Accepted January 18, 2020; Epub April 15, 2020; Published April 30, 2020

Abstract: This study intended to explore the expression of lncRNA-HOTAIR (HOTAIR) in patients with breast cancer and its effect on biological function. Totally 138 patients with breast cancer and admitted to the First Affiliated Hospital of University of South China were included in the research group (RG), and 75 cases of healthy subjects underwent physical examination were included in the control group (CG). The expression of HOTAIR in serum of the two groups was detected by qRT-PCR, and the diagnostic value of HOTAIR in breast cancer patients was assessed by receiver operating characteristic curve (ROC). Independent risk factors for breast cancer were detected using Cox regression analysis. Human normal mammary gland cells and breast cancer cells were purchased. si-HOTAIR and si-NC were transfected into MCF-7 and MDA-MB-231 cells. CCK-8, wound healing assay and flow cytometry were used to determine cell proliferation, invasion and apoptosis. The expression of HOTAIR in serum of patients in the RG was remarkably higher than that in the CG ($P<0.05$). ROC curve analysis exhibited that the AUC of HOTAIR in breast cancer patients was 0.878, the optimal cut-off value was 2.225, the sensitivity was 76.09%, and the specificity was 92%. Cox regression multivariate analysis exhibited that high TNM staging, different molecular typing, lymph node metastasis, and HOTAIR >2.27 were independent prognostic factors for breast cancer ($P<0.05$). Silencing HOTAIR suppressed the proliferation and invasion of breast cancer cells, but increased the apoptosis rate. In summary, HOTAIR is increased in serum of breast cancer patients and is detected to have a high sensitivity and specificity in diagnosis of breast cancer, indicating that the expression of HOTAIR might be correlated with poor prognosis of patients with breast cancer. Silencing HOTAIR could suppress the proliferation and invasion ability of breast cancer cells and improve the apoptosis rate.

Keywords: HOTAIR, breast cancer, expression, prognosis, biological function

Introduction

Breast cancer is one of the most common cancers in women [1, 2]. Due to the highly heterogeneous pathological characteristics of breast cancer, some cases are thought to be aggressive tumors, while others showing good prognosis [3]. Clinical studies have shown that global morbidity and mortality of breast cancer are on the rise, and the high incidence of the disease has led to an urgent need for prevention and treatment measures in society around the world. Although advances in clinical technology have enabled early detection and early treatment of disease [4], and prevented the disease from developing into a metastatic disease, the clinical cause of breast cancer is not fully understood, hence, it is especially important to make early diagnosis of breast cancer patients [5].

LncRNA is a new type of non-coding RNA that can regulate gene expression and protein function [6]. Recently, it has become one of the largest and most diverse RNA families. Clinical studies have shown that it has the function of target-mimetic and sponge/decoy on miRNA [7]. In addition, it has been proved to play a vital role in chromatin modifications, cell differentiation and proliferation, translation, transcription and other biological processes [8-10]. Previous studies have shown that dysregulation of lncRNA is found in multiple tumors, and the dysregulated lncRNA can produce a marked effect on

Table 1. General data between the two groups [n (%)] ($\bar{x} \pm sd$)

Class	Research group (n=138)	Control group (n=75)	t/χ^2	P
Age (years old)			0.004	0.947
≤48	54 (39.13)	29 (38.67)		
>48	84 (60.87)	46 (61.33)		
Residence			2.555	0.110
Cities	67 (48.55)	45 (60.00)		
Countrysides	71 (51.45)	30 (40.00)		
Nationality			0.159	0.690
Han Chinese	55 (39.86)	32 (42.67)		
Minority	83 (60.14)	43 (57.33)		
Education background			0.097	0.756
≥Senior high school	65 (47.10)	37 (49.33)		
<Senior high school	73 (52.90)	38 (50.67)		
Menstruation			0.477	0.490
Pausimenia	54 (39.13)	33 (44.00)		
Premenopausal	84 (60.87)	42 (56.00)		
Number of pregnancies			1.858	0.602
0-1	34 (24.64)	22 (29.33)		
2	42 (30.43)	25 (33.33)		
3	39 (28.26)	15 (20.00)		
≥4	23 (16.67)	13 (17.33)		
Number of miscarriages			0.418	0.811
0-1	60 (43.48)	36 (48.00)		
2-3	45 (32.61)	23 (30.67)		
≥4	33 (23.91)	16 (21.33)		
Family history of breast cancer			2.475	0.115
with	67 (48.55)	28 (37.33)		
without	71 (51.45)	47 (62.67)		
Smoking history			0.924	0.336
Yes	55 (39.86)	35 (46.67)		
No	83 (60.14)	40 (53.33)		
Drinking history			0.283	0.595
Yes	61 (44.20)	36 (48.00)		
No	77 (55.80)	39 (52.00)		
Hypertension history			0.483	0.487
Yes	45 (32.61)	21 (28.00)		
No	93 (67.39)	54 (72.00)		

tumorigenesis by regulating gene transcription and can also act as tumor suppressor genes or oncogenes in tumor diseases [11]. HOTAIR is a 2.2 kb transcript located at the gene boundary of HOXC. Previous studies have reported that it is expressed highly in a variety of cancer types and is bound up with disease metastasis. For example, breast cancer metastasis can be induced by its association with PRC2 complex, thereby retargeting PRC2 genome to hundreds of genes with tumor metastasis [12]. In Dou et al.'s study [13], HOTAIR interacted with polycomb repressive complex 2 and was responsible for the trimethylation of lysine 27 in HOD histone H3, suggesting that metastasis and adverse clinical outcomes in patients with breast cancer were associated with the abnormal expression of HOTAIR. In the study of Wu et al. [14], HOTAIR could modify the progress of cancer, while the missing HOTAIR could inhibit the infiltration of cancer.

In the past, there were few studies on the role of HOTAIR in diagnosis, prognosis and cell biological function of breast cancer. This paper detected the expression of HOTAIR in serum and cell lines of patients to provide reference for the diagnosis and treatment of clinical breast cancer.

Data and methods

General data

Totally 138 breast cancer patients admitted to the First Affiliated Hospital of University of South China from August 2017 to March 2019 were included in the research group (RG), and 75 cases of healthy subjects underwent physical examination were included in the control group (CG). The guardian of the research object can understand the situation of this study and signed the informed consent. The study plan has been submitted to the ethics committee of the First Affiliated Hospital of University of South China for review and implementation after approval. Inclusion criteria were as follows: Patients with histopathological diagnosis of breast cancer [15], with complete clinical

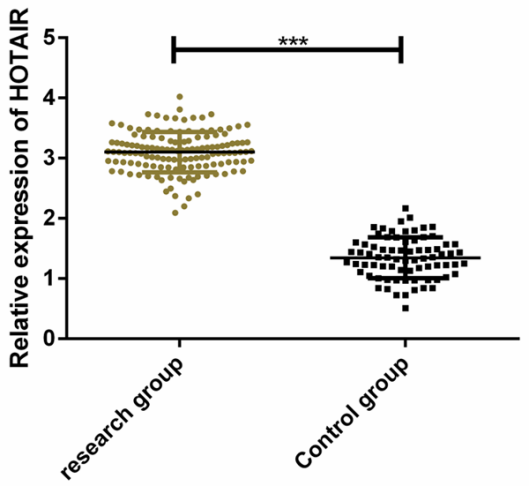

Figure 1. Expression of HOTAIR in serum of two groups of patients. The serum expression of HOTAIR in the RG was significantly higher than that in the CG (P<0.05). Note: ***P<0.05.

general information, expected survival time ≥3 months, and normal function of important organs. Exclusion criteria were as follows: Patients in pregnancy or lactation period. Patients with severe damaged hematopoietic function. Patients with history of other malignant tumors and combined with autoimmune diseases. Patients with severe mental illness. Patients transferred to other hospital halfway. Patients with poor treatment compliance or dropped out of the experiment halfway.

Cell source

Human normal mammary gland cells MCF-10A and breast cancer cells SUM159, SW527, MCF-7, MDA-MB-231 were from ATCC (CRL-10317, CRL-2330, CRL-7940, CRL-3435, HTB-26).

Reagents and instruments

CCK-8 Kit (Bebo-Bestbio, Art. No. BB-4202). Transwell Kit (Shanghai BioGenius Co., Ltd., Art. No. Transwell). qRT-PCR and Reverse Transcription Kit (TransGen Biotech, Beijing, China, AQ141-01, AQ141-01), DMEM, PBS, fetal serum, penicillin-streptomycin (Gibco, USA, 1142802, 10566024, 10010023, 264-00044, 15070063). RIPA reagent, BCA protein kit, ECL luminescence kit, trypsin (Thermo Scientific, USA, 89900, 23250, 32209, 90059). IGF-1 (Shanghai Hengfei Biotechnology Co., Ltd., Art. No. K002504P), β-actin primary antibody, HRP-labeled goat anti-mouse IgG secondary antibody (R&D, US, MAB8929, HAF007). Annexin V/PI apoptosis detection kit (Yeasen Biotechnology Co., Ltd., Shanghai, China, 40302ES20). PCR instrument (ABI, USA, 7500), flow cytometry (BD, USA, FACS Canto II), multifunctional microplate reader (BioTek, USA, DLK0001622). All primers were designed and synthesized by TaKaRa, Dalian, China.

The sample collection

With the consent of the patient, the healthy subjects and the hospital, 5 mL elbow venous blood was extracted from the two groups, centrifuged at 3000 g for 10 min, and the serum was stored for subsequent studies.

Cell culture and transfection

HOTAIR inhibition plasmid (si-HOTAIR) and blank CG (si-NC) were established, respectively, and the established drug-resistant cell lines were transferred to 24-well plates. After 48 h, Lipofectamine 2000 kit was used to transfect the plasmid, and 100 nM si-HOTAIR and si-HOTAIR were transfected into the cells. The operation procedure was strictly in accordance with the kit instructions.

RT-PCR in detecting the expression of HOTAIR

The collected serum was extracted with TRIzol kit for total RNA. An appropriate amount of RNA solution was taken, and the purity and concentration were detected by UV spectrophotometer. Then, PCR amplification was performed with U6 as the internal parameter. PCR reaction system was as follows: 2 × Talent qPCR PreMix 10 μl, upstream primers 1.25 μl, downstream primers 1.25 μl, cDNA 100 ng, and adding water to 20 μl. The PCR amplification conditions were as follows: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 5 s, annealing and extension at 60°C for 15 s, with a total of 40 cycles. The data were analyzed using $2^{-\Delta\Delta CT}$.

WB test

The cultured cells were lysed with RIPA buffer and the protein concentration was detected using BCA. The concentration of protein was adjusted to 4 μg/μL, separated by 12% SDS-PAGE electrophoresis, ionized and transferred to 0.22 μm PVDF membrane. The cells were

Table 2. Relationship between clinicopathological parameters and relative expression of HOTAIR in serum of breast cancer patients ($\bar{x} \pm sd$)

Pathological parameters	n	Relative expression of HOTAIR	t/F	P
Age (years old)			0.322	0.748
≤48	54	3.11±0.35		
>48	84	3.13±0.36		
Residence			0.827	0.410
Cities	67	3.10±0.35		
Countrysides	71	3.15±0.36		
Nationality			0.331	0.742
Han Chinese	55	3.11±0.36		
Minority	83	3.13±0.34		
Education background			0.825	0.411
≥Senior high school	65	3.11±0.35		
<Senior high school	73	3.16±0.36		
Menstruation			1.000	0.319
Pausimenia	54	3.09±0.35		
Premenopausal	84	3.15±0.34		
TNM stage			2.153	0.033
Grade I+II	82	3.20±0.34		
Grade III	56	3.07±0.36		
Hormone receptor (+)			0.496	0.621
ER	68	3.11±0.35		
PR	70	3.14±0.36		
Breast cancer types			0.659	0.511
Ductal carcinoma	84	3.14±0.34		
Lobular carcinoma	54	3.10±0.36		
Family history of breast cancer			0.330	0.742
with	67	3.11±0.34		
without	71	3.13±0.37		
Molecular typing			2.707	0.048
Luminal A	31	3.19±0.38		
Luminal B	55	3.24±0.37		
Overexpressed Her2	23	3.05±0.34		
Triple negative breast cancer	29	3.04±0.35		
Lymph node metastasis			2.108	0.037
with	60	3.06±0.33		
without	78	3.19±0.38		
Local tumor invasion			0.329	0.743
with	63	3.14±0.35		
without	75	3.12±0.36		

sealed with 5% skim milk for 2 h, adding IGF-1, β-actin primary antibody (1:1000) and closed overnight at 4°C. The primary antibody was removed by washing the membrane, adding horseradish peroxidase-labeled goat anti-rabbit secondary antibody at 1:500, incubating at 37°C for 1 h, and rinsing with PBS for 3 times, with 5 min each. Filter paper was used to absorb the excess liquid on the membrane, and ECL was used to illuminate and develop. The protein bands were scanned and the gray level was analyzed using Quantity One software, with GAPDH being used as the internal reference.

Detection of cell invasion (Transwell)

Cells after 24 h of transfection were collected and adjusted to 5×10^4, and inoculated on 6-well plates. They were rinsed twice with PBS and then inoculated. 200 μL DMEM medium was put into the upper chamber, and 500 ml LDMEM (containing 20% FBS) was put into the lower chamber. After culture at 37°C for 48 h, the matrix and cells on the upper chamber that did not cross the membrane surface were wiped. They were rinsed with PBS for 3 times, fixed with paraformaldehyde for 10 min, and rinsed with double distilled water for 3 times. After drying, they were stained with 0.5% crystal violet and observed by a microscope to record the cell invasion.

Apoptosis detection (flow cytometry)

Transfected cells were digested with 0.25% trypsin and rinsed twice with PBS. 100 μL binding buffer was added to prepared 1×10^6/mL suspension. AnnexinV-FITC and PI were successively added, incubating at room temperature and away from light for 5 min. Flow cytometry

Table 3. Diagnostic value of serum HOTAIR in the research group

Diagnostic index	AUC	95% CI	Standard error	Cut-off value	Sensitivity (%)	Specificity (%)
HOTAIR	0.878	0.830~0.94725	0.024	2.225	76.09	92

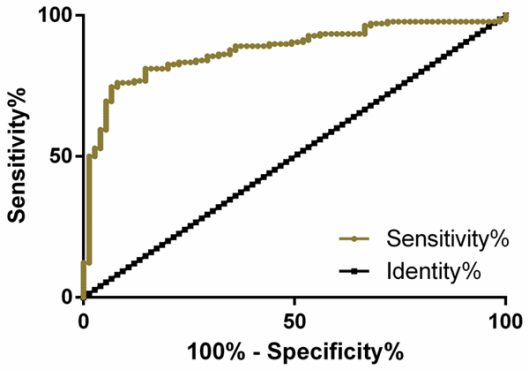

Figure 2. ROC curve of serum HOTAIR for diagnosis of breast cancer. The diagnostic value of serum HOTAIR in RG and CG.

system was used for detection. This experiment repeated 3 times, the data averaged.

Statistical analysis

In this study, statistical analysis of the collected data was performed using SPSS 20.0 Software package, and image rendering of the data was conducted using GraphPad Prism 6 (GraphPad Software, San Diego, USA). Measurement data were represented by mean ± standard deviation (x ± sd). Comparison of measurement data between the two groups was qualified by independent t-test, and enumeration data were expressed as number of cases/percentage [n (%)]. Receiver operating characteristic curve (ROC) was applied to assess the diagnostic value of serum HOTAIR in healthy controls. Independent prognostic factors of breast cancer were examined by Cox regression equation. When P<0.05, there was statistical significance.

Results

General data

There was no notable difference between the RG and CG in the clinical baseline data of age, residence, nationality, education background, menstruation, number of pregnancies, number of miscarriages, family history of breast cancer, smoking history, drinking history and hypertension history (P>0.05). As shown in **Table 1**.

Expression of HOTAIR in serum of two groups of patients

The expressions of HOTAIR in serum of the RG and the CG were 3.12±0.36 and 1.26±0.32, respectively. According to the results of the study, the expression of HOTAIR in serum of the RG was remarkably higher than that in the CG (P<0.05). As shown in **Figure 1**.

Analysis of expression of HOTAIR and clinicopathological characteristics

The relative expression of HOTAIR in breast cancer patients was not significantly different in age, residence, nationality, education background, menstruation, hormone receptor (+), breast cancer type, family history of breast cancer and local tumor invasion (P>0.05). And the clinicopathological characteristics of breast cancer with notable differences in HOTAIR expression were TNM stage, molecular typing, and lymph node metastasis (P<0.05). As shown in **Table 2**.

Diagnostic value of serum HOTAIR in breast cancer

Through ROC curve analysis, it was found that HOTAIR had good clinical diagnostic value in breast cancer patients. The AUC of serum HOTAIR in diagnosing breast cancer patients was 0.878. As shown in **Table 3** and **Figure 2**.

Cox regression analysis of breast cancer

Cox regression univariate analysis showed that high TNM stage, different molecular typing, lymph node metastasis and HOTAIR >2.27 are related factors influencing the prognosis of breast cancer (P<0.05), while age, residence, nationality, educational background, menstruation, hormone receptor (+), types of breast cancer, family history of breast cancer, tumor local infiltration had no notable effect on breast cancer prognosis (P>0.05). We then applied Cox regression analysis, and the results showed that high TNM staging, different molecular typing, lymph node metastasis, and HOTAIR >2.27 were all independent prognostic factors for breast cancer (P<0.05). As shown in **Tables 4**, **5**.

Table 4. Assignment of Logistic multivariate regression analysis

Factors	Variables	Assignment
Age	X1	≤48=0, >48=1
Residence	X2	No=0, yes=1
Nationality	X3	No=0, yes=1
Education background	X4	No=0, yes=1
Menstruation	X5	No=0, yes=1
TNM stage	X6	No=0, yes=1
Hormone receptor (+)	X7	No=0, yes=1
Breast cancer types	X8	No=0, yes=1
Family history of breast cancer	X9	No=0, yes=1
Molecular typing	X10	No=0, yes=1
Lymph node metastasis	X11	No=0, yes=1
Local tumor invasion	X12	No=0, yes=1
HOTAIR	X13	<2.225=0, ≥2.225=1

The expression of HOTAIR in cells and its effect on the biological function of breast cancer patients

According to the detection, the expression of HOTAIR in SUM159, SW527, MCF-7 and MDA-MB-231 cells of breast cancer patients was significantly increased when compared with MCF-10a ($P<0.05$). MCF-7 and MDA-MB-231 with the greatest difference were selected for transfection. After transfection with si-HOTAIR, it could be seen that the expression of HOTAIR in the si-HOTAIR group was remarkably lower than that in the si-NC group ($P<0.05$). After transfection, cell proliferation, invasion and apoptosis were detected, and the proliferation and invasion ability of si-HOTAIR group was remarkably lower than that of si-NC group, while the apoptosis rate was remarkably higher ($P<0.05$). As shown in **Figure 3**.

Discussion

Breast cancer is the most common cancer among women in clinical practice, accounting for about 20% of female deaths [16]. Its morbidity and mortality increases year by year [17]. Breast cancer patients have a risk of recurrence, such as local recurrence in the breast, chest wall or in the lymph nodes [18, 19], and have unique metastatic patterns in regional lymph nodes, bone marrow, liver, and lungs [20].

As a non-protein-coding transcript longer than 200 nucleotides, lcnRNA can target transcription factors, transcribe activators, etc., and produces a marked effective on tumor progression and invasion and cell proliferation, apoptosis [21]. Studies have demonstrated that it plays a significant regulatory part in the occurrence and development of breast cancer [22]. For example, HOTAIR is closely related to the cell metastasis of various cancers such as colorectal cancer, pancreatic cancer and hepatocellular carcinoma, and its expression increases in breast cancer, providing a strong biomarker for tumor metastasis and death of patients with breast cancer [23]. In this paper, the serum expression of HOTAIR in the RG was remarkably higher than that in the CG, and HOTAIR was closely related to TNM staging, molecular typing and lymph node metastasis, indicating that HOTAIR might be a potential analytical marker in the diagnosis of clinical parameters of breast cancer. In the study of Welch G et al. [24], the biological characteristics of tumors were related to the prognosis of breast cancer patients. According to further study, it was found that the AUC of HOTAIR alone diagnosed breast cancer patients was 0.878, the optimal cut-off value was 2.225, the sensitivity was 76.09%, and the specificity was 92%. It suggested that changes in HOTAIR could predict the extent of breast cancer lesions. Univariate analysis of Cox proportional hazard function model for breast cancer patients showed that high TNM stage, different molecular typing, lymph node metastasis and HOTAIR >2.27 were independent prognostic factors for breast cancer. In the research of Deng et al. [25], HOTAIR was significantly up-regulated in breast cancer cell lines CSC-MCF7 and CSC-MB231, suggesting that the migration, invasion and self-renewal of breast cancer cell CSC was in connection with the expression of HOTAIR. In this study, based on the detection of HOTAIR in normal mammary gland cells and breast cancer cells, the expression of HOTAIR in breast cancer cells was found to be significantly higher than that of normal mammary gland cells. Subsequently, we selected cell lines with significant differences for transfection and observed the biological function of the cells. By transfection of si-HOTAIR plasmid into MCF-7 and MDA-MB-231 cells, it

Table 5. Risk factors of breast cancer patients determined by multivariate Logistic regression analysis

Item	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age	1.150 (0.7103-2.028)	0.536		
Residence	1.301 (0.756-2.335)	0.341		
Nationality	0.625 (0.287-1.282)	0.197		
Education background	1.286 (0.376-4.449)	0.673		
Menstruation	1.174 (0.317-4.290)	0.787		
TNM stage	3.036 (1.556-5.913)	0.001	3.670 (1.748-7.271)	0.001
Hormone receptor (+)	1.365 (0.705-3.364)	0.078		
Breast cancer types	1.742 (0.831-3.522)	0.095		
Family history of breast cancer	1.146 (0.276-1.674)	0.797		
Molecular typing	3.044 (1.378-6.430)	0.005	2.487 (1.130-5.675)	0.020
Lymph node metastasis	1.893 (1.217-2.895)	0.008	1.648 (1.045-2.696)	0.034
Local tumor invasion	1.802 (1.056-3.024)	0.083		
HOTAIR	2.749 (1.467-5.212)	0.023	2.186 (1.136-4.226)	0.011

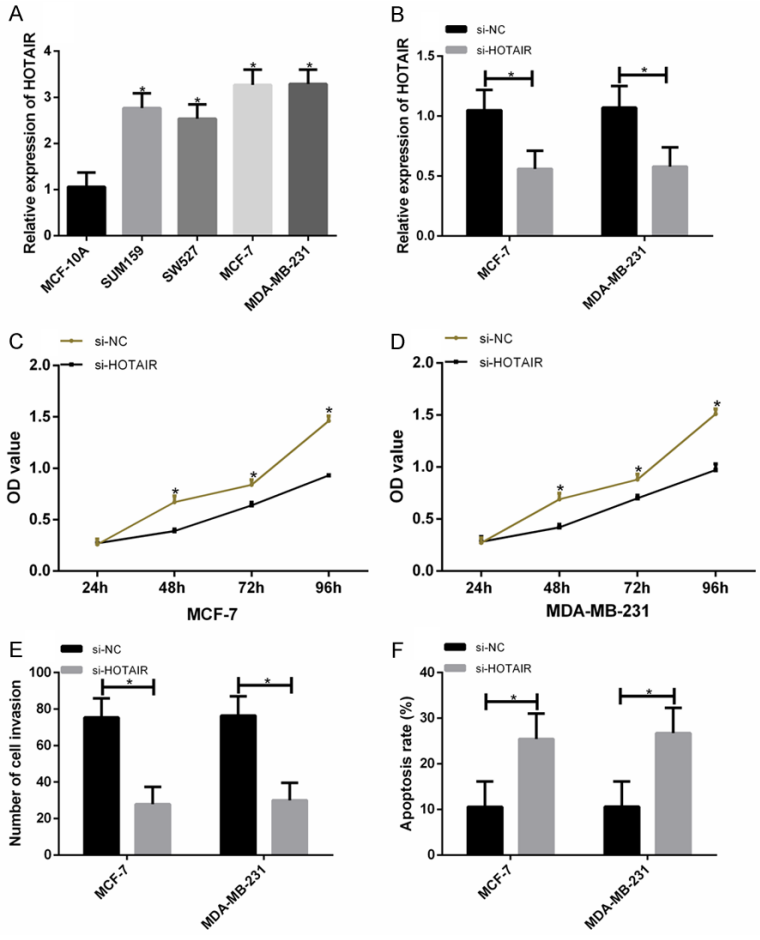

Figure 3. Expression of HOTAIR in cells and its effect on the biological function of breast cancer patients. Expression of HOTAIR in MCF-10A, SUM159, SW527, MCF-7, MDA-MB-231 cells. (A) Expression of PVT1 in MCF-7 and MDA-MB-231 cells after transfection. (B) Proliferation of MCF-7 cells after transfection. (C) Proliferation of MDA-MB-231 cells after transfection. (D) Invasion of MCF-7 and MDA-MB-231 cells after transfection (E). Apoptosis of MCF-7 and MDA-MB-231 cells after transfection (F). Note: *P<0.05.

was found that the proliferation and invasion ability of HOTAIR cells were significantly inhibited after transfection, while the apoptosis rate was significantly increased, suggesting that the down-regulation of HOTAIR expression could suppress the proliferation and invasion ability of cells and promote apoptosis.

This study confirmed that serum HOTAIR could be used as a predictor of prognosis of breast cancer and showed that silencing HOTAIR could suppress the proliferation of breast cancer cells. However, this study still has some limitations. First of all, as a basic research, we can conduct tumor formation in nude experiment and biological cytology experiment by adding the target gene of HOTAIR. In addition, whether the result of HOTAIR in vivo is consistent with the cell test needs to be further verified. Therefore, we hope to carry out more basic studies in future experiments, analyze more potential mechanisms of HOTAIR through bioinformatics, and collect more types (normal people, benign

breast lesions) and different types (serum) samples for verification of our results in this study.

In summary, HOTAIR is increased in serum of breast cancer patients and is detected to have a high sensitivity and specificity in diagnosis of breast cancer, indicating that the expression of HOTAIR might be related to poor prognosis of patients with breast cancer. What's more, silencing HOTAIR could suppress the proliferation and invasion ability of breast cancer cells and improve the apoptosis rate.

Acknowledgements

This study was supported by Research Project of Hunan Provincial Health and Health Committee, No. 20190264; Hengyang Science and Technology Development Plan Project, No. 2017KJ318; Plan of Hunan Provincial Health Department, No. C2019116.

Disclosure of conflict of interest

None.

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