

## Upregulation of *HOTAIR* Transcript Level in Tumor Tissue of Iranian Women with Breast Cancer

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### Abstract

**Background:** Dysregulation of HOX Transcript Antisense Intergenic RNA (*HOTAIR*) has been linked to the etiopathogenesis of several human cancers. According to epidemiological evidences, the risk of susceptibility to breast cancer varies among different populations. This study was designed to determine the transcriptional level of *HOTAIR* in tumor cells of breast cancer patients compared to normal marginal cells and to identify if this molecule has the potential to be considered as a biomarker in the prognosis of breast cancer.

**Methods:** A total of 37 patients with breast cancer were recruited. Tumor and matched normal tumor-free margin samples were collected during surgery from each patient. Following total RNA extraction and cDNA synthesis, quantitative analysis was performed by real-time PCR using the SYBR Green PCR Master Mix to determine the transcript level of *HOTAIR* in samples.

**Results:** It was observed that mRNA expression level of *HOTAIR* was upregulated in tumor cells compared with normal tumor free marginal cells of breast cancer patients (Fold change= +6.9;  $P = 0.0001$ ). However, no statistically significant correlations were observed between the mRNA expression level of *HOTAIR* and clinicopathological manifestations of the patients.

**Conclusions:** The results were in accord with what had previously been reported and therefore, it can be concluded that in Iranian population, too, *HOTAIR* is upregulated in tumor cells of breast cancer patients and has the potential to be considered as a biomarker for the prognosis of breast cancer.

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## Introduction

Breast cancer is considered as the most prevalent cancer among women worldwide (1). Although the incidence of breast cancer was previously low in Iran as well as in other Asian countries, currently it is regarded as one of the most common malignancies among women in Iran (2). In human mammary gland, alteration in gene expression profiling often occurs when normal cells transit to cancerous cells (3). Numerous candidate genes with altered transcript levels have been reported to be associated with human breast carcinogenesis including those involved in cell growth, proliferation, differentiation, apoptosis and invasion (4).

Long non-coding RNAs (lncRNAs) participate in transcription quality and quantity and seem to be important regulators of the epigenome. As one of the first identified lncRNAs, HOTAIR is involved in the development of several malignancies (5). The HOXC locus encodes HOTAIR, which exerts its function in suppressing the expression of genes in the more distal HOXD locus as well as genes on other chromosomes. This results in decreased expression of multiple genes, especially the metastasis-suppressing genes (5-7). Upregulation of HOTAIR expression has been reported in several malignancies such as breast cancer, liver cancer, gastric cancer, and sarcoma (8-11).

since a significant HOTAIR overexpression has been indicated in primary and metastatic breast cancer in comparison with normal breast epithelium, HOTAIR is regarded as an independent biomarker to predict the risk of mortality and metastasis in patients with breast cancer (7). HOTAIR upregulation has been associated with increased invasive ability of tumor cells in breast cancer both *in vitro* and *in vivo* (7). Furthermore, overexpression of HOTAIR has

been observed to be correlated positively with DNA methylation levels in involved cells of primary breast cancer (12). On the other side, DNA hypermethylation has been related to poor disease prognosis (12).

To the best of our knowledge, this study, for the first time, evaluated the expression pattern of HOTAIR mRNA in breast cancer tissue and compared it with the marginal tissues in Iranian population. Furthermore, we determined if the level of HOTAIR mRNA expression level correlated with clinicopathological characteristics of breast cancer patients.

## Material and Methods

### Study Subjects

In this study, 37 patients with breast cancer referred to Noor Nejat Hospital, Tabriz, Iran were selected and their tumorous and matched non-tumoral marginal tissues were collected during mastectomy. Afterward, the collected samples were transferred into RNAlater (Qiagen, Germany) solution, RNA stabilization solution, to stabilize and protect the cellular RNA safely. Samples were then stored at -80 °C until further extraction procedures. All participants filled the informed consent forms. The ethical committee of Tabriz University of Medical Sciences approved this study (50403259/1303092).

### RNA Extraction and cDNA Synthesis

In order to extract RNA from samples, the RNX plus kit (Cinagene) was utilized. Concentrations of the extracted RNA were evaluated using NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA); RNA extraction quality was measured using electrophoresis. In order to generate the first

strand complementary DNA (cDNA), the first strand cDNA synthesis kit (TAKARA) was employed according to the manufacturer's manuals. Reverse transcription implementation was conducted in the final volume of 20µl pre micro-tube. To synthesize the cDNA, briefly, first 4 µl of isolated RNA was mixed with 1 µl of random hexamer primer and 7 µl RNase-free distilled water. This mixture then was incubated at 65°C for 5 minutes. After that, the micro-tubes were placed on ice, and then reaction buffer 4 µl, dNTP mix 2 µl, RNase inhibitor 1 µl, and reverse transcriptase (RT) 1 µl were added to each micro-tube. Immediately then, the samples were incubated at 25°C for 5 minutes, which then followed by 42°C for 60 minutes. Ultimately, the reaction was terminated by heating at 70°C for 5 minutes.

### Quantitative Real-time PCR

Primers applied in this study (Table 1) for transcription quantification of *HOTAIR* were designed using Gene Runner software (<http://www.generunner.net/>). For Specificity and accuracy assessment, all primer sequences were blasted in NCBI website (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Real-time PCR quantification was carried out using *SYBR* green master mix (TAKARA). In order to calibrate the real-time PCR, a serial dilution of human genomic standards was supplied. Following the homogenization of human genome DNA (hgDNA), in order to provide the standards, serial dilution of hgDNA was performed in TRIS-EDTA (TE) buffer. The concentration of hgDNA ranged from 1/10 to 1/10000.

**Table 1.** Primer sequences, amplicon size and melting temperature for each primer

Target	Primer Sequence	Amplicon size (bp)	T <sub>m</sub> (°C)
β2m-F	5'-CTACTCTCTCTTCTGGCCTG-3'		60.3
β2m-R	5'-GACAAGTCTGAATGCTCCAC-3'	191	58.3
HOTAIR-F	5'-CAGTGGGGAAGTCTGACTCG-3'		63.8
HOTAIR-R	5'-GTGCCTGGTGCTCTCTTACC-3'	94	63.5

Quantification of transcripts of target and housekeeping genes was performed via Rotor-gene 6000 Real-time PCR System (Corbett Life Sciences). Micro-tubes of reaction mixtures contained a total volume of 12.5µl (master mix 6.25µl, cDNA 2µl [5ng/ml], primer 0.2 µl forward and reverse each, and H<sub>2</sub>O 3.85 µl). In this study, the real-time PCR conditions were: 50°C for 2 minutes, 95°C for 10 minutes, then 35 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Transcript level of *HOTAIR* was calculated by Pfaffl method (13) after normalization with the correspondence β2-microglobulin (β2M) gene transcript level as the housekeeping gene.

### Statistical Analyses

For statistical analysis of Real-time PCR results, the REST v.2009 and SPSS v.22 (Chicago, IL, USA) softwares were used. First, the Kolmogorov-Smirnov's normality test for evaluating the normal distribution of data was carried out. Independent sample *t*-test for comparing the average expression difference of target genes in tumorous and marginal tissues, Pearson's correlation test for evaluating the correlation between expression of target genes and the patients' clinical profile was employed. All results were

expressed as mean  $\pm$  standard deviation (SD) with statistical significance level at 5%.

## Results

### Clinicopathological Manifestation of Patients

This study was conducted on 37 breast cancer patients (Table 2). Of the studied cases, 25 patients (67.5%) were younger than 55 years and 12 ones (32.5%) were older than 55 years. Patients with left breast involvement (59.5%) were

the major group in comparison to patients with right breast involvement (40.5%). Most of the patients were diagnosed to be in stage II (40.5%); however, 8 (21.5%) and 14 (38%) patients were found to be in stages I and III, respectively. Tumor size of 16 cases (43%) was less than 2cm, while 21 patients (57%) had a tumor size of 2-5cm. In evaluating the pathological state, all of the patients were in the category of Invasive Ductal Carcinoma (IDC).

**Table 2.** Clinicopathological characteristics of breast cancer patients (n=37) and comparison of the HOTAIR1 mRNA expression between different categories

Characteristic of patients	Value	Log of HOTAIR expression in tumor cells	p-value
<b>Age</b>			
<55	25 (67.5%)	0.0039 $\pm$ 0.0090	0.14
>55	12 (32.5%)	0.0011 $\pm$ 0.0014	
<b>Involved side</b>			
Right	15 (40.5%)	0.0040 $\pm$ 0.0092	0.54
Left	22 (59.5%)	0.0024 $\pm$ 0.0063	
<b>Stage of tumor</b>			
I	8 (21.5%)	0.0011 $\pm$ 0.0033	0.75
II	15 (40.5%)	0.0012 $\pm$ 0.0034	
III	14 (38%)	0.0027 $\pm$ 0.0034	
<b>Tumor size (cm)</b>			
<2	16 (43%)	0.0035 $\pm$ 0.0090	0.71
2-5	21 (57%)	0.0027 $\pm$ 0.0064	
<b>Pathology</b>			
IDC2	37 (100%)	0.0026 $\pm$ 0.0058	-

1: HOX transcript antisense RNA; 2: Invasive Ductal Carcinoma

### Transcript Level of HOTAIR Gene

Quantitative real-time PCR analysis revealed a significant upregulation of HOTAIR mRNA in tumorous cells in

comparison to normal tumor free marginal cells (6.9 times upregulated;  $P = 0.0001$ ; Figure 1).

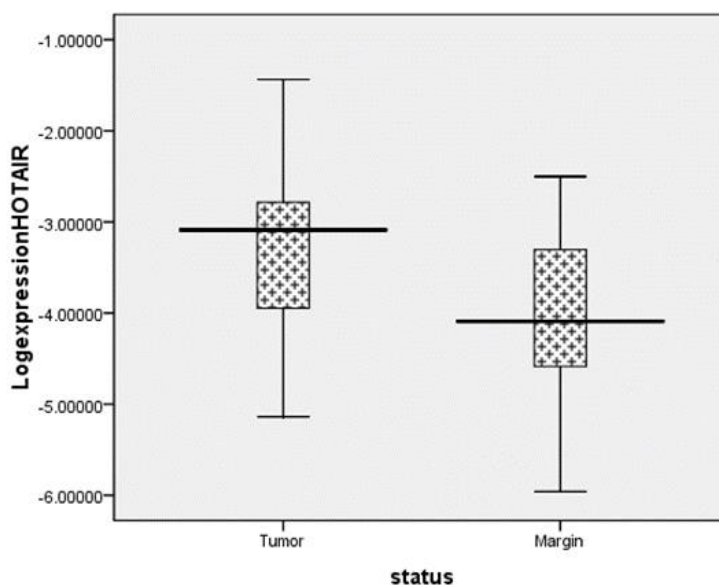


Figure 1. The mRNA expression level of HOTAIR in tumorous and normal marginal tissues (data are shown in logarithmic values)

**HOTAIR Gene Potential as a Breast Cancer Tumor Marker**

Receiver Operating Characteristic (ROC) curve was plotted for *HOTAIR* gene and the area under the ROC curve (AUC) was measured for measuring the specificity and sensitivity of predicting breast cancer and normal tissue by

*HOTAIR* transcript level. *HOTAIR* transcript level presented an AROC of 0.758 (Figure 2.A;  $P < 0.05$ ; CI: 0.64-0.86). To determine the optimal cut-off value, we carried out a post-test from pre-test probability of 0.5 and cost ratio of 1.00. The optimal cut-off point was  $< 10.4$  with 0.92 sensitivity and 0.54 specificity for *HOTAIR* (Figure 2.B).

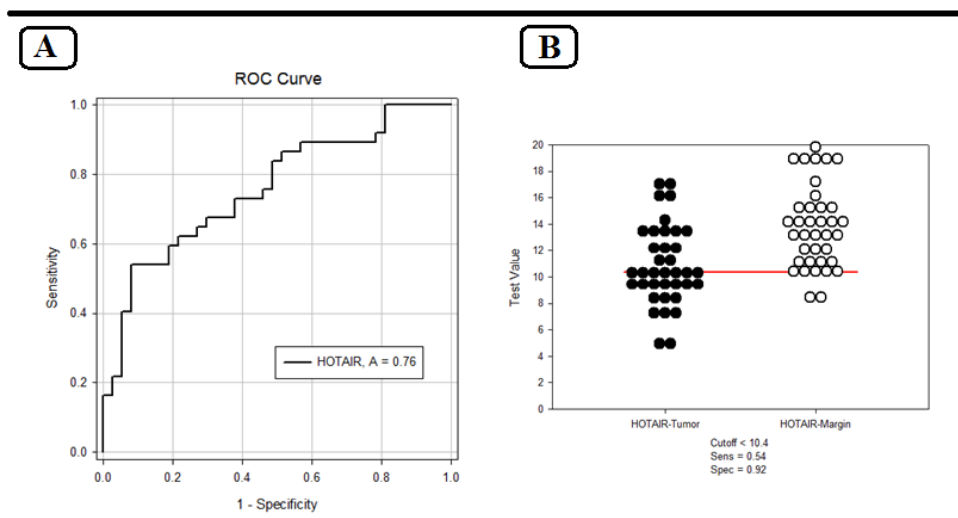


Figure2. A: The ROC curve was automatically generated from 37 points of cut-off values set by Sigma Plot software. The area under the ROC curve (AUC) is 0.758 for *HOTAIR*. B: The dot histogram shows the optimum cutoff points for *HOTAIR* gene.

### Association between HOTAIR mRNA Expression Level and Clinicopathological Characteristics

There was no significant association between mRNA expression levels of HOTAIR gene and clinicopathological traits including age, the involved side of the breast and tumor stage and size (Table 2).

### Discussion

Over the past few years, the expression of HOTAIR has been vastly surveyed in various cancer types; however, the precise molecular mechanism underlying the role of HOTAIR in tumorigenesis has remained obscure. It has been reported that HOTAIR participates in regulating the epigenome and remodeling the chromatin state. HOTAIR is involved in the modification of transcription of genes, including Wnt inhibitory factor 1 (WIF-1), homeobox D10 (HOXD10), matrix metalloproteinase (MMP) 1/3, phosphatase and tensin homolog (PTEN) and snail transcription factor family. Most of these genes play a role in the regulation of cancer cell proliferation and invasion and have been associated with tumor development. On the other hand, HOTAIR regulates expression of genes, which function in cell motility and matrix invasion in tumor cells (14).

Polycomb proteins play a role in the suppression of transcription of several genes, which are involved in cell differentiation (15, 16). These proteins function in protein complexes such as polycomb repressive complex (PRC) 1 and PRC2. As a histone H3 lysine 27 (H3K27) methylase, PRC2 by regulating the H3K27 methylation is involved in gene silencing and has been associated with cancer progression (17, 18). Polycomb proteins and lncRNAs, like HOTAIR,

collaborate to epigenetically induced gene silencing. In fact, HOTAIR binding is mandatory to guide PRC2 to the target regions of the genome, where PRC2 exerts the function of gene expression silencing.

In primary and metastatic breast cancer, there is an overexpression of HOTAIR in comparison to normal breast epithelium. This upregulation is linked to the increased invasive capacity of tumor cells in breast cancer patients (7). On the other hand, increased DNA methylation in primary breast cancer cells has been correlated with highly expressed HOTAIR and associated with poor disease prognosis (12). Significant associations were not reported between the expression of HOTAIR and clinical specifications, suggesting that HOTAIR cannot be an independent prognostic biomarker in breast cancer (12). In accord with previous investigations in breast cancer, our study indicated an up-modulation of HOTAIR in tumor cells in comparison with marginal tumor free cells. Moreover, we did not find any correlations between the expression of HOTAIR and clinicopathological characteristics of breast cancer patients in our study population. On the other hand, with a significant AROC score, HOTAIR expression level could predict the breast cancer or normal tissue.

Given the observation that HOTAIR is overexpressed in a number of human cancers, it provides a promising tool to design anti-cancer therapies. HOTAIR has the potential to be exerted as a biomarker for cancer prognosis and diagnosis. Overexpression of HOTAIR has been associated with promoting tumor cell metastasis, providing a negative prognostic molecule for survival rate of patients. Through monitoring the expression level of HOTAIR in cancers, it is

facilitated to predict the risk of tumor development and progression. Studies in tumor xenograft models of murine have reported that HOTAIR knockout prevents tumor growth *in vivo* (19, 20). Therefore, approaches to decrease the expression level of HOTAIR, such as gene knockout, may hopefully provide therapeutic tools for cancer treatment.

## Conclusion

To sum up all the facts, this study for the first time, to our best knowledge, demonstrated the upregulation of HOTAIR gene in Iranian breast cancer women. However, no significant association was observed between clinicopathological manifestations of breast cancer patients and HOTAIR expression level. Evaluating the alterations in molecular

markers, like HOTAIR, during initiation and progression of cancer underpins the designing of more effective therapies and may provide a tool to prevent cancer progression in early diagnosed cases. To reach this end, further investigations are crucial to reveal the exact molecular mechanism of HOTAIR in various populations.

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## Declaration of Conflict of Interest

The authors report no conflicts of interests to declare.

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