Original Article

Association of *HOTAIR* expression in gastric carcinoma with invasion and distant metastasis

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Abstract

Background: Gastric cancer is the second and fourth most common cancer in Iranian men and women, respectively, but it is the first leading cause of cancer deaths in Iran. Most Iranian patients with gastric cancer are diagnosed at an advanced stage of disease when the conventional treatments have no effect on improving the survival. So, early gastric cancer detection is of high priority in order to decrease its high mortality rate in Iran. *HOTAIR* is a long non-coding RNA which its overexpression has been documented in different types of human cancer and can be considered as a potential cancer biomarker. The aim of this study was to evaluate the clinicopathological relevance of the expression of *HOTAIR* gene in gastric carcinoma.

Materials and Methods: A total of 60 tumoral and non-tumoral gastric specimens were evaluated for *HOTAIR* gene expression using quantitative real-time PCR.

Results: The expression of *HOTAIR* was markedly increased in gastric cancer tissues compared with adjacent non-tumoral tissues. We further showed that there was a positive significant correlation between the *HOTAIR* gene expression, TNM staging, perineural invasion, and distant metastasis, but not with other clinicopathological features of gastric tumors.

Conclusions: These results suggest that *HOTAIR* expression is modulated during gastric cancer progression and therefore may participate in molecular processes relevant to malignant transformation and metastasis in gastric carcinoma.

Key Words: Gastric carcinoma, gene expression, *HOTAIR*, invasion, metastasis

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Received: 13.08.2013, **Accepted:** 14.09.2013

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.133278

INTRODUCTION

Gastric cancer is the second most common cancer worldwide and the second leading cause of cancer mortality.[1] Gastric cancer is the second and fourth most common cancer in Iranian men and women, respectively, but it is the first leading cause of cancer deaths in Iran.[2] The main environmental factors

How to cite this article: Emadi-Andani E, Nikpour P, Emadi-Baygi M, Bidmeshkipour A. Association of *HOTAIR* expression in gastric carcinoma with invasion and distant metastasis. Adv Biomed Res 2014;3:135.

for the high incidence of gastric cancer in Iran are *H. pylori* infection, high intake of salt, and smoking. Most Iranian patients with gastric cancer are diagnosed at an advanced stage of disease when the conventional treatments have no effect on improving the survival. So, early gastric cancer detection is of high priority in order to decrease its high mortality rate in Iran.[3]

A large part of the genome is transcribed for non-coding RNAs (ncRNAs) with various biological functions.[4] Long non-coding RNAs (lncRNAs) have a length of >200 nucleotides, with direct effect on the transcription or employing histone modification complexes regulating expression of different genes.[5,6] *Hox transcript antisense intergenic RNA* (*HOTAIR*) is a lncRNA with 2158 nucleotides in length which is expressed from HOXC locus on 12 chromosome.[7] It interacts with Polycomb Repressive Complex 2 (PRC2) and causes targeting it to the HOXD locus.[7,8] Several studies display an active role for *HOTAIR* in cancers of the breast,[9,10] colorectal,[11] hepatocellular,[12-14] pancreatic,[15] gastrointestinal stromal tumors,[16] nasopharyngeal carcinoma (NPC),[17] laryngeal squamous cell carcinoma (LSCC),[18] and sarcoma.[19] In all studies, increased expression of *HOTAIR* is associated with malignant progression and poor survival. Hence, *HOTAIR* may be considered as a potential target for diagnosis and treatment of various cancer types.[9-19]

Based on these findings, we tested if *HOTAIR* also shows a similar pattern in gastric cancer. To this aim, we evaluated *HOTAIR* expression in tumoral and non-tumoral gastric tissue samples by using quantitative real-time RT-PCR. Our results demonstrated that the expression of *HOTAIR* was markedly increased in gastric cancer tissues compared with adjacent normal tissues. We further showed that there was a positive correlation between the *HOTAIR* gene expression, TNM (T, N, and M stand for tumor, lymph node, and metastasis, respectively) staging, perineural invasion, and distant metastasis, but not with other clinicopathological features of gastric tumors.

MATERIALS AND METHODS

Tumor and non-tumor tissues

All experimental procedures were approved by the Ethics Committee of Isfahan University of Medical Sciences. A total of 60 specimens of gastric cancer tissues and adjacent benign tissues (paired) were obtained from the Iran Tumoral Bank (Tehran, Iran) as described previously.[20-22] All patients provided written informed consent to the Iran Tumoral Bank prior to the participation.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from gastric cancer tissues using Qiazol reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity of RNA was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide. The quality and quantity of RNA were determined by ultraviolet spectroscopy. cDNA was synthesized using random hexamer primers and an M-MLV reverse transcriptase (Fermentas, Vilnius, Lithuania) as described elsewhere.[20-22]

Quantitative real-time PCR

The expression level of *HOTAIR* gene was determined by quantitative real-time RT-PCR with *TBP* (TATA box binding protein) as a reference gene.[23] The primers for the target gene were as follows: 5´-AGACGGCGGCGAGAGGAA-3´ and 5´-CTGAAATGGAGGACCGGCG-3´ with an amplicon size of 126 bp. PCR was performed using Maxima™ SYBR Green/ROX qPCR Master MIX (Fermentas, Vilnius, Lithuania) and an Applied Biosystems StepOnePlus™ instrument. The PCR amplification conditions consisted of an initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C and 58°C for *TBP* and *HOTAIR* genes, respectively, then extension for 15 seconds at 72°C. All samples were measured in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative levels of gene expression.

Statistical analysis

All data are expressed as means ± standard error of mean (SEM) from at least three separate experiments. Statistical analyses were performed using SPSS version 16.0. Differences between groups were analyzed using a paired t-test or one-way ANOVA with post hoc multiple comparisons. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Optimization of PCR amplification

In order to obtain a specific amplicon for *HOTAIR*, both conventional and real-time PCR was done with a temperature gradient. As it was shown in a previous study that *HOTAIR* is expressed in MCF-7 breast cancer cell line,[9] we used the cDNA of this cell line for optimization procedures. Gel electrophoresis of amplified product of *HOTAIR* with the designed primers showed a specific band with expected size (126 bp) in 58°C [Figure 1]. Analysis of melting curves of real-time PCR also showed a unique melting curve for this amplicon without primer dimmers (data not shown).

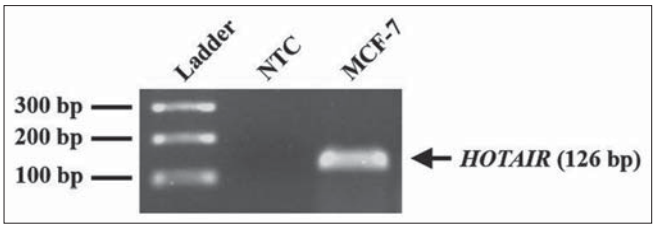

Figure 1: Optimization of PCR conditions for *HOTAIR* gene expression. RT-PCR products were separated on agarose gel electrophoresis as follows: lane 1: DNA size marker, lane 2: Non-template control (adding no cDNA), and lane 3: adding cDNA of MCF-7 cell line

HOTAIR gene expression in tumoral and non-tumoral gastric specimens

To examine the expression of *HOTAIR* in human gastric tissues, cDNA was synthesized for all samples and real-time PCR was performed using specific primers for both *HOTAIR* and *TBP* genes. The relative expression of *HOTAIR* showed statistically significant overexpression in pooled tumoral specimens compared to the paired non-tumoral samples [Figure 2, $P = 0.028$].

Correlation of *HOTAIR* expression with clinicopathological features in gastric carcinoma

In order to examine the clinical importance of the *HOTAIR* overexpression, the correlation between clinicopathological status of gastric tumor samples and level of *HOTAIR* expression was investigated. Analyses showed a significant and positive association between the expression levels of *HOTAIR*, TNM staging, perineural invasion, and distant metastasis. A trend was also evident toward the same pattern for lymph node metastasis (N classification) and invasion depth although not reaching statistical significance. No significant correlation was found for other clinicopathological features of gastric tumors [Table 1].

DISCUSSION

Our study showed for the first time that *HOTAIR* expression is significantly correlated with perineural invasion and distant metastasis in gastric cancer. An increased *HOTAIR* expression in gastric carcinomas compared to their non-tumoral adjacent tissues was also documented.

Until now, altered expression of *HOTAIR* gene has been documented in different types of human cancer. Gupta *et al.*[9] showed increased expression of *HOTAIR* in primary breast tumors as well as metastases. They showed that high expression of *HOTAIR* is an independent prognostic factor for metastasis and death in breast cancer patients. In another study by Milhem *et al.*,[19] increased expression of *HOTAIR*

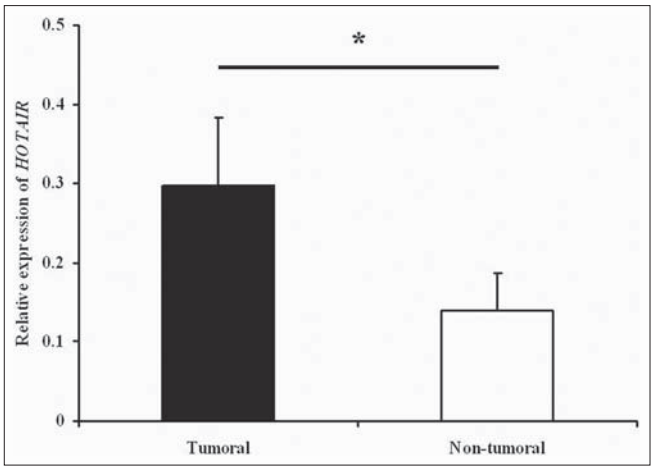

Figure 2: The relative expression levels of *HOTAIR* in tumoral vs non-tumoral gastric samples. Error bars represent standard error of mean (SEM) and the asterisk represents a statistically significant difference ($p \leq 0.05$)

Table 1: Relationship between *HOTAIR* expression levels and clinicopathological parameters of tumoral gastric samples

Characteristics	Numbers (%)	*HOTAIR* relative expression (mean±SEM*)	*P* value
Sex			0.13
Male	18 (60)	0.15±0.05	
Female	12 (40)	0.51±0.28	
Age (years)			0.31
≥70	15 (50)	0.36±0.23	
<70	15 (50)	0.23±0.09	
Depth of invasion			0.07
T1-T2	3 (10)	0.11±0.06	
T3-T4	27 (90)	0.31±0.13	
N classification			0.06
N0	9 (30)	0.12±0.03	
N1-N3	21 (70)	0.37±0.17	
M classification			0.04
Mx	15 (50)	0.20±0.07	
M0	11 (36.6)	0.19±0.08	
M1	4 (13.3)	0.94±0.36	
TNM stage			0.03
I-III	26 (86.7)	0.19±0.05	
IV	4 (13.3)	0.94±0.36	
Perineural invasion			0.04
Negative	6 (20)	0.08±0.03	
Positive	24 (80)	0.35±0.11	
Lymphatic invasion			0.11
Negative	16 (55.17)	0.14±0.04	
Positive	13 (44.82)	0.50±0.27	
Tumor size (cm)			0.45
≥5	20 (68.7)	0.31±0.17	
<5	9 (31. 3)	0.29±0.14	
Tumor grades			0.21
I	10 (33.3)	0.47±0.34	
II	8 (26.6)	0.21±0.12	
III	12 (40)	0.20±0.09	
Tumor types			0.16
Diffuse	15 (50)	0.17±0.07	
Intestinal	15 (50)	0.42±0.23	

*SEM: Standard error of mean

was detected in most of the primary and metastatic sarcoma patient tumor samples in such a way that its expression was correlated with the likelihood of metastasis in primary sarcoma tumors. In 2011, Geng *et al.* measured *HOTAIR* gene expression in hepatocellular carcinoma (HCC) tissues and showed that the expression of *HOTAIR* is significantly higher in tumoral vs. non-tumoral HCC tissue samples. Furthermore, they reported a positive correlation between the *HOTAIR* gene expression and lymph node metastasis. However, they did not observe any significant correlation between *HOTAIR* expression levels and other clinicopathological features of patients like age, gender, tumor size, tumor number, and portal invasion.[13] Kogo *et al.* also showed *HOTAIR* overexpression in colorectal cancerous tissues compared to the noncancerous ones. They divided their patients into two groups, one with high and one with the low *HOTAIR* expression. They found a strong correlation between *HOTAIR* expression and histological grade, depth of tumor and liver metastasis but not with age, gender, lymph node metastasis, lymphatic or venous invasion. The group with higher *HOTAIR* expression had also poorer prognosis.[11] In another study of HCC patients, *HOTAIR* expression level was shown to be higher in tumor tissues than their adjacent non-tumoral ones. Furthermore, the higher level of *HOTAIR* was linked to shorter survival and more probability of recurrence after liver transplantation.[12] In contrast to the other previous reports, a study by Lu *et al.* in 2012 demonstrated that high levels of *HOTAIR* are correlated with the lower chance of recurrence and mortality in 348 examined tissues from patients with breast cancer. They also found no significant correlation between *HOTAIR* levels and various pathological and clinical features of breast cancer samples like tumor stage, grade, histological type, tumor size, and nodal status.[24] In 2012, a study by Chisholm *et al.*[10] was performed to analyze *HOTAIR* gene expression in formalin-fixed paraffin-embedded breast cancer tissues. They showed a positive correlation between *HOTAIR* levels and ER/PR (estrogen receptor/progesterone receptor) positivity and also with worse survival rates. In 2013, overexpression of *HOTAIR* was also shown in LSCC tissue samples compared to their adjacent non-tumoral tissues. In that study, a positive association between *HOTAIR* gene expression levels and T classification, neck nodal metastasis, and clinical stage was reported.[18] Nie *et al.* examined *HOTAIR* gene expression using *in situ* hybridization and real-time PCR in NPC samples. Similar to other studies, they also showed increased expression of *HOTAIR* in cancerous samples in comparison to non-cancerous tissues. They also reported a positive association of *HOTAIR* gene expression with tumor size, clinical stage, lymph node tumor burden, and distant metastasis.[17]

Nearly all studies that have examined the *HOTAIR* expression levels in tumoral and non-tumoral samples of different cancer types have reported *HOTAIR* elevated levels in cancerous specimens.[9-19] Taken together our data are in accord with other studies[9-19] that showed *HOTAIR* expression increased significantly in cancerous tissues. We also observed a positive significant correlation between *HOTAIR* expression, staging, invasion, and metastasis; in a same way to the other studies.[9-11,13,17-19] Moreover, a trend was also evident toward the same pattern for lymph node metastasis (N classification) and invasion depth although not reaching statistical significance. This is consistent with findings of previous studies.[11,13,17,18] However, we did not observe a significant association between the target gene expression and other clinicopathological parameters, like age, gender, tumor size, grades, histological types, and lymphatic invasion consistent with other studies.[11-13,17,18,24] Finally, during processing of the manuscript, two relevant studies appeared in the Pubmed in which they also showed that *HOTAIR* is overexpressed in gastric cancer and is associated with TNM staging, lymph node metastasis, and poor overall survival.[25,26]

In summary, this is the first study showing a positive and significant correlation between *HOTAIR* gene expression profile, perineural invasion, and distant metastasis in gastric tumor samples. These results suggest that *HOTAIR* expression is modulated during gastric cancer progression and therefore may participate in molecular processes relevant to malignant transformation and metastasis in gastric carcinoma.

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Source of Support: The study was supported in part by a research grant from Isfahan University of Medical Sciences, Isfahan and Razi University, Kermanshah, Iran, **Conflict of Interest:** None declared.