

Investigation of the Association of HOTAIR Single Nucleotide Polymorphisms and Risk of Breast Cancer in an Iranian Population

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Abstract

Background: Long non coding RNAs (lncRNAs) are of functional non coding RNAs which have been shown to be involved in several important pathways in cancer development and progression. Among them is Hox transcript antisense intergenic RNA (HOTAIR) whose overexpression has been detected in several cancer types. In addition, its functional polymorphisms have been shown to be associated with breast cancer risk in certain populations.

Objectives: The aim of the present study was to investigate the effects of three HOTAIR polymorphisms (rs12826786, rs1899663 and rs4759314) and their haplotypes on breast cancer risk in a sample of Iranian population.

Methods: This study is a case-control study which consisted of 122 unrelated breast cancer patients from Hamadan University hospital and 200 normal females who were referred to a routine health survey in 2015. Genomic DNA was extracted from blood samples of all participants using the standard salting out method. Tetra-primer ARMS-PCR method was used for analyses of rs12826786, rs1899663, and rs4759314 genotypes. Comparison of genotype and allele frequency between the breast cancer patients and the control group was performed using Pearson chi-square test considering odds ratio (OR) and 95% confidence intervals (CI) for calculation of the relative risk. Haplotype frequencies for HOTAIR were calculated using SNPStats online program.

Results: No significant difference has been found in allele and genotype frequencies of polymorphisms between case and control groups. Furthermore, no specific HOTAIR haplotype was shown to be associated with breast cancer risk in the analyzed population.

Conclusions: These polymorphisms do not seem to be associated with breast cancer risk in this population. However, further research is needed to evaluate the results of the present study in larger patient samples.

Keywords: HOTAIR, Long Non Coding RNA, Breast Cancer, Iran

1. Background

Long non coding RNAs (lncRNAs) are an important group of functional non coding RNAs with a defined role in critical cellular pathways implicated in normal development as well as cancer (1). For instance, they participate in chromatin rearrangement, histone modification, and modification of alternative splicing genes as well as regulation of gene expression (1). All these functions are implicated in cancer development as well as metastasis. Considering the immense burden of breast cancer and challenges in its screening programs (2), determination of genetic factors which contribute to breast cancer risk among women is of value. In order to find their contribution in breast cancer risk, lncRNAs role in breast cancer has been evaluated in different studies with focus on their differential expression (3) as well as their variants at DNA sequence (4). Hox transcript antisense intergenic RNA (HOTAIR) is

among lncRNAs with a defined role in regulation of cancer stem cell (CSC) plasticity and a possible target for anti-CSC treatments (1). Considering the role of CSC in breast cancer pathogenesis and development (5), evaluation of its significance in breast cancer is of value. HOTAIR is transcribed from HOXC locus and inhibits transcription in trans across long distance of the HOXD locus (6). HOTAIR interaction with the polycomb repressive complex 2 (PRC2) is implicated in regulation of the methylation at histone H3K27 and subsequent modulation of gene expression (7). In addition, HOTAIR binds to the LSD1/CoREST/REST complex and influences the demethylation of histone H3K4, through which it regulates genes expression (8). It inhibits the expression of numerous tumor suppressor genes namely the protocadherin family, such as HOXD10 and PGR, in addition to several metastasis suppressor genes such as PCDH10, PCDHB5, and JAM2 (9). HOTAIR overexpression

has been associated with tumor invasiveness in a broad range of cancers (10). In breast cancer, its overexpression has been demonstrated in primary tumors and metastases. Notably, its expression level in primary tumors is predictive of ultimate metastasis and death. While its overexpression changes gene expression pattern of breast epithelial cells to an expression pattern associated with embryonic cells, its downregulation would result in decreased invasiveness via epigenetic modulations (7). In addition, functional polymorphisms within this gene have been shown to be associated with breast cancer (4) as well as cervical cancer (11) and esophageal squamous cell carcinoma risk (12) in certain populations. However, to the best of our knowledge, HOTAIR polymorphisms and their relation with cancer risk have not been evaluated in Iranian population yet. In the present study, we investigated the association between three single nucleotide polymorphisms (SNPs) in HOTAIR gene (rs12826786, rs1899663 and rs4759314) and breast cancer risk in an Iranian population. The rationale for selection of these SNPs was the observed association with breast cancer risk as well as patients' clinicopathological characteristics in other populations (4, 13, 14).

2. Objectives

The aim of the present study was to investigate the effect of three HOTAIR polymorphisms and their haplotypes on breast cancer risk in a sample of Iranian population considering the fact that these SNPs have been shown to be associated with breast cancer risk in some other populations.

3. Methods

3.1. Study Population

This study is a case-control study and has been approved by the local ethical committee. The patient group consisted of 122 unrelated breast cancer patients from the department of surgery, Hamadan University hospital whose breast cancer diagnosis had been confirmed by pathologic study. None of the patients received chemotherapy or radiotherapy prior to sampling. The control group included 200 normal females who were referred to a routine health survey during 2015. Informed consent was acquired from all participants. Clinical and pathological data of patients were obtained from their medical records.

3.2. Genotype Determination

Genomic DNA was extracted from blood samples of all participants using the standard salting out method.

Tetra-primer ARMS-PCR method was used for analyses of rs12826786, rs1899663 and rs4759314 genotypes. In brief, 100 ng of genomic DNA was amplified in a total volume of 25 μ L reaction mixture by Taq DNA polymerase master mix red (Ampliqon, Denmark). The PCR condition consisted of a preliminary denaturation at 94°C for 4 minutes, subsequent 35 cycles of 94°C for 45 seconds, specific annealing temperatures for 45 seconds, and 72°C for 55 seconds, with the final extension of 72°C for 5 minutes. Specific annealing temperatures were as follows: 60°C for rs12826786, 57°C for rs1899663, and 54.5°C for rs4759314 respectively. The primers used in the genotyping analysis are listed in Table 1.

3.3. Statistical Analysis

The genotype frequencies were determined by direct counts, while the allele frequencies were calculated by dividing the overall allele counts with the total sum of chromosomes. Goodness of fit to Hardy-Weinberg equilibrium was assessed. For this purpose, the expected frequencies of each genotype were compared with the observed values by means of Chi-square test. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Comparison of genotype and allele frequency between the breast cancer patients and the control group was performed using Pearson chi-square test considering odds ratio (OR) and 95% confidence intervals (CI) for calculation of the relative risk. Haplotype frequencies for HOTAIR were calculated using SNPStats online programme (<http://bioinfo.iconcologia.net/SNPstats>). Differences were considered significant when $P < 0.05$.

4. Results

The frequencies of HOTAIR alleles in both patients and control groups have been shown to be in agreement with the assumption of Hardy-Weinberg equilibrium ($P > 0.05$), which shows that they were randomly distributed. No significant difference has been demonstrated in age between cases and controls (mean age of patients: 38.9 ± 2.1 and mean age of healthy controls: 39.1 ± 1.8). The allele and genotype frequencies of the polymorphisms in both groups, and the statistical analysis data are demonstrated in Table 2. No significant difference has been found in allele and genotype frequencies of polymorphisms between case and control groups. Distribution of HOTAIR haplotype frequencies in breast cancer patients and controls are shown in Table 3. No specific HOTAIR haplotype was shown to be associated with breast cancer risk in the analyzed population.

Table 1. Sequence of Primers

Primer Position	Primer Sequence	PCR Product Size (bp)
rs12826786		
Forward inner primer (C allele)	CGCAGGACGGGCTTCGTGAATTAAC	for C allele: 203, for T allele: 145, by outer primers: 293
Reverse inner primer (T allele)	CGGCAGAGGGAAGGAGCTTAGGATAATGA	
Forward outer primer (5' - 3')	ATCTGTCCAGTCGCTCGTACCTGAG	
Reverse outer primer (5' - 3')	TGTGTTGGTCTGTGTCGAGGTCAGTTT	
rs1899663		
Forward inner primer (T allele)	CCATTATCCAGTTGAGGAGGGTGAAT	for T allele: 226, for G allele: 284, by outer primers: 457
Reverse inner primer (G allele)	CCAAAAGCCTCTAATTGTTGTCGCC	
Forward outer primer (5' - 3')	TGAAAGCCACGATCATTAAACATAACCA	
Reverse outer primer (5' - 3')	TATCTACGGAGGACTTACCTTATTCCTG	
rs4759314		
Forward inner primer (A allele)	GCATGGAAGAGATATAAACAGGCGAA	for A allele: 181, for G allele: 121, by outer primers: 24
Reverse inner primer (G allele)	TTATCACGTTTATTAACCTGCATCCTCC	
Forward outer primer (5' - 3')	AAACCATATCCTGACAGAAGCCAAATAC	
Reverse outer primer (5' - 3')	CCAAGGTAGGGAAGTCTCTATTCTCTG	

5. Discussion

HOTAIR has been shown to exert an active role in altering the cancer epigenome and has been proposed as an important target for cancer detection and treatment (7). In addition, HOTAIR has been demonstrated to regulate expression of many oncogenes (HER2, MMP3/9), tumor suppressor genes (PTEN and P21), and key signaling pathways (PI3K/AKT/S6/mTOR, STAT3, and wnt/b-catenin) all of which implicated in cancer development and progression (11). Further supports for the role of HOTAIR in cancer development has originated from a study which has shown that HOTAIR could indirectly downregulate Mir-7 expression and consequently promotes epithelial-mesenchymal transition (EMT) and contributes to tumor invasion and metastasis in breast cancer cell lines (15). Accordingly, evaluation of association of functional polymorphisms within this gene with cancer risk is of practical significance. In the present study, we evaluated the association of three HOTAIR SNPs with breast cancer risk in an Iranian population. However, we could not find any significant difference between patients and the control group in allele and genotype frequencies of these three SNPs. Previous studies have evaluated the association between these functional polymorphisms in HOTAIR and risk of breast cancer in different populations such as Chinese (14) and Turkish (4, 13). For instance, rs12826786 polymorphism has been shown to be associated with an increased risk of breast cancer in a Turkish population. Besides, the same polymorphism has

been shown to be significantly associated with advanced TNM stage, larger tumor size, and distant metastasis, as well as poor histological grade (13). Such interethnic differences in association of certain SNPs with a specific disorder can be attributed to several factors including linkage disequilibrium between the analyzed SNP and another variant or the existence of different haplotypes in diverse populations. Another study which has been conducted in a Chinese population has revealed that rs1899663 genotypes have been associated with breast cancer risk only in subgroups of breast cancer patients whose age at menarche was above 14 and patients whose number of pregnancies were more than two. In addition, rs4759314 genotypes have been linked with breast cancer risk only in a subgroup of patients whose age at menopause was below 50 (14). As a result of the limited number of patients in the present study, it was not possible to analyze associations in certain patient subgroups. However, although we could not find any significant relationship between these polymorphisms and breast cancer risk in our patient population, patient follow up may reveal an association with these polymorphisms and patients' survival or resistance to chemotherapeutic agents as revealed for cervical cancer patients (11). Furthermore, evaluation of the effects of these polymorphisms on expression of HOTAIR in tumor samples would help in better recognition of their role in cancer progression which should be undertaken in future studies.

Table 2. Allele and Genotype Frequencies of the HOTAIR Gene Polymorphisms in the Case and Control Groups

Allele/Genotype	Patients	Controls	P Value	OR (95%CI)
rs4759314				
Alleles	N = 244	N = 400		
A	216 (0.89)	345 (0.86)	0.403	1.23 (0.757 - 1.999)
G	28 (0.11)	55 (0.14)		
Genotypes	N = 122	N = 200		
AA	96 (0.79)	148 (0.74)	0.341	1.297 (0.759 - 2.218)
AG	24 (0.2)	49 (0.24)	0.316	0.755 (0.435 - 1.309)
GG	2 (0.01)	3 (0.01)	0.922	1.094 (0.18 - 6.644)
rs12826786				
Alleles	N = 244	N = 400		
C	133 (0.55)	212 (0.53)	0.71	1.063 (0.772 - 1.462)
T	111 (0.45)	188 (0.47)		
Genotypes	N = 122	N = 200		
CC	37 (0.3)	61 (0.3)	0.974	0.992 (0.608 - 1.618)
CT	59 (0.48)	90 (0.45)	0.557	1.145 (0.729 - 1.797)
TT	26 (0.21)	49 (0.24)	0.511	0.835 (0.486 - 1.432)
rs1899663				
Alleles	N = 244	N = 400		
G	136 (0.56)	237 (0.59)	0.381	0.866 (0.628 - 1.195)
T	108 (0.44)	163 (0.41)		
Genotypes	N = 122	N = 200		
GG	36 (0.3)	75 (0.38)	0.143	0.698 (0.43 - 1.131)
GT	64 (0.52)	87 (0.44)	0.118	1.433 (0.912 - 2.252)
TT	22 (0.18)	38 (0.19)	0.829	0.938 (0.524 - 1.677)

Table 3. Haplotype Frequencies of the HOTAIR Polymorphisms in the Case and Control Groups^a

Haplotypes	Patients	Controls	P Value	OR (95%CI)
ACG	96 (0.39)	140 (0.35)	0.99	0.99 (0.55 - 1.5)
ATT	70 (0.28)	104 (0.26)	0.98	0.99 (0.65 - 1.53)
ATG	25 (0.10)	56 (0.14)	0.33	1.34 (0.75 - 2.41)
ACT	25 (0.10)	44 (0.11)	0.83	1.07 (0.60 - 1.88)
GCG	14 (0.06)	28 (0.07)	0.47	1.38 (0.58 - 3.28)
GTT	12 (0.05)	16 (0.04)	0.81	0.89 (0.32 - 2.44)
GTG	2 (0.01)	12 (0.03)	0.29	2.11 (0.53 - 8.43)

^aLoci chosen for hap-analysis: Site1 (rs4759314), Site2 (rs12826786), Site3 (rs1899663)

Similar to other functional polymorphisms within other genes, the effect of these polymorphisms on expression of HOTAIR would be modified by other factors within this gene or even other genes and should be interpreted

in a population specific basis. Other studies are needed to be conducted in larger patient samples with regards to certain clinical and pathological subgroups. In addition, evaluation of association of other functional polymorphisms

within this gene with breast cancer risk may result in determination of practical marker for cancer prediction.

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Footnotes

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References

1. Soudyab M, Iranpour M, Ghafouri-Fard S. The Role of Long Non-Coding RNAs in Breast Cancer. *Arch Iran Med.* 2016;**19**(7):508-17. [PubMed: 27362246].
2. Haghghat S, Akbari ME, Yavari P, Javanbakht M, Ghaffari S. Cost-Effectiveness of Three Rounds of Mammography Breast Cancer Screening in Iranian Women. *Iran J Cancer Prev.* 2016;**9**(1):5443. doi: 10.17795/ijcp-5443. [PubMed: 27366315].
3. Iranpour M, Soudyab M, Geranpayeh L, Mirfakhraie R, Azargashb E, Movafagh A, et al. Expression analysis of four long noncoding RNAs in breast cancer. *Tumour Biol.* 2016;**37**(3):2933-40. doi: 10.1007/s13277-015-4135-2. [PubMed: 26409453].
4. Bayram S, Sumbul AT, Batmaci CY, Genc A. Effect of HOTAIR rs920778 polymorphism on breast cancer susceptibility and clinicopathologic features in a Turkish population. *Tumour Biol.* 2015;**36**(5):3863-70. doi: 10.1007/s13277-014-3028-0. [PubMed: 25586347].
5. Tabarestani S, Ghafouri-Fard S. Cancer stem cells and response to therapy. *Asian Pac J Cancer Prev.* 2012;**13**(12):5951-8. [PubMed: 23464384].
6. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell.* 2007;**129**(7):1311-23. doi: 10.1016/j.cell.2007.05.022. [PubMed: 17604720].
7. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature.* 2010;**464**(7291):1071-6. doi: 10.1038/nature08975. [PubMed: 20393566].
8. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, et al. Long noncoding RNA as modular scaffold of histone modification complexes. *Science.* 2010;**329**(5992):689-93. doi: 10.1126/science.1192002. [PubMed: 20616235].
9. Axelsdottir E. The rna hotair promotes chromatin alteration in cancer. *Undergraduate Res J.* 2014.
10. Nikpayam E, Tasharrofi B, Sarrafzadeh S, Ghafouri-Fard S. The Role of Long Non-Coding RNAs in Ovarian Cancer. *Iran Biomed J.* 2017;**21**(1):3-15. doi:10.6091/21.1.24. [PubMed: 27664137].
11. Qiu H, Liu Q, Li J, Wang X, Wang Y, Yuan Z, et al. Analysis of the association of HOTAIR single nucleotide polymorphism (rs920778) and risk of cervical cancer. *APMIS.* 2016;**124**(7):567-73. doi: 10.1111/apm.12550. [PubMed: 27229487].
12. Zhang X, Zhou L, Fu G, Sun F, Shi J, Wei J, et al. The identification of an ESCC susceptibility SNP rs920778 that regulates the expression of lncRNA HOTAIR via a novel intronic enhancer. *Carcinogenesis.* 2014;**35**(9):2062-7. doi:10.1093/carcin/bgu103. [PubMed: 24788237].
13. Bayram S, Sumbul AT, Dadas E. A functional HOTAIR rs12826786 C>T polymorphism is associated with breast cancer susceptibility and poor clinicopathological characteristics in a Turkish population: a hospital-based case-control study. *Tumour Biol.* 2016;**37**(4):577-84. doi: 10.1007/s13277-015-4430-y. [PubMed: 26577852].
14. Yan R, Cao J, Song C, Chen Y, Wu Z, Wang K, et al. Polymorphisms in lncRNA HOTAIR and susceptibility to breast cancer in a Chinese population. *Cancer Epidemiol.* 2015;**39**(6):978-85. doi: 10.1016/j.canep.2015.10.025. [PubMed: 26547792].
15. Zhang H, Cai K, Wang J, Wang X, Cheng K, Shi F, et al. MiR-7, inhibited indirectly by lincRNA HOTAIR, directly inhibits SETDB1 and reverses the EMT of breast cancer stem cells by downregulating the STAT3 pathway. *Stem Cells.* 2014;**32**(11):2858-68. doi: 10.1002/stem.1795. [PubMed: 25070049].