



Original Article

Evaluation of *HOTAIR*, *HOXD8*, *HOXD9*, *HOXD11* gene expression levels in Turkish patients with acute and chronic myeloid leukemia: A single center experience

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Abstract



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Homeobox (*HOX*) transcript antisense RNA (*HOTAIR*) and *HOX* genes are reported to be more expressed in various cancers in humans in recent studies. The role of *HOTAIR* and *HOXD* genes in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) is not well known. In this study, expression levels of *HOXD8*, *HOXD9* and *HOXD11* from *HOXD* gene family and *HOTAIR* were determined from peripheral blood samples of 30 AML and 30 CML patients and 20 healthy volunteers by quantitative Real Time PCR. We determined that the expression levels of *HOXD9* and *HOXD11* in the AML patients were significantly lower than the control group ($p < 0.001$ and $p = 0.002$, respectively). There was no significant difference in the expression levels of *HOTAIR* and *HOXD8* when compared to the control group. In the CML patients there was a significant increase in the expression level of *HOTAIR* when compared to the control group ($p = 0.002$). The expression levels of *HOXD9* and *HOXD11* were found to be significantly lower than the control group ($p < 0.001$). Our study showed that *HOTAIR* may not be a biomarker in the diagnosis and is not significantly correlated with the clinicopathological prognostic characteristics of AML. Additionally; it can be said that *HOTAIR* is oncogenic by suppressing the expression of *HOXD9* and *HOXD11* but not *HOXD8* in CML patients. The expression profiles of *HOTAIR* may be a potential biomarker in the diagnosis of CML patients in predicting and monitoring drug resistance.

Keywords: *HOTAIR*, *HOXD8*, *HOXD9*, *HOXD11*, AML, CML

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic stem cells that lose normal differentiation ability. The disease is characterized by various cytogenetic and molecular abnormalities with different prognoses and gene expression profiles. It is the most common type of leukemia in adults and has the lowest survival rate among all leukemias. The prevalence of AML is 3.8 per 100000 people and increases to 17.9 per 100000 adults aged 65 years and over. The most appropriate treatment should be chosen according to the patient's genetic profile. For this purpose, long non-coding (lnc) RNAs have recently been used as biomarkers in the diagnosis of cancer [1].

Chronic myeloid leukemia (CML) is a myeloproliferative

disorder of hematopoietic stem cells carrying the Philadelphia chromosome and oncogenic *BCR-ABL1* fusion gene. The peptide p210, the product of the *BCR-ABL*, has structurally activated tyrosine kinase activity that is involved in the pathogenesis of CML [2]. CML is a malignancy that occurs in 1 or 2 cases in approximately 100000 people in adults every year and it is mostly seen between 25-60 years of age [3]. Studies have been conducted to investigate the profile of micro RNA and lnc RNA sequences that have been validated at different stages of CML to better understand the disease and to improve therapeutic intervention in CML patients [4].

lnc RNAs with a nucleotide number of more than two hundred are also referred to as macro RNA and long intergenic non-coding RNA and are not involved in protein

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generation [4]. While many of the lnc RNAs have been reported to vary in expression levels in different cancers, there is insufficient information about their role in cancer development. Therefore, the explanation of the mechanisms of these molecules is one of the current research topics. Although the function of lnc RNAs is not fully known, increasing numbers of lnc RNA and accumulated evidence for its involvement in many biological processes have shown that they have important functions in normal and malignant cells [5]. For this purpose, lnc RNAs have recently been used as biomarkers in the diagnosis of cancer [6]. It has been stated that the expression profiles of lnc RNAs are associated with recurrent mutations, clinical features and outcomes in AML, some of these lnc RNAs may have a functional role in leukemogenesis and may also be used as biomarkers in AML [7]. Studies have been conducted to investigate the profile of micro RNA and lnc RNAs sequences that have been validated at different stages of CML to better understand the disease and to improve therapeutic intervention in CML patients [8].

Homeobox (*HOX*) transcript antisense RNA (*HOTAIR*) was first introduced in 2007 by Rinn et al. as a lnc RNA that suppresses expression of *HOXD* genes on the 2nd chromosome. [6]. The relationship between lncRNAs and polycomb repressive complex 2 (PRC2), including *HOTAIR*, is considered a common mechanism in the gene silencing event in epigenetic regulation. *HOTAIR* binding is required to direct PRC2 to specific regions of the genome. Thus, PRC2 correlates with gene expression and silences epigenetically [9]. It is clear that *HOTAIR* reprograms chromatin to promote metastasis and investigates the molecular mechanisms of tumor formation, metastasis and drug resistance [10].

Because *HOX* genes play a critical regulatory role in many cellular processes, changes in the expression of these genes play an important role in the development of cancer by affecting many mechanisms such as proliferation, growth and differentiation in mutations [11]. Studies have reported changes in the expression of *HOX* genes in tumors such as lung carcinoma, neuroblastoma, glioma, ovarian cancer and leukemia [12].

HOXD gene contains between 9-11 genes arranged in a homologous sequence organization located on chromosome 2 (2q31) [13].

HOXD8 is a gene belonging to the *HOX* gene family consisting of 2 exons, located on the 2nd chromosome (2q31.1). Deletions occurring in the entire *HOXD* gene group or at the 5' end of this gene have been associated with severe limb and genital abnormalities. Negative and positive regulation of transcription from the RNA polymerase II promoter in the embryo plays a role in skeletal development processes. In addition to its regulatory effects during embryogenesis, it has been reported that this particular gene may play a role in adult urogenital system func-

tion [14].

HOXD9 is one of the *HOXD* genes, which is part of the developmental regulatory system located on the chromosome regions 2q31-2q37 [15]. *HOXD9* is important for embryonic segmentation and limb development throughout development. This gene participates in the development and modeling of the forelimb and axial skeleton [16].

HOXD11 is one of the belonging to the *HOX* gene family, which regulates the development and control of many cellular processes such as cell development and shaping, proliferation, migration, and apoptosis. It is a homeodomain consisting of 2 exons that encode a protein of 338 amino acids located on the 2nd chromosome (2q31.1) [17]. It has been reported that the *HOXD11* plays a role in anterior dorsal morphology in mice [18].

There is no information in the literature about how *HOXD8*, *HOXD9* and *HOXD11* gene expression levels change in AML and CML. Therefore, we aimed to investigate the changes in the expression levels of these genes simultaneously with *HOTAIR* in relevant patient groups.

2. Materials and methods

2.1. Clinical sample collection

A total of 30 AML and 30 CML patients and 20 healthy volunteers were enrolled in this study. Clinical and laboratory data and consent forms of the patients were also obtained from Erciyes University Medical Faculty Gevher Nesibe Hospital Medical Genetics Laboratory, The study was carried out in accordance with the principles of the Helsinki Convention on Human Rights. The Ethics Committee of Erciyes University Faculty of Medicine gave ethical approval for this work. (Approval number: 2015/222, Decision date: 08/05/2015).

2.2. RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was isolated from four milliliter EDTA blood samples using the Trizol method [19]. cDNA synthesis was made with the transcriptor High Fidelity cDNA Synthesis Kit and PreAmp cDNA synthesis was performed using the real-time ready cDNA Pre-Amp Master kit according to the manufacturer's instruction (Roche Diagnostics). *Beta-actin* as housekeeping gene was used for normalization in gene expression analysis. Sequences of specific primers belonging to *HOTAIR*, *HOXD8*, *HOXD9*, *HOXD11* and *Beta-actin* genes are shown in Table 1. Gene expression measurements using the LightCycler 480 Probes Master kit were performed by LightCycler 480 II (Roche Diagnostics Ltd. Rotkreuz, Switzerland) polymerase chain reaction instrument. Analyzes were evaluated with LightCycler 480 Software (release 1.5.0 SP4). Each of the patient and control samples was repeated three times. Two negative controls and calibrators were used in each study.

Table 1. Sequences of specific primers belonging to *HOTAIR*, *HOXD8*, *HOXD9*, *HOXD11* and *Beta-actin* genes.

Primer Name	Forward Sequence	Reverse Sequence
Beta-actin	5'-CACCATTGGCAATGAGCGGTTC-3'	5'-AGGTCTTTGCGGATGTCCACGT-3'
HOTAIR	5'-GGGTGTTGGTCTGTGGAAC-3'	5'-CAGTGG-GAACTCTGACTCG-3'
HOXD8	5'-GTCCAGTGGTAATATTGGCGAGG-3'	5'-GCGACTGTAGTTTGTCTTCTC-3'
HOXD9	5'-AGGAGGAGAAGCAGCATTCGCA-3'	5'-CTTTCTCCAGCTCAAGCGTCTG-3'
HOXD11	5'-CAGTCCCTGCACCAAGGCGAC-3'	5'-GGTATAGGGACAGCGCTTTTTCC-3'

The crossing points (Cp) values in the target genes of each sample were normalized to a ratio of the *Beta-actin* housekeeping gene Cp of the same samples. In this manner, relative quantitation values were obtained [20].

2.3. Statistical analysis

In our study, the statistical difference between the gene expression levels measured in patient and control samples were compared by using T test method and p values were calculated. The data were analyzed with SPSS 22.0 statistical program (Chicago, ILL, USA). $p < 0.05$ was considered significant. Data are expressed as Mean \pm Standard Error Mean (SEM).

3. Results

3.1. Clinical and demographic characteristics of patients

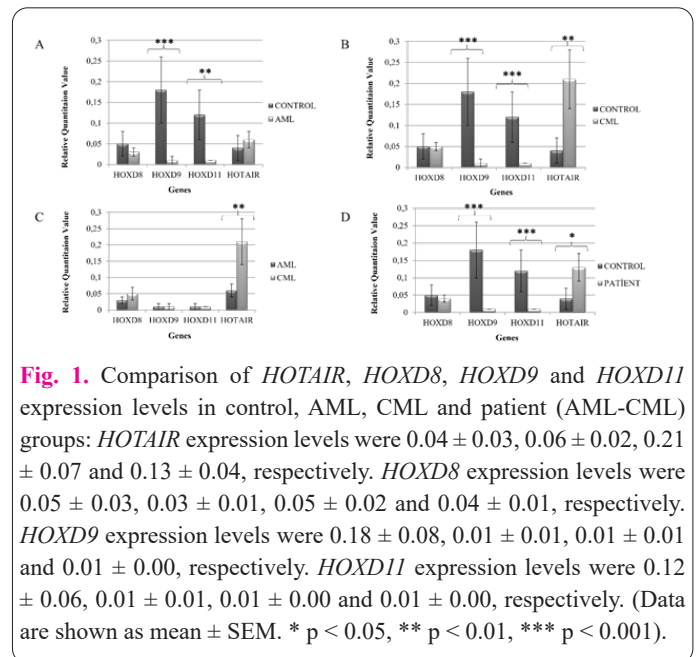
Newly diagnosed AML and CML patients, aged 18 to 75 years, who came to Erciyes University Medical Faculty Gevher Nesibe Hospital Medical Genetics Laboratory, consisted of 60 patients (30 AML, 30 CML), 35 of whom were male (20 AML, 15 CML) and 25 were female (10 AML, 15 CML), with a mean age of 42.07 ± 18.18 (AML) and 48.03 ± 19.90 (CML). 20 healthy individuals as a control group consisted of 13 of whom were male and 7 were female, with a mean age of 34.95 ± 9.59 . Healthy volunteers who did not use any drug were chosen as controls. The age and sex distribution of the AML and CML patient groups were similar to the control group.

Classification of AML patients according to their clinicopathological characteristics are shown in Table 2 and clinical information of the CML patients are shown in Table 3.

3.2. Comparison of target gene expression levels in AML, CML and control groups

The expression levels of *HOTAIR*, *HOXD8*, *HOXD9* and *HOXD11* were compared among AML patients, CML patients and healthy volunteers. The results are demonstrated in Figure 1.

When AML patients were compared with the control group, there was no statistically significant difference in the expression levels of *HOTAIR* and *HOXD8* ($p=0.661$ and $p=0.242$, respectively). The expression levels of *HOXD9* and *HOXD11* were significantly lower than the control group ($p < 0.001$ and $p=0.002$, respectively) (Figure



1A).

In CML patients, the expression level of *HOTAIR* was found to be significantly higher than the control group ($p=0.002$). The expression levels of *HOXD9* and *HOXD11* were significantly lower than the control group ($p < 0.001$). When the expression level of *HOXD8* was compared with the control group, there was no statistically significant difference ($p=0.463$) (Figure 1B).

HOTAIR expression level of CML patients was statistically higher than AML patients ($p < 0.01$). There was no statistically significant difference in the expression levels of *HOXD8*, *HOXD9* and *HOXD11* between both groups ($p=0.396$, $p=0.725$ and $p=0.940$, respectively). (Figure 1C).

HOTAIR expression level of the patient group (AML-CML) was statistically higher than the control group ($p=0.032$). The expression levels of *HOXD9* and *HOXD11* were significantly lower in the patient group than in the control group ($p < 0.001$). There was no statistically significant difference between the groups in the expression level of *HOXD8* ($p=0.216$) (Figure 1D).

4. Discussion

While lnc RNAs contribute to epigenetic gene regula-

Table 2. Clinicopathological characteristics of AML patients.

Clinicopathological features	N (%)	Prognostic Criteria
Gender		
Female	10 (33.3%)	
Male	20 (66.7%)	
Cytogenetic anomalies		
t(8;21) (AML-M2)	3 (10%)	
t(15;17) (AML-M3)	12 (40%)	Low risk
inv(16) (AML-M4)	8 (26.7%)	
Molecular anomalies		
<i>NPM1</i> Mutation	8 (26,7%)	Medium Risk Group
<i>FLT3-ITD</i> Heterozygous Mutation	3 (10%)	High risk Group

NPM1: Nucleophosmin 1, **FLT3-ITD:** Fms-like tyrosine kinase 3 internal tandem duplication

Table 3. The clinical information of CML patients.

Patient	Gender	Medicine	Major Molecular Response	EUTOS	Prognostic Score	
					SOKAL	HASFORD
P1	Male	Imatinib	No	Low	Intermediate	Intermediate
P2	Female	Imatinib	No	Low	Intermediate	Low
P3	Male	Imatinib	No	Low	Low	Intermediate
		Nilotinib	Yes			
P4	Male	Imatinib	Yes	Low	Intermediate	Low
P5	Male	Imatinib	Yes	Low	High	Intermediate
P6	Female	Imatinib	No	Low	High	Intermediate
P7	Female	Imatinib	Yes	Low	Intermediate	Low
P8	Female	Imatinib	No	Low	Low	Intermediate
		Nilotinib	Yes			
P9	Female	Imatinib	No	Low	High	Intermediate
P10	Female	Imatinib	Yes	Low	Low	Low
P11	Male	Imatinib	No	Low	High	High
P12	Female	Imatinib	Yes	Low	Intermediate	Low
P13	Male	Imatinib	Yes	Low	Intermediate	Low
P14	Female	Imatinib	Yes	Low	Intermediate	Intermediate
P15	Male	Imatinib	Yes	Low	Low	Low
P16	Female	Imatinib	No	Low	High	High
P17	Male	Imatinib	Yes	Low	Intermediate	Intermediate
P18	Female	Imatinib	Yes	Low	Low	Low
P19	Female	Imatinib	No	Low	High	Intermediate
P20	Male	Imatinib	No	Low	High	Intermediate
P21	Female	Imatinib	Yes	Low	High	Low
P22	Male	Imatinib	No	Low	Intermediate	Intermediate
	Female	Imatinib	No			
P23		Imatinib	No	Low	Intermediate	Intermediate
		Nilotinib	Yes			
P24	Male	Imatinib	Yes	Low	Intermediate	Low
P25	Male	Imatinib	No	Low	High	Intermediate
		Nilotinib	No			
P26	Male	Imatinib	No	Low	Intermediate	Intermediate
P27	Female	Imatinib	Yes	Low	Intermediate	Intermediate
P28	Male	Imatinib	No	Low	Low	Intermediate
		Nilotinib	Yes			
P29	Male	Imatinib	Yes	Low	Intermediate	Intermediate
P30	Female	Imatinib	No	Low	Low	Intermediate
		Nilotinib	Yes			

EUTOS: European Treatment and Outcome Study for CML

tion, metastases and prognosis of solid tumors, their relationship with hematopoietic cancers has also started to be demonstrated in recent years [21]. *HOTAIR*, characterized by increased expression of various human solid tumors, is a lnc RNA that is considered a potential cancer biomarker. Recent studies in many cancer types have shown that *HOTAIR* has a high expression level. *HOTAIR* has been reported to be overexpressed in various cancers such as colon, colorectal, gastric adenocarcinoma tissues, nasopharyngeal carcinoma, primary hepatocellular carcinoma, and renal carcinoma cells in humans [22-24]. *HOTAIR* suppresses the expression of *HOXD8*, *HOXD9*, *HOXD11* located on the *HOXD* locus of 2nd chromosome [6, 9].

There is insufficient information about the role of *HOTAIR* and *HOXD* in hematological malignancies. In addition,

it is the first time study investigating the mRNA expression levels of *HOXD* genes such as *HOXD8*, *HOXD9* and *HOXD11* in patients with acute and chronic myeloid leukemia.

In studies conducted to investigate *HOTAIR* expression profile in AML patients and to evaluate their clinical significance; *HOTAIR* has been suggested that bad prognosis can be represented as a biomarker and may be a potential therapeutic target for the treatment of AML. The results showed that elevated *HOTAIR* expression level was significantly correlated with the high-risk group of the disease [25-27].

Sayad et al. [28] reported that *HOTAIR* is not a biomarker for Iranian AML patients. They have suggested that *HOTAIR* expression level cannot be considered a defini-

tive diagnostic and therapeutic biomarker for AML and should be confirmed by future studies and the correlation between *HOTAIR* and AML should still be investigated.

In our study, peripheral blood samples of 30 patients with AML and 20 healthy controls were evaluated. According to the findings, the expression levels of *HOTAIR* in AML patients were not statistically different when compared to the control group. Our investigations with AML patients are similar to the findings of Sayad et al. [28] and supports that *HOTAIR* expression level cannot be considered as a definitive diagnostic or therapeutic biomarker for AML.

A hybrid *BCR-ABL* oncogene is formed on chromosome 22, and its product, the p210 peptide, has constitutively activated tyrosine kinase activity that plays a role in the pathogenesis of CML [2]. The widely used and highly effective treatment for CML is administration of imatinib mesylate, one of the tyrosine kinase inhibitors that inhibit the kinase activity of the *BCR-ABL* oncoprotein. Tyrosine kinase inhibitors targeting the *BCR-ABL* are the first-line therapy for most CML patients and have greatly improved the CML prognosis. However, imatinib resistance is emerging as a major challenge in the treatment of CML. Several molecular pathways have been associated with resistance to tyrosine kinase inhibitor therapy, but the precise mechanism for the development of drug resistance remains unclear. Therefore, in recent years, emphasis has been placed on the expression profiles of lnc RNAs, which can predict clinical outcomes in diagnosis, help prognostic classification of patients, and predict and manage drug resistance [29].

In our study, it was seen that the expression levels of *HOTAIR* increased compared to the control group in the CML patients ($p=0.002$). In addition, clinical route and drug use of CML patients were followed. No significant difference was observed when the expression levels of the target genes measured at the time of diagnosis of CML patients were compared between patients who responded to treatment and those who did not and were resistant to imatinib.

Aghamohammadhossein et al. [30] have shown that *HOTAIR* expression levels in patients with positive CML for *BCR-ABL* increased compared to healthy individuals. They stated that changes in the expression levels of the *HOTAIR* may play a role in the biology of CML. In addition to these findings, in the present study, it was shown that *HOTAIR* expression levels increased while *HOXD9* and *HOXD11* expression levels decreased in CML patients compared to the healthy control group.

In previous studies, it was stated that *HOXD8*, *HOXD9*, and *HOXD11* expression level profiles change in various cancer types [17, 31-35]. In our study, for the first time, the expression levels of these genes were evaluated together with *HOTAIR* and demonstrated in AML and CML patients.

In the present study, it has been shown that *HOTAIR* epigenetically suppresses *HOXD9* and *HOXD11*, but *HOXD8* does not and may have an oncogenic effect on the development of CML.

5. Conclusion

As a result, our study showed that *HOTAIR* may not be a biomarker in the diagnosis and is not significantly correlated with the clinicopathological prognostic character-

istics of AML. Additionally; it can be said that *HOTAIR* is oncogenic by suppressing the expression of *HOXD9* and *HOXD11* but not *HOXD8* in CML patients. The expression profiles of *HOTAIR* may be a potential biomarker in the diagnosis of CML patients in predicting and monitoring drug resistance.

Conflict of Interests

The authors have no competing interests to declare.

Consent for publications

All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Erciyes University Faculty of Medicine. (Approval number: 2015/222, Decision date: 08/05/2015).

Informed Consent

Clinical and laboratory data and consent forms of the patients were obtained from Erciyes University Medical Faculty Gevher Nesibe Hospital Medical Genetics Laboratory.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Esma Saraymen: Data collection and processing, analysis and interpretation, literature search, critical reviews, writing; Yakut Erdem: data collection and processing, analysis and interpretation, literature search, critical reviews; Hilal Akalım: data collection and processing, analysis and interpretation; Nazife Taşçıoğlu: data collection and processing, analysis and interpretation; Berkay Saraymen: data collection and processing, literature search; Serhat Çelik: materials; data collection and processing; Yeşim Özdemir: data collection and processing; Leylagül Kaynar: supervision, materials, analysis and interpretation; Mustafa Çetin: supervision, materials, analysis and interpretation; Yusuf Özkul: concept, design, supervision, resource, materials, data collection and processing, analysis and interpretation, literature search, critical reviews.

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The findings of this study were presented as oral presentation at the 7th International Congress on Leukemia Lymphoma Myeloma (ICLLM), İstanbul, Türkiye with title of "Long Noncoding RNA *HOTAIR* Is A New Potential Biomarker For Chronic Myeloid Leukemia (CML) Not For Acute Myeloid Leukemia (AML) In Turkish Patients" and as poster presentation at the 6th International Participation Experimental Hematology Congress, Gaziantep, Türkiye with title of "Can long non-coding RNA *HOTAIR* be a novel biomarker in the diagnosis of CML".

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