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Original Article

Comparison of the Overexpression of HOTAIR lncRNAs and the Downregulation of HOXD10 Between Familial and Sporadic Coronary Artery Diseases

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ABSTRACT

Background: Coronary artery disease (CAD) is a multifactorial disorder and one of the major causes of death all over the world. Both genetics and the environment are responsible for CAD occurrence. Recent studies have shown the considerable role of epigenetics in various diseases. *HOTAIR* is a circulating long noncoding RNA (lncRNA) in the blood with an epigenetic regulation role in transcriptional pathways in different diseases. Recent investigations have shown that *HOTAIR* could be a potential biomarker for diagnosis and therapeutic targets in CAD.

Methods: In the present study, we sought to evaluate the expression of *HOTAIR* lncRNAs in the blood samples of 30 patients with a family history of CAD and 30 sporadic CAD samples with coronary angiography-confirmed CAD. The expression level was examined using the semiquantitative reverse transcriptase-polymerase chain reaction technique. For the epigenetic validation of *HOTAIR* function, the expression level of the *HOXD10* gene as the main target of *HOTAIR* lncRNAs in expression modulation was evaluated.

Results: The expression level of *HOTAIR* was higher in patients with familial CAD than in sporadic CAD patients, whereas the expression level of *HOXD10* in the familial CAD group was lower than that in the sporadic group. Notably, the average age of the familial CAD group was lower than that of the sporadic group.

Conclusions: The high expression level of *HOTAIR* in patients with a family history of CAD in comparison with sporadic CAD patients shows the role of genetics and epigenetics in the expression level of *HOTAIR*. High expression levels of *HOTAIR* increase susceptibility to CAD and have a positive correlation with age at CAD onset. ***(Iranian Heart Journal 2021; 22(4): 127-134)***

KEYWORDS: Coronary artery disease, Epigenetics, *HOTAIR* long noncoding RNA, *HOXD10*

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Epigenetic regulation is one of the most frequent processes that happen in cells. Even though multicellular organisms are homogeneous in genetic codes, they are heterogeneous in structure and function, which is due to various gene expression patterns. Different gene expression patterns are retained through the cell cycle during development. [1] Epigenetic regulation is a sustained alteration that is heritable and could pass from parents to offspring. DNA alteration sequences are not in epigenetics. Research over the past few years has focused on the molecular mechanisms that mediate epigenetic phenomena such as chromatin remodeling and different forms of histone modifications. [2] Of the genes that have altered expression in human developmental system disorders are *HOX* family genes. *Homeobox* genes are master genes that act at the top of genetic hierarchies regulating aspects of proliferation, organogenesis, adhesion, and migration. Humans have 39 *HOX* genes in 4 clusters on 4 different chromosomes. *HOX* genes comprise a family of regulatory molecules that encode highly conserved transcription factors. The genetic codes of the *HOX* family are expressed in anterior-posterior axes to regulate cell differentiation and organogenesis. [3] *HOX* genes are responsible for the development of individual zones of the heart. [4, 5] In humans, only around 2% of the whole genomic data are coding parts and have functional proteins. Approximately, 98% of the entire genomic data are noncoding parts that regulate the expression of coding parts. [6] Transcripts produced by gene expression are categorized into 2 main features: coding and noncoding. Coding RNAs encode proteins with biological functions in cells and signaling pathways. Noncoding RNAs are classified into 2 regulatory and structural categories. Structural noncoding RNAs consist of ribosomal and transfer RNAs (rRNAs and tRNAs, respectively). Regulatory noncoding RNAs are categorized by their length into 3 clusters: short, intermediate, and long (Fig. 1). Short noncoding RNAs have a famous member known as “microRNA (mRNA)”, which is less than 20 nt in length. Long noncoding RNAs (lncRNAs) are over 200nt in length and are abundantly transcribed in the human genome. Nonetheless, their exact physiological and pathological roles need further elucidation. Human lncRNAs play a critical role in the diversity of signaling pathways and physiological tasks, including the epigenetic regulation of gene expression, RNA decay, microRNA regulation, RNA splicing, and protein folding. The activation or inactivation of chromatin by lncRNAs is also a major topic in epigenetic regulation. [7] The disfigurement of lncRNAs can lead to human disorders such as cancer. [8] HOX (homeobox) transcript antisense RNA (*HOTAIR*, primarily HOX antisense intergenic RNA) is a lncRNA that is 2337 nucleotides in length. This gene is located within the *HOXC* (homeobox C) gene cluster in the 12q13 cytogenetic region. It is transcribed by RNA polymerase II from the antisense strand of the *HOXC* (homeobox C) gene cluster, capped, and polyadenylated, in the same manner as all other transcripts. The human *HOTAIR* gene has 6 exons separated by 5 introns. It has 5 transcripts due to alternative splicing. The transcript is found to be unstable with a half-life of less than 4 hours in human Hela cells. [9] *HOTAIR* can transregulate the *HOXD* cluster, especially *HOXD10* as well as nearby genes and transcriptional factor regions. [10] In terms of molecular mechanisms, *HOTAIR*-bound polycomb repressive complex 2 (PRC2), which displays histone methylase activity, and trimethylated H3 histone (H3K27met3) target a region in the chromatin to be silenced. That methylase activity is provided by histone methyltransferase EZH2 (enhancer of zeste 2 polycomb repressive complex II

subunit), which is a member of the polycomb group family. *HOTAIR* can actively recruit PRC2 or may play the role of a platform to bind for PRC2. [11] *HOTAIR* has an interaction with histone demethylase LSD1 (lysine-specific demethylase 1A), which is another chromatin modifier critical for gene silencing. *HOTAIR* directs the LSD1-mediated demethylation of histone H3 at lysine 4 (H3K4demet). Therefore, *HOTAIR* acts as a biochemical scaffold that interacts with PRC2, LSD1, and chromatin, while the *HOXD10* gene downregulates the expression of *HOXD* loci. *HOTAIR* binds a GA-rich polypurine DNA motif (GA-rich polypurine motif, the *HOTAIR* motif) and regulates gene transcription. This effect can be extended for other sites in the genome and other regulatory lncRNAs. [12] Recent studies have shown that *HOTAIR* lncRNAs have an epigenetic role and are a potential novel biomarker in cardiovascular disease. [13, 14]

None of the previous studies has compared sporadic patients without a record of coronary artery disease (CAD) in their family and patients with a family history of CAD (at least 2 affected patients in different generations or in a row). Indubitably, a thorough evaluation of genetic or epigenetic factors requires the consideration of genetic conditions as well.

METHODS

Study Population

The samples were composed of 30 CAD sporadic patients with no family history records and 30 CAD patients with a family history of CAD who had at least 2 CAD-affected patients in their family with different generations presenting as premature CAD. The study population was recruited from Shariati General Hospital and Mehrad General Hospital, Tehran, Iran, from 2018 through 2020. The demographic characteristics of the study participants are provided in Table 1. CAD in all the patients was confirmed with coronary angiography. Stenosis of 50% or more in any coronary vessel was identified as CAD. The severity of coronary artery narrowing was estimated with quantitative coronary angiography to avoid inter and intraobserver variability. The indication for coronary angiography was acute coronary syndrome, myocardial infarction, and positive noninvasive tests in patients with chronic stable angina. Informed consent was obtained from all the subjects.

Table 1. Clinical phenotypes of the samples

Features	Sporadic CAD	Familial CAD
Age Average	61.2	47.4
Sex		
male	19	21
female	11	9

CAD, Coronary artery disease

Blood Sample Collection

Generally, 2 mL of peripheral blood was collected in EDTA blood tubes from all the patients. The samples were prepared immediately for the next procedure.

Total RNA Extraction and cDNA Synthesis

Peripheral blood mononuclear cells were isolated from the peripheral blood of the patients according to the protocol.

Total RNA was isolated with the NucleoSpin Kit and treated with the DNase I RNase Free Kit (Fermentas) to remove genomic DNA contamination. Two µg of total RNA was used for reverse transcription reaction with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and random hexamer primer, according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) tests were set up in duplicate with the Power SYBR Green Master Mix (Applied Biosystems) and analyzed with the 7500 real-time PCR system (Applied Biosystems).

Reverse Transcriptase-Polymerase Chain Reaction

The reaction mixture for PCR (20 µL) contained 1 µL of the cDNA template and 500 nM of each of sense and antisense primers, amplified as follows: initial denaturation at 95 °C for 5 minutes, and amplification by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds, followed by 5 minutes of final extension. The band intensity of the PCR product was verified by electrophoresis on 1.2% agarose gel. Agarose gel with GelRed nucleic acid stain and band density was quantified by ultraviolet exposure.

Primer Design and Amplification Efficiency

For PCR amplification, specific primers were used to identify *HOTAIR* and *HOXD10* as object genes and the *GAPH* gene as a housekeeping gene. The primers were designed by the Primer3 online webpage and the PerlPrimer software and evaluated. The specification of the primers was assessed by the Gene Runner software and the University of California Santa Cruz (UCSC) Genome Browser Database. The primer sequences are presented in Table 2.

Biostatistics Analysis

Quantitative real-time PCR is an important technique in medical and biomedical applications. The PCR package provides a unified interface for quality assessing, analyzing, and testing qPCR data for statistical significance. The present study aimed to describe the different methods and modes used to relatively quantify the gene expressions of qPCR and their implementation in the PCR package. In this study, we used the ΔΔCT, which is the assumption of the amplification efficiency and is critical for the reliability of the model, in particular, the slope and the R^2 of the line between the log input amount and ΔCT or the difference between the CT value of the target *HOTAIR* and *HOXD10* genes and the housekeeping gene (*GAPDH*) as an endogenous control. Typically, the slope should be very small (<0.01). The slope here was appropriate (0.0264562). A value of the amplification efficiency itself is given by 10−1/slope, so the assumption holds true. [15] In contrast with the absolute quantification of the amount of mRNA in a sample, relative quantification uses an internal control (the reference gene) and/or a control group (the reference group) to quantify the mRNA of interest relative to these references. This relative quantification is sufficient to draw conclusions in most biomedical applications involving qPCR. A few methods have been developed to perform this relative quantification. These methods require different assumptions and models. The 2 most common of these methods are explained here.

Comparative CT Methods

The comparative CT methods assume that the cDNA templates of the gene/s of interest, as well as the control/reference gene, have similar amplification efficiency and that this amplification efficiency is near perfect. In other words, at a certain threshold during the linear portion of the PCR reaction, the amount of the gene of interest and the control doubles each cycle. Another assumption is that the expression difference between 2 genes or 2 samples can be captured by subtracting one (the gene or sample of interest) from the other (the reference). This final assumption requires that these references not change with the treatment or the course in question. The formal derivation of the ΔΔCT model is described in a project by Livak et al. [18]

Table 2. Sequences of forward and reverse primers designed for RNA expression evaluation

	Forward Sequences	Reverse Sequences	Amplicon Size bp	TM ˚C
HOTAIR	AGTTCCACAGACCAACACC	GCTTCTAAATCCGTTCCATTC	137	60
HOXD10	GCCTTACACTAAGCACCA	AACCAAATCTTTTGACCTGCCT	130	60
GAPDH	CTCATTTCCTGGTATGACACCGA	CTTCCTCTTCCGCTCTTGCT	123	60

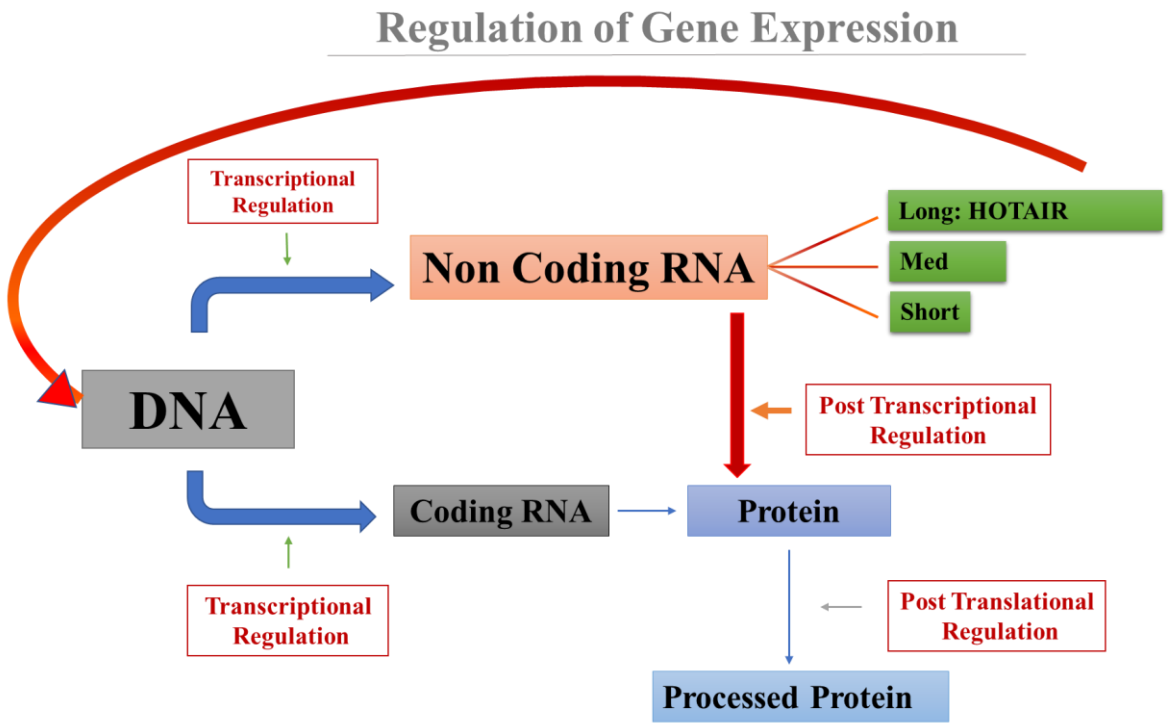

Figure 1. The image depicts the regulation of gene expression. Coding and noncoding RNA categories are shown with different states of regulation.

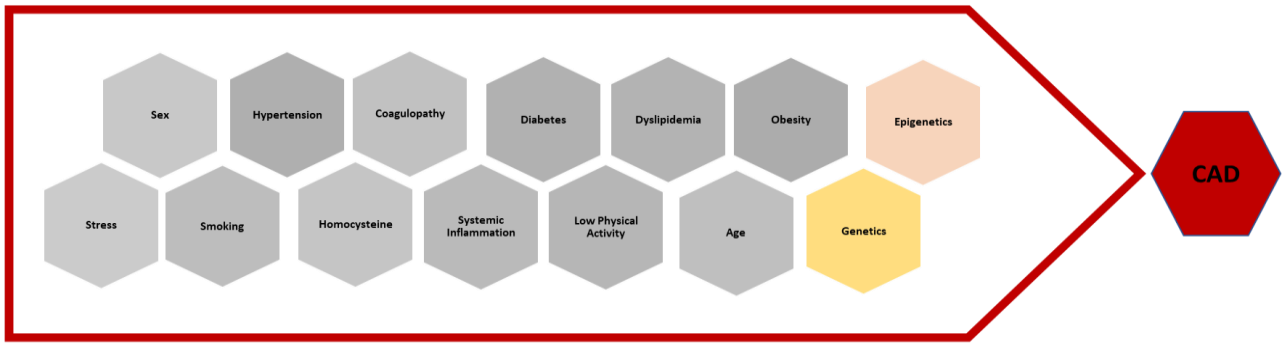

Figure 2. The image illustrates the etiology and risk factors of coronary artery disease (CAD). CAD is a multifactorial disorder that has environmental and inheritance causes.

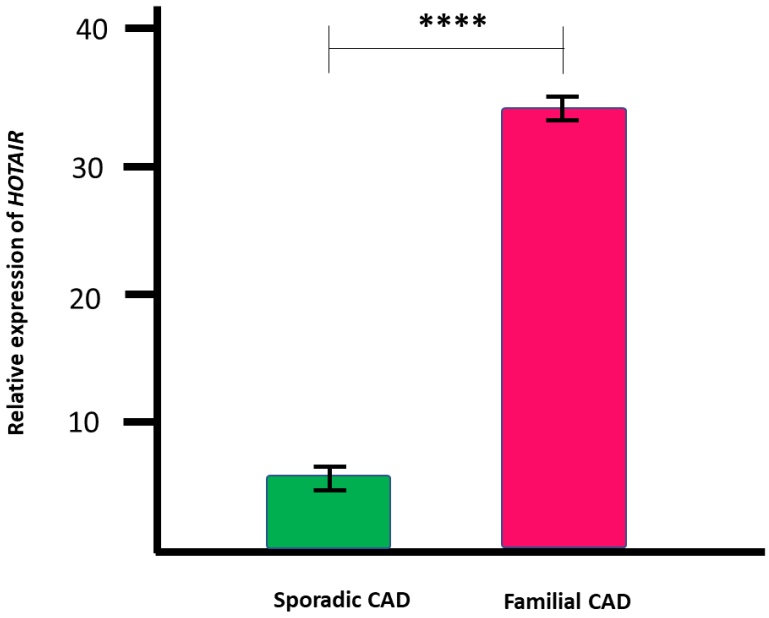

Figure 3a. The image demonstrates the relative expression of *HOTAIR* lnc*RNA* in patients with sporadic CAD and familial CAD samples. The *t* test analysis indicates that the expression of *HOTAIR* in patients with a family history of CAD is significantly higher than normal (****P<0.0001 for *HOTAIR*). Stars denote significant differences between the groups. CAD, Coronary artery disease

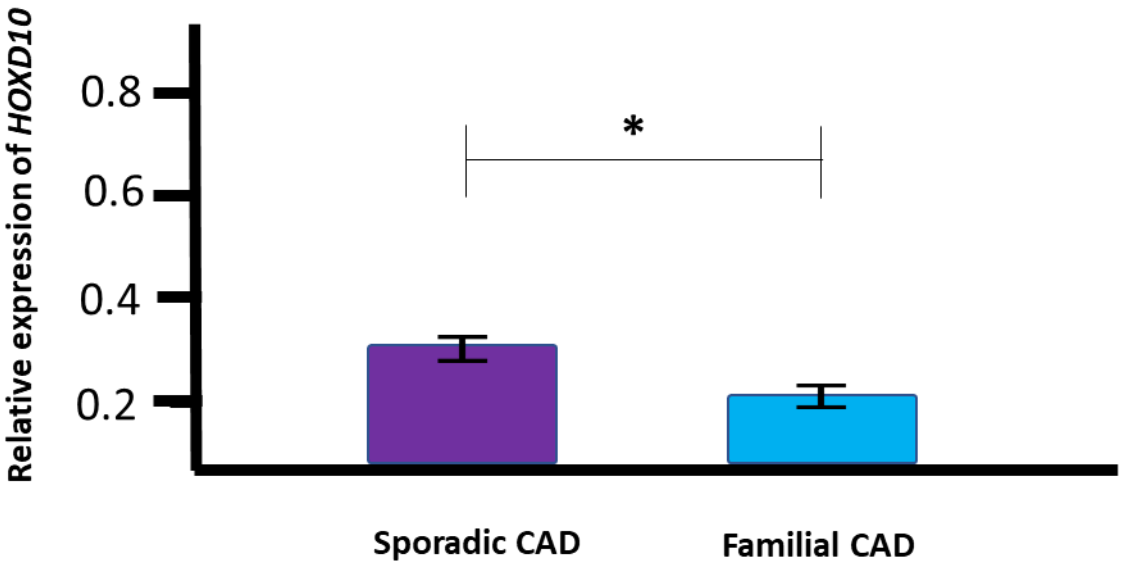

Figure 3b. The image demonstrates the relative expression of *HOXD10* in patients with sporadic CAD and familial CAD samples. The *t* test analysis indicates that the expression of *HOXD10* in patients with a family history of CAD is significantly higher than normal (*P<0.05 for *HOTAIR*). Stars denote significant differences between the groups. CAD, Coronary artery disease

RESULTS

The results of the current investigation demonstrated that the expression level of the *HOTAIR* lncRNA gene was altered in patients with premature CAD (Fig. 2a).

For the epigenetic validation of the *HOTAIR* role, the expression level of *HOXD10* as the

main target of *HOTAIR* lncRNA was determined. The results showed that the expression level of *HOXD10* in familial CAD was significantly lower than that in sporadic CAD (Fig. 2b).

DISCUSSION

CAD, as a multifactorial disorder, is a serious condition caused by a buildup of plaques in the coronary arteries (Fig. 3). Multifactorial diseases are caused by a combination of the effects of multiple genes or by interactions between genes and the environment. Multifactorial diseases have both genetic and environmental components, which contribute in different proportions in each patient or family. Disease appearance in epidemiology has different levels such as pandemic, endemic, epidemic, and sporadic levels. In epidemiology, "sporadic" is a term used to refer to a disease that occurs only infrequently, haphazardly, irregularly, or occasionally from time to time in a few isolated places with no discernible temporal or spatial pattern, as opposed to a recognizable epidemic or endemic pattern. [16] Genetics contributes to 2 subtypes of sporadic and familial diseases. [17] A sporadic genetic disease is not inherited from parents but arises via a genetic alteration. However, a sporadic genetic disease becomes inheritable to the children of the person who has acquired the genetic disease via genetic variation. A familial disease is hereditary and passed on from 1 generation to the next. Furthermore, in familial disease pedigree, there are at least 2 affected patients in a row or 1 generation or different generations. Epigenetic regulation is a major regulatory system that can manage the expression condition of genes. Exposure to various environmental stimuli can also induce epigenetic alterations heritable to the next generation. Recent years have witnessed a rise in the number of studies focused on epigenetic regulatory systems, especially lncRNAs. A similar trend has occurred vis-à-vis CAD. Be that as it may, none of the previous studies has compared sporadic patients without a record of CAD in their family and patients with familial CAD who have at least 2 affected patients in different generations or in a row. A meticulous assessment of a genetic or epigenetic factor should be in tandem with a thorough evaluation of genetic conditions. In the present study, we evaluated both the genetic and epigenetic conditions of CAD development. Our results demonstrated that the expression levels of *HOTAIR* lncRNAs, which have already been recognized as a malignancy biomarker, were elevated in familial CAD cases by comparison with sporadic CAD cases. It means that in premature CAD, at the epigenetic level of the disease, elevated expression levels of *HOTAIR* lncRNAs are correlated with higher malignancy forms of the disease. The expression level of the *HOXD10* gene as one of the main outcomes of *HOTAIR* function was downregulated in our familial CAD group, which is a validation of *HOTAIR* molecular function. We observed that age at CAD onset in the familial CAD group was lower than that in the sporadic CAD group. Genetic assessments of CAD indicate that when there is a familial accumulation of the disease in the family, genetic alteration is likely. Therefore, more studies using genetic and epigenetic evaluations should be undertaken.

CONCLUSIONS

The results of the present study showed that *HOTAIR* expression in patients with a family history of CAD was higher than that in a group of samples with sporadic CAD. Our findings suggest a significant correlation between the altered expression of the *HOXD10* gene and *HOTAIR* lncRNAs as an epigenetic regulator in CAD. *HOTAIR* lncRNAs could play genetic and epigenetic roles in CAD development. The results of

the current investigation can provide a fresh insight into the molecular diagnosis of CAD.

Conflict of Interest

The authors declare no conflicts of interest.

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