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Original Article

Increased Expression Level of Long Noncoding RNA H19 in Plasma of Patients with Myocardial Infarction

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Long noncoding RNAs (lncRNAs) are lengthy noncoding transcripts which are actively involved in crucial cellular pathways. Tissue-specific expression of lncRNAs besides its secretion into the body fluids, has made lncRNAs in attention as biomarkers of the diseases. According to the role of lncRNAs, especially *H19* in cardiac regeneration, it is not surprising if their altered expression levels lead to cardiac diseases. In the present study, the relative expression of H19 was compared in the plasma of atherosclerotic myocardial infarction and control individuals by real time-PCR, and data were normalized using *GAPDH*. The association of plasma level of lipid and homocystine with *H19* expression was also considered. The potential of *H19* to discriminate the case from control was studied using the ROC analysis. We found that the plasma level of *H19* transcript significantly increased in the plasma of patients in comparison with the control group. Additionally, the relative expression level of *H19* was directly associated with the plasma homocystine level. The relative expression of *H19* at threshold of 0.3 showed 70% sensitivity and 94% specificity to discriminate cases from controls. This study revealed that the expression level of *H19* may be considered as a biomarker of myocardial infarction, although further studies are needed to generalize this finding.

Key words: Atherosclerosis, blood-based biomarker, homocysteine, long noncoding RNA, myocardial infarction, plasma

Among the cardiovascular diseases (CVDs), coronary atherosclerosis is the most prevalent cause of death worldwide (1). Atherosclerosis is initiated by lipid deposition, oxidation and modification inside the blood arteries triggering the inflammatory responses which can eventually lead to stenosis, stroke, and heart attack (2). Variety of risk factors have been introduced for CVDs among which the role of sedentary lifestyle, arterial hypertension, smoking, hyperlipidemias, obesity, diabetes mellitus, and familial antecedents are well-established (3). In spite of many advances in the

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treatment of CVDs, the disease still has a definite outbreak. Therefore, development of new approaches for early diagnosis of CVDs is urgent especially in case of families with history of the disease (4). Understanding of the involved molecular mechanisms of the disease could be promising to achieve this goal (5). Long noncoding RNAs (lncRNAs) are lengthy noncoding transcripts which are actively involved in the regulation of variety of biological pathways including cell cycle, growth, and apoptosis (6, 7). These RNAs are in the attention due to their regulatory roles at transcriptional, and post transcriptional levels (8). Additionally, tissue-specific, developmental stage-specific expression of lncRNAs besides their long-term stability in biofluids including plasma and urine made these transcripts as potent diagnostic biomarkers of diseases (9). The latter characteristic has made these molecules easily detectable by quantitative molecular methods (10). A growing body of evidence showed that lncRNAs play an important role in cardiac cell proliferation, growth and differentiation, so their altered expression levels may be connected with CVDs (4). The association of some lncRNAs with cardiac development,atherosclerosis, myocardial infarction, heart failure, hypertension, and aneurysms has been reported recently (8). LncRNA *H19*, is a maternally expressed gene on chromosome 11p15.5 (11). The gene is expressed during the 6^{th} to 8^{th} week of gestation from both parental alleles; however, it is exclusively expressed from maternal allele on the 10^{th} week of pregnancy (12). The expression of *H19* has been detected in liver, tongue, heart, muscle, kidney, and intestine; however, this expression is restricted to skeletal muscle and heart after birth (13). It has been evidenced that *H19* is a negative regulator of body weight and apoptosis (14). The gene is also induced by increased level of homocysteine, an approved risk factor of CVDs (15). In addition, during cardiac muscle injuries, cardiac cells become leaky and release their contents into the bloodstream (16). Regarding to such history, this study was aimed to evaluate the expression level of *H19* in plasma samples of patients suffering from myocardial infarction due to coronary atheroscle-rosis diseases.

Materials and methods

Patient's recruitment

Human subjects were recruited at the Department of Cardiology at Golestan Hospital (Ahvaz, Iran) after meeting the following criteria by the cardiologist: the presence of at least one atherosclerotic vessel which was confirmed through the computed tomography scan (CT) angiography, and early detection of myocardial infarction. The patients with of CVDs or history of gastric cancer, NSCLC, and breast cancer were excluded from this study. The patients with negative results in CT angiography were considered as control group. Totally, 32 cases and 30 control individuals were collected from 2017 to 2018. All human participants gave their written informed consent before joining the project. A whole blood sample (5 ml) was collected from all individuals into the ethylenediaminetetraacetic acid (EDTA)-containing tubes. Table 1 represents the clinical presentation of atherosclerotic and normal individuals. The present study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences Research Affairs, Ahvaz, Iran (Grant number: CMRC-9520 and code of ethics: IR.AJUMS. REC. 1395.818), and was experimentally performed in the Department of Medical Genetics.

Plasma isolation

To isolate the plasma, blood samples (5 mL) were centrifuged at 2000 g for 10 min at room temperature. The tubes were removed from the centrifuge, and plasma was located at the top of the specimen. The isolated plasma samples were stored at -70 °C until use.

RNA extraction

Within 1 h after blood drawing, the RNA was

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Table 1. Clinical presentation of atherosclerotic and normal individuals.

Parameters	Patients (n=32)	Controls (n=30)
Sex ratio (F/M)	(14/18)	(13/17)
Age	56±9.7	45±11
Artery stenosis	In 3 vessels (n=11) In 2 vessels (n=14) In 1 vessel (n=7)	None
TG (mg/dl)	133.5±67.04	-
TC (mg/dl)	181.94±59.9	158±46.7
LDL (mg/dl)	106.5.58±48.6	82.4±30
HDL(mg/dl)	47.11±10.46	45.85±6.9
Hcys (mg/dl)	26.7±12.81	29.81±26

TG: triglyceride; LDL: low-density lipoprotein; HDL: high-density lipoprotein; VLDL: very-low-density lipoprotein; Hcys: homocysteine.

Table 2. List of the primer sets and related amplicon.

Gene	Primer	Sequence	Amplicon size (bp)
H19	*H19*-Forward	5'-TGAGGTGATCATGACTGGTAC-3'	101
	H19-Reverse	5'-TGGCTTCAACTGATTCCGTG-3'	
GAPDH	*GAPDH*-Forward	5'-GTGAACCATGAGAAGTATGACAAC-3'	123
	GAPDH-Reverse	5'-CATGAGTCCTTCCACGATACC-3'	

quickly extracted using RNX-plus solution (CinnaGen, Iran) according to a protocol provided by the manufacturer. The concentration and purity of the RNAs were evaluated by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) reading at 230, 260, and 280 nm wavelengths. It is necessary to mention that to avoid contaminations containing RNAs such as intact cells, apoptotic cells or cell fragments, after thawing, the plasma samples were centrifuged at 3000 g for 5 min at room temperature to pellet debris (17).

Complementary DNA (cDNA) synthesis and real-time polymerase chain reaction (PCR)

Complementary DNA was synthesized by PrimeScript™ RT reagent kit (Takara Bio Inc, Shiga, Japan) based on the manufacturer's protocol. The list of primers for *H19* gene and reference gene *GAPDH* are illustrated in Table 2. Of note, *GAPDH* was selected as internal control since a variety of previously published experiments validated it for normalization of mRNA targets in serum and plasma samples (18). Real-time PCR was performed using the SYBR® Premix Ex Taq™ II (Takara, Japan) as described by the manufacturer. Relative gene expression was calculated as $2^{-\Delta Ct}$.

Evaluating the plasma lipid concentration

Plasma concentration of cholesterol, triglyceride (19), low-density lipoprotein (LDL), high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), and homocysteine were measured in patient and control groups based on diagnostic Ltd Axis-Shield protocols (Axis-Shield, Scotland).

The significance of dyslipidemia on *H19* expression level

As dyslipidemia has been observed in atherosclerotic patients (20), the association of factors including VLDL, LDL, and HDL with relative expression of *H19* lncRNA was studied. Additionally, as homocysteine has been reported as one the risk factors of atherosclerosis (21), the

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association of this parameter with relative *H19* expression level was also considered.

Receiver operating characteristic (ROC) curve analysis

To determine the sensitivity and specificity of the *H19* expression level to distinguish people with the disease from normal ones, GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA) was applied to calculate the cut-off value and presented it as graph of 100%-Specificity% versus 77- 95% confidence interval (CI) was considered for each possible cut-off between normal and abnormal levels. The area under a ROC curve (AUC) quantifies the overall ability of the test to discriminate between those individuals with the disease and those without the disease. A good ROC curve is indicated by an AUC close to one and the AUCs less than 0.5 are not acceptable.

Statistical analysis

Each study was performed in 3 independent experiments. Data were presented as mean±SD into the Graphpad Prism version 7. The statistical difference between case and control groups was evaluated using student T-test. The statistical significance threshold was considered as 0.05.

Results

Long noncoding RNA *H19* was increased in plasma of myocardial infarction patients

Comparing the expression level of *H19* in plasma of myocardial infarction individuals showed the relative expression of *H19* in plasma of patients with atherosclerotic vessels was equal to 0.540227 ±0.05 while the corresponding value in normal individuals was 0.16±0.02. This indicates that *H19* lncRNA is significantly more detectable in plasma of patients than the control individuals, and the fold-change was equal to 3.3 ($P<10^{-4}$) (Figure 1).

The significance of homocysteine concentration with increased expression level of *H19* in plasma

It was observed that some of the patients have normal range of plasma homocysteine, therefore, patients were divided into two groups including those with normal range of homocysteine (6-12 mmol/ml) and individuals with abnormal level of homocysteine (>12 mmol/mL). Comparing the relative expression in two groups showed that the increased levels of homocysteine was significantly associated with higher expression values of *H19* (Figure 2) (P = 0.002).

Regarding lipid profile association with *H19* expression level, no statistical association was observed between LDL, HDL and cholesterol level and *H19* expression level (P>0.05) (data not shown).

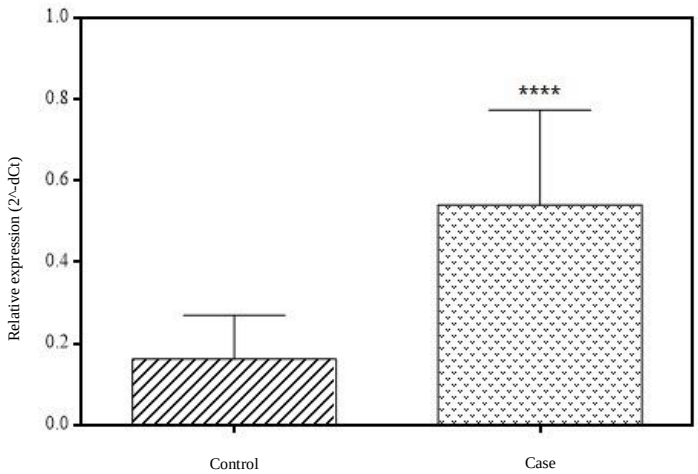

Fig. 1. The relative *H19* lncRNA expression level in the plasma of atherosclerotic patients and non-atherosclerotic control counterparts. Data are presented as mean±SD. The asterisk"*" indicates the statistical significance less than 10^{-4}.

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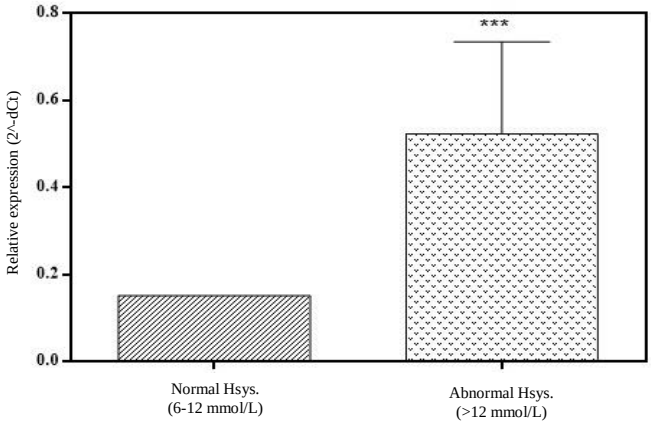

Fig. 2. The association of homocysteine concentration with relative expression of H19 in plasma of atherosclerotic patients. Data are presented as mean±SD. The asterisk"***" indicates the statistical significance less than 10^{-3}.

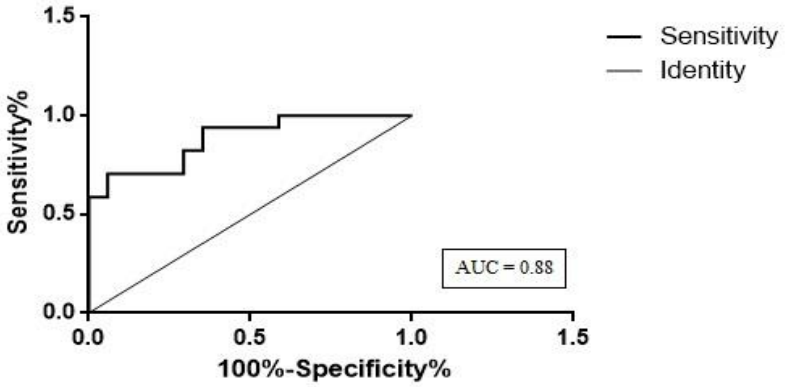

Fig. 3. ROC curve analysis. Overall ability of plasma *H19* expression level to discriminate the atherosclerotic patients from non-atherosclerotic individuals by computing the AUC index in ROC curve.

Increased level of *H19* in plasma can be considered as a biomarker for atherosclerosis

The ROC curve is indicated in Figure 3. The calculated AUC was equal to 0.88 ± 0.05 for *H19* which shows that the increased level of *H19* in plasma of patients can discriminate these individuals from normal counterparts at cutoff point of 0.3 (sensitivity = 70% and specificity = 94%, P = 0.0001).

Discussion

Tissue specific feature of lncRNAs besides their presence in body fluids including saliva, urine, and bloodstream made these lengthy transcripts as novel and promising biomarkers of diseases (22). Additionally, lncRNAs are stable transcripts probably due to their broad biological functions in the cells (23). The lncRNA *H19* was previously reported as noncoding transcript which is preferentially expressed in heart tissue, so it is not surprising if its altered expression was connected with cardiovascular diseases (24). In addition, during cardiac muscle injuries, cardiac cells become leaky and release their contents into the bloodstream (16). This is why cardiac-specific molecules are great candidates as biomarkers of the heart diseases. About half of all myocardial infarctions are caused by atherosclerosis which is partly due to narrowing and hardening of the vessels, and restriction of the blood flow. This

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eventually leads to rupture of cardiac cells, and cardiac muscle cells necrosis (25-27). The RNA biomarkers are recently in the attention of researchers as these molecules can be easily quantified by molecular methods like realtime-PCR. The latter feature is valuable as the protein-based methods for plasma samples are very expensive probably due to the antibodies' cost (28).

The present study is the first pilot report showing the importance of lncRNA *H19* in patients suffering from atherosclerosis. The 3.2-fold increased level of lncRNA *H19* was observed in the plasma of patients with atherosclerotic vessels in comparison with the normal counterparts. In a similar study , Zhang et al. showed that *H19* and long intergenic noncoding RNA predicting cardiac remodeling (*LIPCAR*) increased in plasma of CVD patients (29). Myocardial infarction was diagnosed through electrocardiogram (ECG) in all of the included patients in this study and the blood samples were collected immediately before any drug medications. The obtained data showed that the abnormal level of homocysteine was directly connected with high expression score of *H19* in patients. In other words, the patients with high plasma level of homocysteine showed high expression level of *H19* in comparison with patients with normal range of homocysteine. It has previously been demonstrated that the increased level of homocysteine in plasma can promote atherosclerosis through the induction of oxidative responses or endothelial dysfunction (30). Castro et al. showed that homocysteine concentration can modulate the expression level of *H19* in endothelial cells (31). In a study on cystathionine-beta-synthase (*Cbs*) gene deficient mice by Delvin and his colleagues, they showed that hyperhomocysteinemia led to 2.5 fold increase in *H19* expression through its impact on differentially methylated domain methylation in aorta and the level of *H19* transcripts was positively correlated with plasma homocysteine concentration (32). Li et al. consequently found that homocysteine induces the hypomethylation of the sixth CCCTC-binding factor (CTCF)-binding sites located upstream of *H19* gene (15). The CTCF regions are the crucial regulatory sites to control the imprinting expression of insulin-like growth factor 2 (*IGF2*) and *H19* genes (33). We also observed that such increase in *H19* level led to 70% sensitivity and 94% specificity to discriminate atherosclerotic individuals from non-atherosclerotic ones. These data demonstrate that plasma *H19* level can be considered as a potential biomarker of myocardial infarction. However, there are still some limitations connected to this study to generalize results. Totally, 32 cases and 30 control samples were collected through 1 year based on angiography. This sample size was inadequate for statistical analysis especially in case of subgroup analysis, and may affect and hide the significance of the results. As an example, only two cases with normal range of homocysteine existed among atherosclerotic cases. In conclusion, the results of the present study, for the first time, demonstrated that the expression level of *H19* in plasma can be considered as a biomarker of myocardial infarction, although more experiments are needed.

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Conflict of Interest

Authors declare no conflict of interest.

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