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Diagnostic potential of lncRNAs-ANRIL and MIAT in the blood of patients with cerebral venous thrombosis

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Venous Thrombosis; Stroke; Long Non-Coding RNA; Myocardial Infarction Associated Transcript; Gene Expression

Abstract

Background: Long non-coding ribonucleic acids (lncRNAs) have been implicated as possible circulating stroke indicators. This study focused on the expression status of antisense non-coding ribonucleic acid in the INK4 locus (ANRIL) and myocardial infarction associated transcript (MIAT) in patients with cerebral venous thrombosis (CVT).

Methods: In this study, fifty patients with CVT and one hundred age/gender-matched individuals as controls were included. The circulating levels of ANRIL and MIAT in the first 24 hours after admission were evaluated using the quantitative real-time polymerase chain reaction (RT-PCR) method. We compared the expression levels of ANRIL and MIAT between patients and controls using the independent two-sample t-test. Subgroup analysis was used to investigate the association of lncRNAs with clinical characteristics in patients with CVT. Receiver operating characteristic (ROC) curve analyses were conducted to evaluate the diagnostic value of two lncRNAs in patient assessment.

Results: The relative expression of lncRNAs ANRIL and MIAT significantly decreased in patients compared to the control. ANRIL and MIAT were shown as potential markers for discriminating patients with CVT from the healthy controls with an area under the curve (AUC) of 0.98 and 0.99, respectively.

Conclusion: For the first time, we found down-regulation and diagnostic potential of lncRNAs-ANRIL and MIAT in the blood of patients with CVT.

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Introduction

Cerebral venous thrombosis (CVT) is an uncommon but potentially fatal cerebrovascular disease. It mainly affects young to middle-aged individuals, and more patients are women; approximately 60% of 812 patients with CVT from 9 Asian countries were women.[1] However, timely diagnosis and treatment may lead to a less dismal prognosis.[2,3] CVT is often multifactorial, including female hormones, trauma surgery, pregnancy, puerperium, malignant disease, thrombophilia, inflammation, or idiopathic factors.[4] Inflammation is considered an exacerbating and predisposing factor for CVT. The inflammatory reaction leads to localized endothelial injury and hyper-coagulation, which may trigger CVT onset. The aggravation of brain tissue ischemic injury following CVT-induced inflammation can lead to poor clinical outcomes.[5,6]

Long non-coding ribonucleic acids (lncRNAs) are ribonucleic acids (RNAs) with more than 200 nucleotides without protein-coding capacity, which have shown critical roles in gene regulation.[7] LncRNA antisense non-coding RNA in the INK4 locus (ANRIL) is located on the 9p21.3 loci. ANRIL accelerates atherosclerosis development,[8] which plays a prominent role in angiogenesis and thrombosis.[9]

A previous study showed the positive association of lncRNA ANRIL expression with inflammatory responses in patients with coronary artery disease (CAD),[10] intracranial aneurysm, and type 2 diabetes.[11] In the pathogenesis of CVT, prothrombotic conditions and inflammatory responses are identified as two important risk factors for the development and progression of CVT disease.[12]

LncRNA-myocardial infarction associated transcript (MIAT) exhibits up-regulation in ischemic stroke (IS), myocardial infarction (MI), and down-regulation in schizophrenia, diabetic nephropathy, and bone disease.[13] MIAT expression was positively correlated with the pro-inflammatory cytokine levels in MI,[14] and it was suggested that MI was also associated with a transient increased venous thromboembolism (VTE) risk.[15] Considering the key role of ANRIL and MIAT in the pathogenesis of inflammation and thrombosis, we can assume that dysregulation of these lncRNAs may be involved in developing CVT.

We aimed to investigate the circulating level of ANRIL and MIAT lncRNAs in patients with CVT in countries with a high prevalence of CVT.[16]

Materials and Methods

Study subjects: In this prospective study, a total of 50 consecutive patients with symptoms of CVT who were admitted to the department of neurology of Namazi Hospital, Shiraz City, Iran, from June 2020 to June 2021, were selected as study subjects. Inclusion criteria were as follows: (a) definite diagnosis of first acute CVT, (b) in the first week after the onset of CVT symptoms, (c) being over 18 years of age, and (d) written informed consent prior to participation.

The diagnosis of CVT was based on the presence of relevant clinical symptoms by expert neurologists/radiologists, and it was confirmed by computed tomography (CT), CT venography (CTV), magnetic resonance imaging (MRI), and/or MR venography (MRV). Exclusion criteria were: (a) all patients with CVT with incomplete medical records, (b) patients with other neurological disorders rather than CVT such as hypertensive intracranial hemorrhage (HICH), arterial infarcts, brain arteriovenous malformation, cerebral vasculitis without venous-sinus thrombosis, brain aneurysms, reversible vasoconstriction syndrome, toxic and metabolic encephalopathies, and idiopathic intracranial hypertension (IIH) without evident venous-sinus thrombosis.

The control group was composed of a representative sample of Shiraz population which were sex and age-matched with cases. The following variables were assessed: demographic data, predisposing factors for CVT, previous history of venous thrombotic events, and family history of CVT/deep vein thrombosis (DVT).

Routine laboratory tests and specific tests were conducted to find the most probable cause of CVT. These causes include infection, anemia, thrombophilia, hematology, rheumatology, malignancy, sex-specific causes, mechanical trauma, and dehydration.

The Institutional Review Board (IRB) and Ethics Committee of Islamic Azad University, Arsanjan Branch, Arsanjan, Iran, approved the study protocol (Approval Code: IR.IAU.A.REC.1399.021). This study follows the ethical standards of the institutional and national research committee and the Declaration of Helsinki or comparable ethical standards.[17] Data can be shared with other centers upon the approval of the Ethics Committee.

RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis: The patient's blood samples (3 ml) were collected in the

first 24 hours after admission.

Total RNA was isolated from each sample using a RiboEx 100 ml TRIzol Kit (GeneAll, South Korea) according to the manufacturer's protocol. The quality of the RNAs was assessed by the optical density (OD) (A260/A280 nm ratio). The concentration of the extracted RNAs was measured at 260 nm by NanoDrop (Thermo Scientific Company, USA). All samples had high quality (OD 260/280 = 1.8-2.1). Then extracted RNAs were reverse transcribed to the cDNA pool using the AddScript cDNA Synthesis Kit (AddBio, South Korea) in a mixture of oligo-dT and random hexamer primers under the provider's instructions, then cDNAs were stored in -80 °C.

Quantitative real-time polymerase chain reaction (RT-PCR): Primers for the interesting genes and actin beta gene (ACTβ, used as a housekeeping gene) were designed using AlleleID 7.5 (Premier Biosoft International, Palo Alto, CA, USA). The primer sequences are shown in table 1. These primers were specific to messenger RNAs (mRNAs) and failed to amplify genomic deoxyribonucleic acid (DNA).

At first, the standard curve was considered for all genes. Quantitative RT-PCR was performed using StepOne Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA) in a 20-µl total volume using 1X qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems, UK), 100 ng cDNA, and 10 µmol/µl of primers. RT-PCR amplifications were done as follows: A pre-amplification denaturation was applied at 95 ˚C for 10 minutes (1 cycle), accompanied by 40 cycles of 95 ˚C for 20 seconds (denaturation), 58 ˚C for 20 seconds (annealing), and 72 °C for 30 seconds (extension). All samples were analyzed in duplicate. Melting curve analysis was performed, ramping from 75 ° to 95 °C and rising 1 degree per step to confirm the precision of the polymerase chain reaction (PCR). The relative mRNA levels for each individual (patients and controls) were calculated according to the $2^{-\Delta Ct}$ method based on the threshold cycle (Ct) values.[18]

Data were presented as mean ± standard error (SE). Statistical analysis was surveyed using SPSS software (version 19, SPSS Inc., Chicago, IL, USA). For all tests, a $P < 0.05$ was considered statistically significant. Expression levels of lncRNAs were compared between patients and controls using an independent Student's t-test. The Student's t-test or Mann-Whitney U test was applied to determine the correlation between the patients' characteristics and gene expression level. Receiver operating characteristic (ROC) curve analyses were conducted to evaluate the diagnostic value of circulating ANRIL and MIAT in patient assessment.

Results

Demographic and clinical characteristics of patients with CVT and controls: Fifty patients with CVT (28 women) and 100 healthy controls (56 women) were recruited for this study. Table 2 shows demographics and the relative expression of ANRIL and MIAT in patients and controls.

The levels of lncRNAs ANRIL and MIAT in patients with CVT relative to controls: As shown in figure 1, the relative expression of lncRNA ANRIL in patients with CVT significantly decreased compared to controls (0.204 ± 0.038 vs. 1.302 ± 0.049, $P < 0.001$). We also found significant MIAT down-regulation in patients with CVT in comparison with controls (0.256 ± 0.039 vs. 1.158 ± 0.037, $P < 0.001$).

Association between lncRNAs ANRIL and MIAT with clinical parameters in patients with CVT: The MIAT level showed a significant increase in patients with sex-specific risk factors (women, oral contraceptive, and pregnancy) compared to patients without these risk factors (0.364 ± 0.070, n = 18, vs. 0.205 ± 0.040, n = 32; P = 0.040) (Table 3).

There were no significant differences between men and women patients in ANRIL and MIAT expression levels after CVT.

Table 1. Primer sequences of two selected long non-coding ribonucleic acids (lncRNAs) and actin beta (ACTβ) gene

Genes	Gene ID		Sequences (5′→3′)	Product length (bp)
ANRIL	100048912	Forward	GAGGGTTCAAGCATCACTGTTAG	136
		Reverse	CCCGTCTCTACTGTTACCTCTG	
MIAT	440823	Forward	GGAGGCTGCGGACGAGTG	227
		Reverse	AGGAACTTGCTGCTCTCTTGGT	
ACTβ	60	Forward	GCCTCGCCTTTGCCTATCC	236
		Reverse	TCTCTTGCTCTGGTCCTCGTC	

ANRIL: Antisense non-coding RNA in the INK4 locus; MIAT: Myocardial infarction associated transcript; ACTβ: Actin beta

Table 2. Demographic and clinical characteristics of the participants in the case and control groups

Variables	Cases (n = 50)	Controls (n = 100)	P
Age (year)	43.44 ± 1.80	43.52 ± 1.20	0.971**
Sex			> 0.999*
Women	28 (56.0)	56 (56.0)	
Men	22 (44.0)	44 (44.0)	
ANRIL relative expression (fold change)	0.204 ± 0.038	1.302 ± 0.049	< 0.001**
MIAT relative expression (fold change)	0.256 ± 0.039	1.158 ± 0.037	< 0.001**

Categorical and continuous data are listed as mean ± standard error (SE) or as number (%).
*Chi-square test, **Independent two-sample t-test; P-value < 0.05 was regarded as statistically significant.
ANRIL: Antisense non-coding RNA in the INK4 locus; MIAT: Myocardial infarction associated transcript

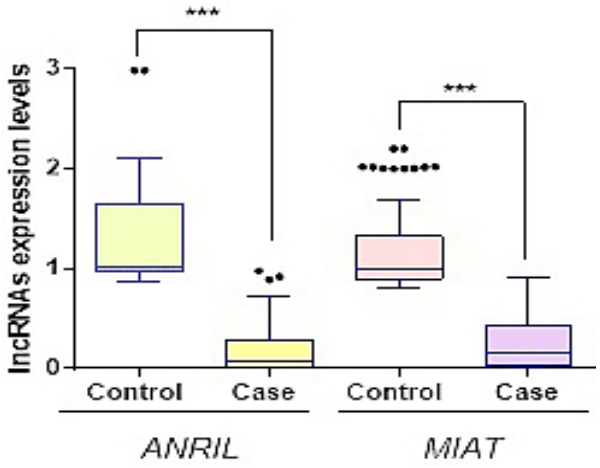

Figure 1. Comparison of the long non-coding ribonucleic acids (lncRNAs) of antisense non-coding RNA in the INK4 locus (ANRIL) and myocardial infarction associated transcript (MIAT) genes expression in the patients with cerebral venous thrombosis (CVT) and controls
[Data were shown as median (minimum-maximum). The data were analyzed using Student's t-test. ***P < 0.001 significant differences vs. control subjects]

Two of the patients had a positive family history for CVT while 48 patients had negative family history for CVT.

Diagnostic value of circulating lncRNAs ANRIL and MIAT in patients with CVT: ANRIL level showed a potential marker for discriminating patients with CVT from the healthy controls with an area under the curve (AUC) of 0.98 [95% confidence interval (CI): 0.970-1.005, P < 0.001; Figure 2a]. The corresponding sensitivity and specificity for ANRIL were 94.00% and 99.99%, respectively. For MIAT levels, we also found significant discrimination with an AUC score of 0.99 (95% CI: 0.980-1.004, P < 0.001; Figure 2b), and the corresponding sensitivity and specificity for MIAT were 98.00% and 100%, respectively.

Discussion

In this study, we reported for the first time the down-regulation of lncRNAs ANRIL and MIAT in 50 patients with CVT relative to controls. Moreover, two lncRNAs (ANRIL and MIAT) were identified as potential markers for CVT diagnosis with an AUC of 0.98 and 0.99, respectively, for CVT. In our study, there was no significant association between ANRIL expressions with demographic and CVT risk factors in our patients. Unfortunately, data for identifying CVT severity with the modified Rankin Scale (mRS) were overlooked for many patients.

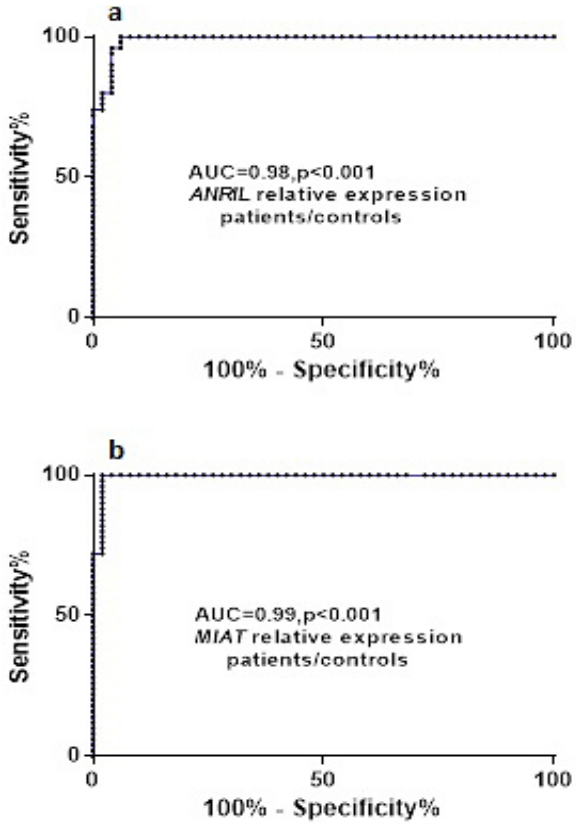

Figure 2. Pearson correlation between relative expression of of antisense non-coding RNA in the INK4 locus (ANRIL) (a) and myocardial infarction associated transcript (MIAT) (b) in 50 patients with cerebral venous thrombosis (CVT) with modified Rankin Scale (mRS) at admission

Table 3. Association between long non-coding ribonucleic acids (lncRNAs) of antisense non-coding RNA in the INK4 locus (ANRIL) and myocardial infarction associated transcript (MIAT) with demographic and clinical parameters in patients with cerebral venous thrombosis (CVT)

Variable		Patients (n = 50)	ANRIL	P	MIAT	P
Age (year)	≤ 40	26	0.165 ± 0.040	0.173	0.315 ± 0.050	0.140
	> 40	24	0.239 ± 0.060		0.205 ± 0.050	
Gender	Men	22	0.179 ± 0.050	0.519	0.221 ± 0.050	0.340
	Women	28	0.217 ± 0.050		0.295 ± 0.050	
Hypertension	No	43	0.188 ± 0.040	0.460	0.261 ± 0.040	0.950
	Yes	7	0.279 ± 0.110		0.269 ± 0.120	
Diabetes	No	45	0.188 ± 0.030	0.489	0.263 ± 0.040	0.940
	Yes	5	0.312 ± 0.150		0.254 ± 0.130	
Smoking	No	40	0.198 ± 0.040	0.900	0.269 ± 0.040	0.690
	Yes	10	0.210 ± 0.100		0.234 ± 0.070	
Alcohol	No	48	0.195 ± 0.030	0.680	0.257 ± 0.030	0.490
	Yes	2	0.336 ± 0.260		0.391 ± 0.360	
Infectious disease	No	39	0.202 ± 0.040	0.770	0.292 ± 0.040	0.145
	Yes	11	0.196 ± 0.060		0.157 ± 0.030	
Anemia	No	38	0.175 ± 0.040	0.288	0.245 ± 0.040	0.480
	Yes	12	0.281 ± 0.080		0.316 ± 0.080	
Hematology disorders	No	43	0.212 ± 0.040	0.357	0.235 ± 0.040	0.070
	Yes	7	0.132 ± 0.070		0.431 ± 0.100	
Rheumatology disorders	No	46	0.206 ± 0.040	0.959	0.263 ± 0.030	0.970
	Yes	4	0.139 ± 0.080		0.256 ± 0.170	
Malignancy	No	43	0.207 ± 0.040	0.956	0.265 ± 0.040	0.870
	Yes	7	0.162 ± 0.090		0.247 ± 0.120	
CVT family history	No	48	0.207 ± 0.030	0.040	0.263 ± 0.030	0.960
	Yes	2	0.043 ± 0.030		0.254 ± 0.230	
Sex-specific	No	32	0.188 ± 0.040	0.650	0.205 ± 0.040	0.040
	Yes	18	0.223 ± 0.060		0.364 ± 0.070	
Mechanical trauma	No	45	0.191 ± 3.120	0.900	0.255 ± 0.030	0.560
	Yes	5	0.284 ± 3.900		0.330 ± 0.160	
Dehydration	No	46	0.196 ± 0.030	0.800	0.263 ± 0.030	0.970
	Yes	4	0.255 ± 0.210		0.258 ± 0.170	
Unrecognized	No	43	0.195 ± 0.030	0.784	0.277 ± 0.040	0.350
	Yes	7	0.235 ± 0.140		0.174 ± 0.040	

CVT: Cerebral venous thrombosis; ANRIL: Antisense non-coding RNA in the INK4 locus; MIAT: Myocardial infarction associated transcript

Therefore, we could not estimate the genetic correlation between gene expressions with CVT severity to determine the inflammatory or anti-inflammatory effects of ANRIL and MIAT expression after CVT. According to previous reports, ANRIL gene polymorphisms associate to the IS risk and play a critical role in IS pathogenesis.[19] Moreover, ANRIL rs10965215 showed a good diagnostic accuracy for IS.[20] Further, several studies have identified the potential use of ANRIL as diagnostic biomarker for other diseases such as stroke,[19,21] coronary heart disease (CHD),[22,23] CAD,[24,25] and cancer.[26,27]

ANRIL overexpression is associated with inflammatory response in IS.[28] The possible mechanisms involved in pro-inflammatory effect of ANRIL include increase in nuclear factor kappa B (NF-κB) expression,[29] type I interferon (IFN)-mediated signal transduction pathway, and innate immune response.[28] On the other hand, the ANRIL down-regulation with anti-inflammatory effects has been suggested in inflammatory bowel disease (IBD),[30] attenuating the atherosclerosis progression in patients with CAD,[8] and acute IS.[31] Additionally, the association of ANRIL expression on endothelial dysfunction and promotion of thrombosis[8,9] can represent the possible diagnostic potential of ANRIL for CVT.

We also found significant MIAT down-regulation in patients with CVT compared to controls. Moreover, MIAT level in our patients with positive sex-specific risk factor was significantly higher relative to patients with negative sex-specific risk factor. Data from several

sources have identified the positive association between sex-specific risk factors and CVT severity.[32,33] These results indicated the possible inflammatory role of ANRIL in CVT.

Previous results confirmed the inflammatory effect of MIAT up-regulation with a positive correlation with disease severity in IS,[34,35] CAD,[36] multiple sclerosis (MS),[37] and cancer.[38] The inflammatory mechanism of MIAT has been suggested to participate in endothelial inflammation, vascular dysfunction,[39] vascular leakage, and up-regulation of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6).[40] However, Wang et al. reported an anti-inflammatory effect of lncRNA MIAT in macrophages in synovium and myocardial tissues of collagen-induced arthritis mice.[41]

We analyzed the circulating ANRIL and MIAT expression in patients with CVT to identify a novel biomarker for CVT. Our results showed that circulating lncRNAs ANRIL and MIAT could be considered CVT biomarkers with an AUC score of 0.98 and 0.99, respectively, with the corresponding high sensitivity and specificity. This pilot study with 50 patients and 100 controls well demonstrated ANRIL and MIAT down-regulation after CVT. However, despite several substantial limitations, such as retrospective design, small sample size, data obtained from one center, and not evaluating the mRS in admission and 3 months after CVT, we cannot definitively report an association between these lncRNAs and CVT severity.

Conclusion

Collectively, this study outlines a possible role for ANRIL lncRNA in CVT. We firstly observed significant ANRIL down-regulation in patients with CVT compared to controls. This is a novel finding for CVT disease, which might guide a new direction for future research. We recommend a study with a larger sample size for better evaluation of gene expression and outcome assessment.

Conflict of Interests

The authors declare no conflict of interest in this study.

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