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Expression of Long Non-Coding RNAs (UCA1 and CCAT2) in the Blood of Multiple Sclerosis Patients: A Case - Control Study

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Abstract

Background: Multiple sclerosis (MS) is an autoimmune and multifactorial disease, and its pathogenesis is associated with many genetic and environmental factors. Long Non-coding RNA (lncRNAs) are a group of genes that have recently been identified as predisposing genetic factors for the development of many cancers.

Objectives: This is a case–control study to evaluate the expression of two lncRNAs including Urothelial Carcinoma Associated 1 (UCA1) and Cancer-Associated Transcript 2 (CCAT2) in Relapsing-Remitting Multiple Sclerosis (RRMS) patients compared to healthy control group.

Methods: In this case-control study, the expression of UCA1 and CCAT2 was evaluated in 50 RRMS patients (37 females, 13 males with a mean age of 36.2 ± 2.9 years) compared to 50 healthy controls (38 females, 12 males with a mean age of 35.3 ± 2.1), using the TaqMan real-time PCR technique. This study was conducted during 2017 and 2018 at Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Results: There was no significant difference between the overall expression of UCA1 ($P = 0.282$) and CCAT2 ($P = 0.983$) among the case and control groups. However, there was a significant difference in the expression of UCA1 in female patients older than 40 years in comparison with healthy age-matched females ($P = 0.013$). In addition, there was a significant correlation between the expression of UCA1 and CCAT2 ($P < 0.0001$).

Conclusions: These results suggest the synergistic effects of UCA1 and CCAT2 on pathogenic aspects of MS, by affecting cellular signaling pathways such as WNT and NF-kB.

Keywords: Cancer-Associated Transcript 2, Case-Control Studies, Gene, Long Non-Coding RNA, Multiple Selection, Urothelial Carcinoma Associated 1

1. Background

Multiple sclerosis (MS) is a neurodegenerative disorder that is associated with chronic inflammation and demyelination in the central nervous system, along with axonal degeneration and gliosis. The pathogenesis of MS involves multiple cellular pathways and genetic factors (1-3). In the development and progression of MS, long noncoding RNAs (lncRNAs) appear to have a pivotal role (4, 5).

Over the last several decades, genomic studies have evidenced the presence of a large portion of DNA that was transcribed to RNAs (called non-coding RNAs) but did not encode proteins. A class of non-coding RNAs is known as lncRNAs and through various mechanisms, regulate numerous aspects of cells such as cell proliferation, apoptosis, and so on (6, 7).

Moreover, lncRNAs may play a crucial role in the activation, differentiation, and imbalanced proliferation of immune cells (T cells, B cells, macrophages, and NK cells) observed in autoimmune diseases (8). One of the important causes of MS pathogenesis is the T cell ability to escape apoptosis (9). The inability of the immune system to remove self-reactive lymphocytes is due to a defect in cell death machinery which ultimately results in autoimmune reactions. Based on a study which has been conducted in

RRMS patients by cDNA microarrays, more than 80% of the top 30 most significantly altered genes have been shown to be associated with apoptosis signaling pathway (10).

UCA1 (Urothelial Carcinoma Associated 1) is a member of the lncRNAs family which has gained increasing attention in recent years. UCA1 was first identified in human bladder carcinoma, where it increased bladder cancer cell proliferation and progression. The gene that encodes this lncRNA is located on chromosome 19p13.12. Several studies have reported this lncRNA as a multiplier for increasing cell proliferation, cell resistance to apoptosis, invasion, and induction of drug resistance in numerous cancer cells such as the bladder, breast and colorectal cancer (11, 12). UCA1 has been found to affect the proliferation, apoptosis and cell cycle progression in colorectal cancer cells (13). However, the role of UCA1 in autoimmune diseases, especially MS, is completely unknown.

Cancer-Associated Transcript 2 (CCAT2) is a new lncRNA mapped to the 8q24 gene. This lncRNA was initially known as an oncogene in colon cancer, and it appears to play an important role in tumor growth, metastasis, chromosomal instability, and inability to apoptosis (14, 15).

Although the role of CCAT2 and UCA1 in different cancers and cellular pathways has been investigated, no study has been conducted to assess the expression level of these two lncRNAs in RRMS patients.

2. Objectives

The present study investigated the expression levels of UCA1 and CCAT2 in RRMS patients compared to healthy subjects. It also evaluated the correlation between expression levels of UCA1 and CCAT2 and the clinicopathological features of MS like the Expanded Disability Status Scale (EDSS), disease duration and age at onset.

3. Methods

3.1. Subject and Control Group

The present investigation is an association study, which was conducted to measure the levels of the expression of two lncRNAs (UCA1, CCAT2) in 50 RRMS patients compared to 50 healthy match controls. As there was no data regarding expression of these lncRNAs in MS patients, we have used information of Eftekharian et al. article to calculate sample size of the study using the formula (4).

$$n = \frac{\left(Z_{1-\alpha}\sqrt{\bar{p}\left(1-\bar{p}\right)} + Z_{1-\beta}\sqrt{p_1(1-p_1)+(1-p_2)}\right)^2}{(P_1-P_2)^2} \quad (1)$$

Considering the study power of 80% and type I error of 5%, the appropriate sample size of the study is 50. So we included 50 patients and 50 healthy subjects in the study.

The blood samples were taken in Farshchian Hospital, Hamadan, Iran, in summer 2017. All patients who participated in this study were in a stable phase and were clinically diagnosed by McDonald's criteria by an expert neurologist (16).

Clinical diagnosis with MRI (Magnetic Resonance Imaging) criteria was confirmed. Vitamin D levels were also evaluated in both case and control groups, and its normal levels, were considered as inclusion criteria. Based on the significant role of HLA-DRB1*15 in MS pathogenesis (17), in order to have a more pure selection of patients, we excluded HLA-DRB1*15 positive patients from the study. In addition, all of the patients were clinically responsive to interferon-beta and took CinnovexTM (Cinnagene, Iran) as treatment (18, 19). Inclusion criteria for the control group were as follows: 1) Sufficient Vitamin D levels, 2) No family history of autoimmune disease in first-degree relatives; 3) lack of corticosteroid injections; 4) and lack of a history of a lately viral infection. The clinical variables of patients like EDSS and disease was measured by an expert neurologist using international criteria (20). The consent form was received from all participants in this study. The study was approved by the local Ethics Committee of Shahid Beheshti University of Medical Sciences (Code of ethical approval: IR.SBMU.MSP.REC.1396.635, Date of ethical approval: 2018.11.19). The Ethical Considerations included in this study are: non-imposition of additional costs to participants, Preservation of personal information of participants, the implementation of the study in coordination with the physician.

3.2. Blood Sampling and RNA Extraction

In our study, 3 mL of peripheral blood was taken from each participant and placed in the EDTA-coated tube. Total RNA was extracted using Gene All Hybrid-RTM blood RNA extraction kit (cat No.305-101, Korea). Applied Biosystems High-capacity cDNA reverse transcription kit (PN: 4375575, USA) was used for cDNA synthesis, and the manufacturer's instructions were executed. Synthesized cDNAs were stored at -20°C until PCR. Allele ID7 (Premier Biosoft, Palo Alto, USA) software was used to design specific probes and primers of each gene. HPRT1 gene was selected as the reference gene. Table 1 presents the sequences of designed probes, primers and the length of the PCR products. Real-time quantitative PCR was conducted in duplicates by using Applied Biosystems TaqMan Universal PCR Master Mix (PN: 4304449, USA). Corbett Rotor gene 6000 machine (Corbett Life Science, USA) was used to run the re-

actions. For quality control and detection of contamination, a negative control was included in each run.

All the equipment that used in this study including the centrifuge, thermocycler and Corbett Rotor gene 6000 were calibrated for avoiding the bias in the final results.

3.3. Statistical Analysis

To determine the significant difference in means between two groups, the Kruschke's Bayesian estimation was implemented to fit the two-sample Bayesian t-test. A normal prior distribution was assumed for parameters with 200000 iterations. Spearman's correlation coefficient was applied for assessment of correlations between the level of genes expression and clinical or demographic variables. The level of significance was fixed at < 0.05. All data were analyzed using SPSS Statistical Software for Windows, version 18.0 (SPSS Inc., Chicago, ILL., USA).

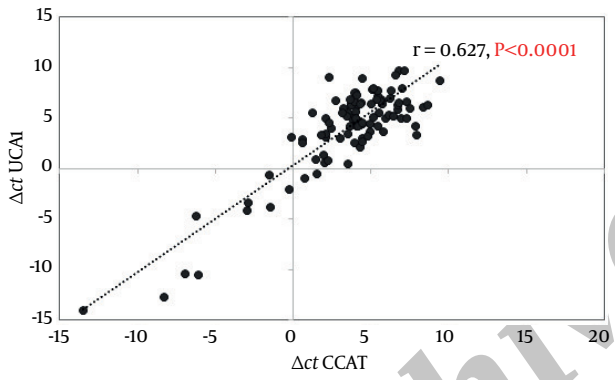

Figure 1. ΔCT Spearman's correlation between Δct UCA1 relative quantitation and Δct CCAT. Abbreviations: CCAT, cancer associated transcript; UCA1, urolthelial carcinoma associated.

4. Results

This study was performed on 50 patients (37 females, 13 males, mean age ± SD: 36.2 ± 2.9 years) and 50 healthy subjects (38 females, 12 males, mean age ± SD: 35.3 ± 2.1). Table 2 presents a summary of the demographic data and clinical characteristics of RRMS patients, including age, disease duration, EDSS, as well as the mean age of the control group.

4.1. Expression Levels of UCA1 and CCAT2 in RRMS Patients and Controls

Based on the obtained data, there were no significant differences in the expression levels of UCA1 (Table 3) and CCAT2 (Table 4) between the patient's group and the healthy controls. To find a correlation between the expression of mentioned lncRNAs among the case and control groups based on gender and age, samples were categorized into several subgroups. A significant change in the expression of UCA1 gene was observed in female patients over 40 years old (n = 15) compared to healthy females over 40 years of age (n = 20) (P-value = 0.013). However, in other age and sex subgroups, there was no significant correlation between UCA1 and CCAT2 expressions among the case and control groups.

4.2. Correlation Between UCA1 and CCAT2 Transcript Levels

In this study, there was a significant correlation between the lncRNA levels of UCA1 and CCAT2.

4.3. Correlation Between Genes Expression Level and Expanded Disability Status Scale, Disease Duration and Age at Onset

The results of the present study revealed no correlation between the expression of UCA1 and CCAT2 genes and EDSS, disease duration and age at onset.

5. Discussion

UCA1 and CCAT2 are two lncRNAs that have been proven to vary in their expression levels in a number of cancers such as breast, ovarian, urinary tract, colon, esophageal squamous cell carcinoma and lung (21-30). However, the expression level of these two lncRNAs in multiple sclerosis are yet to be studied. One limitation of our study may be the sample size. The larger sample sizes allow more accurate statistics and give more reliable results.

In previous studies, there have been numerous reports about changes in the expression levels of UCA1 in women with breast and ovarian cancer.

One of the important female cancer pathways is the Wnt signaling pathway, and some lncRNAs such as UCA1 and CCAT2 are known as the main Wnt target genes (31). Since multiple sclerosis is more prevalent in women, the significant difference in expression of UCA1 in women over 40 years of age compared with the healthy age-matched women, can be attributed to the role of the Wnt pathway in this subgroup of patients. In 2012, Yuan et al. (32), reported the role of the Wnt signaling pathway in Experimental autoimmune encephalomyelitis (EAE) mice. Since there is a significant relationship between the expression of UCA1 and CCAT2 in RRMS patients, it appears that these two lncRNAs have some interactions with each other in the Wnt signaling pathway.

Several lines of studies have shown that the activation of the NFKB gene causes inflammation in MS patients (33). In a study conducted in 2015 (34), higher expression of NF-kB and UCA1 genes in the brain tissue of epilepsy-treated rats was observed in comparison to the control group.

Table 1. The Nucleotide Sequence of Primers and Probes

Gene	Primer and Probe Sequences	Primer and Probe Length	Product Length
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM -CATCTGGAGTCCTATTGACATCGC- TAMRA	24	
CCAT2	F: GCGCTGACAGAGATTGCTTAC	21	141
	R: CCAGAGTAGAACAGGGGAAGC	21	
	FAM- TGTGCTCCAAGTGCTTGCCAGGCT-TAMRA	24	
UCA1	F: TCTCCATTGGGTTCACCATTCC	22	100
	R: GCTCTCGGCCTAATCTTGTGG	21	
	FAM- AGCCATGCCCATCAGACAGCCAGC- TAMRA	24	

Abbreviations: CCAT2, cancer associated transcript; HPRT1, hypoxanthine-phosphoribosyltransferase; UCA1, urolthelial carcinoma associated.

Table 2. General Clinical and Demographic Data of Participants[a]

Variables	Multiple Sclerosis Patients	Healthy Subjects
Female/Male	37 (74)/13 (26)	39 (78)/11 (22)
Age, y	36.2 ± 2.9	35.3 ± 2.1
Age range, y	17 - 55	22 - 60
Age of onset, y	31.41 ± 2.8	-
Relapsing-remitting course	100 (100)	-
Duration, y	4.58 ± 3.2	-
EDSS	3.07 ± 2.7	-

Abbreviation: EDSS, expanded disability status scale.
[a] Values are expressed as mean ± SD or No. (%).

Moreover, the expression of UCA1 in the peripheral blood of affected rats was higher compared to that of healthy rats (34).

Therefore, it can be concluded that there is a relationship between the expression of UCA1 and NF-kB in female patients over 40 years of age, and more studies are required in this area. Based on previous studies, the expression of CCAT2 is associated with inflammation (28). Therefore, the significant correlation between the expression levels of UCA1 and CCAT2 in this study may be suggestive of the synergistic effect of these two lncRNAs on the promotion of the inflammation process.

In different cancers, lncRNA UCA1 regulates the proliferation of cells via PI3K-AKT, ERK1/2 and MAPK pathways (35, 36). Therefore, a significant change in the expression of UCA1 in women over 40 years of age in this study may indicate the key role of these pathways in the pathogenesis of MS in this group of patients.

One of the most important genes involved in cell cycle regulation is p27, and it acts as a cell cycle inhibitor. In a study conducted in 2016 (37), UCA1 was found to suppress the expression of p27 and thus, increases the proliferation of breast cancer cells. Therefore, one of the possible pathogenesis pathways in female patients over 40 years of age in the present study is the increased expression of UCA1, which causes excessive T-cell proliferation and ultimately, may lead to an autoimmune disorder like MS.

5.1. Conclusion

This report was conducted for the first time on the whole blood of multiple sclerosis patients to reveal the role of two lncRNAs (UCA1 and CCAT2) in the pathogenesis of MS as an autoimmune disease. UCA1 is one of the genetic factors which can be involved in this disorder through mechanisms such as inhibition of cell cycle arrest and promotion of inflammation. The over-expression of CCAT2 can have a synergistic effect on the mentioned pathways.

Footnotes

Authors' Contribution: All of the authors have the same contribution in this study.

Conflict of Interest: The authors declare they have no conflict of interest.

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Table 3. Relative Expression of UCA1 in Distinct Sex and Age-Based Subgroups of MS Patients and Healthy Subjects

UCA1 Expression	Control No.	MS Patient No.	Expression Ratio	ΔΔCT	Se[a]	P Value[b]	95% CrI[c]
Total	50	50	1.6629	-0.38	0.54	0.282	[-1.45, 0.7]
Male	12	13	3.5606	-4.01	3.02	0.098	[-10.06, 1.82]
Female	38	37	1.6688	-0.12	0.61	0.771	[-1.36, 1.08]
< 30 y							
Male	2	5	3.24	-	-	-	-
Female	13	10	0.6174	0.58	1.68	0.446	[-2.73, 3.91]
30 – 40 y							
Male	6	3	0.359	-1.07	10.29	0.714	[-21.33, 18.92]
Female	5	12	0.4505	2.46	2.1	0.234	[-1.87, 6.38]
> 40 y							
Male	4	5	6.5197	-8	6.41	0.19	[-21.14, 5.15]
Female	20	15	1.7454	-1.45	0.59	0.013	[-2.64, -0.31]

Abbreviation: UCA1, urolthelial carcinoma associated.
[a] SE: standard error.
[b] Estimated from frequentist method.
[c] 95% credible intervals, ΔΔCT: case-control.

Table 4. Relative Expression of CCAT2 in Distinct Sex and Age-Based Subgroups of the Multiple Sclerosis Patients and Healthy Subjects

CCAT2 Expression	Control No.	MS Patient No.	Expression Ratio	ΔΔCT	Se[a]	P-Value[b]	95% CrI[c]
Total	50	50	1.4015	0.04	0.51	0.983	[-0.99, 1.06]
Male	12	13	0.8918	-1.46	2.47	0.936	[-6.32, 3.48]
Female	38	37	1.7299	-0.07	0.57	0.916	[-1.15, 1.05]
< 30 y							
Male	2	5	1.8000				
Female	13	10	0.8368	0.3	1.85	0.483	[-3.43, 3.86]
30 - 40 y							
Male	6	3	0.1670	1.66	10.58	0.714	[-15.54, 21.7]
Female	5	12	2.1297	1.04	1.61	0.383	[-1.94, 3.85]
> 40 y							
Male	4	5	2.3865	-5.35	6.19	> 0.999	[-18.15, 5.94]
Female	20	15	1.4253	-0.94	0.57	0.064	[-2.05, 0.18]

Abbreviation: CCAT2, cancer associated transcript.
[a] SE: standard Error.
[b] Estimated from frequentist method.
[c] 95% credible intervals, ΔΔCT: case-control.

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