



Original Article

Evaluation of *LncRNA uc.173* and *Occludin* in Iranian Patients with Inflammatory Bowel Disease Compared with Healthy IndividualsChaleshi Vahid¹, Oloumi Kalantar Saeideh², Salahshourifar Iman^{2*}, Shahrokh Shabnam¹, Asadzadeh AghdaeiHamid^{1*}, Zali Mohammad Reza³

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Abstract

Background & Objective: Inflammatory bowel disease (IBD) is characterized by the chronic gastrointestinal inflammation. The two common forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD) that are distinguished by their location and depth of involvement in the diffuse inflammation of the colonic mucosa and affects the rectum (proctitis). A novel class of *LncRNAs* transcribed from ultra-conserved regions (UCRs) is a recently identified ultra-conserved region (T-UCR) transcript that is involved in the cellular function in a variety of pathways. However, the regulation of *LncRNA uc.173* in IBD remains to be fully elucidated. In this study, we aimed to examine the expression of *LncRNA uc.173* and *Occludin* genes in an Iranian population with inflammatory bowel disease.

Materials & Methods: This case-control study was performed on 33 inflammatory bowel disease patients including 13 Crohn's disease, 20 ulcerative colitis and 20 healthy controls. The mRNA levels of *LncRNA uc.173* and *Occludin* genes were assessed using the quantitative Real-time polymerase chain reaction. The B2M was used as an internal control. The $2^{-\Delta\Delta C_q}$ method was used to determine the expression fold changes.

Results: Statistically, the level of the *LncRNA uc.173* gene expression between the UC and normal tissues increased significantly ($P=0.0024$). Also, the expression analysis revealed no significant difference between the samples of CD patients compared to the controls ($P>0.05$). In order to further evaluate the role of *LncRNA uc.173* in IBD, the associations between the transcript levels of the *LncRNA uc.173* and *Occludin* mRNA demonstrated significant difference in the CD tissue ($R=0.59$; $P=0.002$). In our study, the mRNA expression of *Occludin* gene did not show any changes in the IBD patients compared to the healthy controls.

Conclusion: The increased expression of *LncRNA uc.173* in the tissues of UC patients may be considered as a diagnostic or prognostic biomarker. Also, there was no correlation found between *Occludin* and *LncRNA uc.173* expressions in the IBD patients' tissues.

Keywords: Inflammatory bowel disease, Ulcerative colitis, Crohn's disease, *LncRNA uc.173*, *Occludin*

Introduction

Inflammatory bowel disease (IBD) is characterized by the chronic gastrointestinal inflammation

that can affect the entire digestive tract (1). Crohn's disease (CD) and ulcerative colitis (UC) are the two common different forms of IBD (2). The UC is characterized by the chronic inflammation of the colon and rectum, while the entire gastrointestinal tract is involved in CD (3). One of the most important events about IBD is its prevalence in Iran and the other parts of the world (4, 5). The etiology of IBD is not well understood yet. The genetic alteration determined an interplay between intestinal microbiome, the immune response, and disease pathogenesis (6, 7). Dysregulated mucosal immune response can damage the intestinal mucosal barrier

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and stimulate the luminal surface of the intestine by increasing the intestinal permeability that augments more intense immune responses (8). The genetics studies have shown that ultra-conserved regions (UCRs) in human, mouse and rat have functional role and may be involved in human disease (9). Studies have shown that T-UCRs (a new subset of non-coding RNAs (ncRNAs)) are involved in CD pathogenesis and participate in the intestinal mucosa barrier by affecting the intestinal tight junctions (TJs) (10, 11). Tight junction proteins play a key role in the epithelial barrier function (12) such as occludin, claudins, junctional adhesion molecule (13) and tricellulin (14). The *Occludin* gene located on human chromosome 5 at q13.1 location plays an important role in the intestinal epithelial integrity and affects the cell permeability (15, 16). Remarkably, the mRNA of the *Occludin* gene is unstable and this process is affected by the post-transcriptional regulation (17). The *LncRNA uc.173* is one of the long noncoding RNAs with the length of 276 bp, which is transcribed from the UCR region of the *UBE2B* gene on human chromosome 5 (18, 19). *UC.173* was prevalingly found in the cytoplasm. *UBE2B* mRNA transcribed from the host gene of *uc.173* was scattered in both the cytoplasm and the nucleus. The results illustrate that growth inhibition of the intestinal epithelium is attended by a reduction in the levels of *uc.173* (19).

Recent studies have suggested that *LncRNA uc.173* may play an important role in the intestinal homeostasis by affecting intestinal regeneration and permeability (19, 20).

The aim of this study was to evaluate the expression of *LncRNA uc.173* and *Occludin* genes between two groups of UC and CD patients compared to the healthy individuals to gain further knowledge about the diagnostic and prognostic values of the selected genes.

Materials & Methods

Patients

In this case-control study, 33 patients with inflammatory bowel disease including 13 Crohn's and 20 ulcerative colitis patients, and 20 healthy

controls were studied. The healthy and patient volunteers who were referred to the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran between September 2018 and September 2019 were enrolled in the present study. Pathology and colonoscopy results were performed by a specialist physician and after evaluating the inclusion and exclusion criteria, participants were included in the study. Healthy control volunteers with personal or family history of cancer or inflammatory diseases including gastritis, ulcerative colitis, and/or Crohn's disease were excluded. History of drug use was also received from patients in which none of the patients had used any drug that was related to or interfered with the results of the study. The participants were from one of the Iranian ethnicities and provided written informed consent for the present study prior to the sampling procedure. This study was approved by the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RIGLD.REC.1395.120). The provided tissue samples were frozen in liquid nitrogen immediately.

RNA extraction and cDNA synthesis

Total RNA was extracted from the biopsy samples of the patients using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Germany) according to the instruction. The RNA concentration was quantified by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and its quality was measured by the A260/A280 and A260/A230 ratio. The concentration of the samples was normalized and 1 µg of total RNA was reverse transcribed to cDNA using the RevertAid RT kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Quantitative real-time PCR analysis

QRT-PCR was performed using a PCR cycler (Rotor-Gene Q MDx; Qiagen GmbH) and cDNA fragments as templates

to amplify the *LncRNA uc.173* and *Occludin* genes using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) according to the manufacturer protocol. The experimental protocol was performed as follows: i) Thermocycling conditions consisted of an initial activation step for 30 sec at 94°C, 35 cycles at 94°C for 5 sec and 60°C for 35 sec ii) Melting curve analysis.

The primer sequences were designed with GeneRunner Software and their specificity was confirmed by the Primer-BLAST (NCBI). The primer sequences of the qRT-PCR are listed in Table 1. The *B2M* gene was used as a normalizer endogenous gene. The $2^{-\Delta\Delta Cq}$ method was used to determine the expression fold changes (patient vs. normal).

Table 1. Primer sequences used for Real-time PCR

Primer's name		Sequence (5'→3')	GC%	Tm	Reference
lncRNA uc.173	Forward	ACTTTTATTGCATGGTGTGAACT	33.3	60	(21)
	Reverse	CACTTGGAATAAATAACAAACAGG	33.3		
Occludin	Forward	GGTGTTTCTGTTGGCGTTGG	55	60	Designed
	Reverse	TCAGAAATGGAAGGGATGTCG	47.6		
β2M	Forward	TGCTGTCTCCATGTTTGATGTATCT	40	60	(22)
	Reverse	CTCTGCTCCCCACCTCTAAGT	57.1		

Statistical analysis

Statistical analysis was performed using SPSS 21 (IBM Corp., USA) and data was plotted using GraphPad Prism (v.5.04; GraphPad Software, Inc.). The significance level was determined using unpaired t-test and one-way analysis of variance test, with a Tukey's multiple comparison post-hoc test in which $P < 0.05$ was considered as significant. The association between *LncRNA uc.173* and *Occludin* genes expression was assessed via linear regression. The receiver operating characteristics (ROC) curve was constructed to describe diagnostic specificity.

Results

General statistical information

In this study, 33 patients with inflammatory bowel disease and 20 healthy controls were included. All Iranians were selected and non-Iranians such as Afghans and other ethnicities were excluded. The mean age and BMI of the patients were 33.40 ± 13.364 and 22.62 ± 4.89 , respectively and for controls were 55 ± 12.732 and 25.68 ± 4.26 , respectively. Among the patients, 20 (60.6%) and 13 (39.4%) were diagnosed as UC and CD, respectively. The gender of IBD patients was equal to 15 (46.9%) males and

17 (53.1%) females. The gender for controls was 9 (45%) males and 11 (55%) females.

Expression of *LncRNA uc.173* and *Occludin* in the IBD tissue samples

QRT-PCR data analysis of the *LncRNA uc.173* gene showed a significant difference between UC samples compared to the controls ($P=0.0024$, 95% CI, 1.306 ± 5.604). However, the gene expression analysis showed no significant difference between CD samples compared to the controls ($P>0.05$). Also, there was no significant difference between the samples of CD patients compared to the UC ($P>0.05$) (Chart. 1A). The differences in the expression levels of *Occludin* in the biopsy

samples between patients with UC, CD and normal controls were not statistically significant ($P>0.05$) (Chart. 1B)

Relative expression of *LncRNA uc.173* and *Occludin* in individual samples

In order to determine the association between the expression of *LncRNA uc.173* and *Occludin* genes, the relative expression of these genes was compared in each set of the samples. No significant association was observed between the levels of *LncRNA uc.173* and *Occludin* in UC tissues ($R=0.06$; $P=0.298$) (Chart. 1C), but, a significant association was observed between the levels of *LncRNA uc.173* and *Occludin* in CD tissues ($R=0.59$; $P=0.002$) (Chart. 1D).

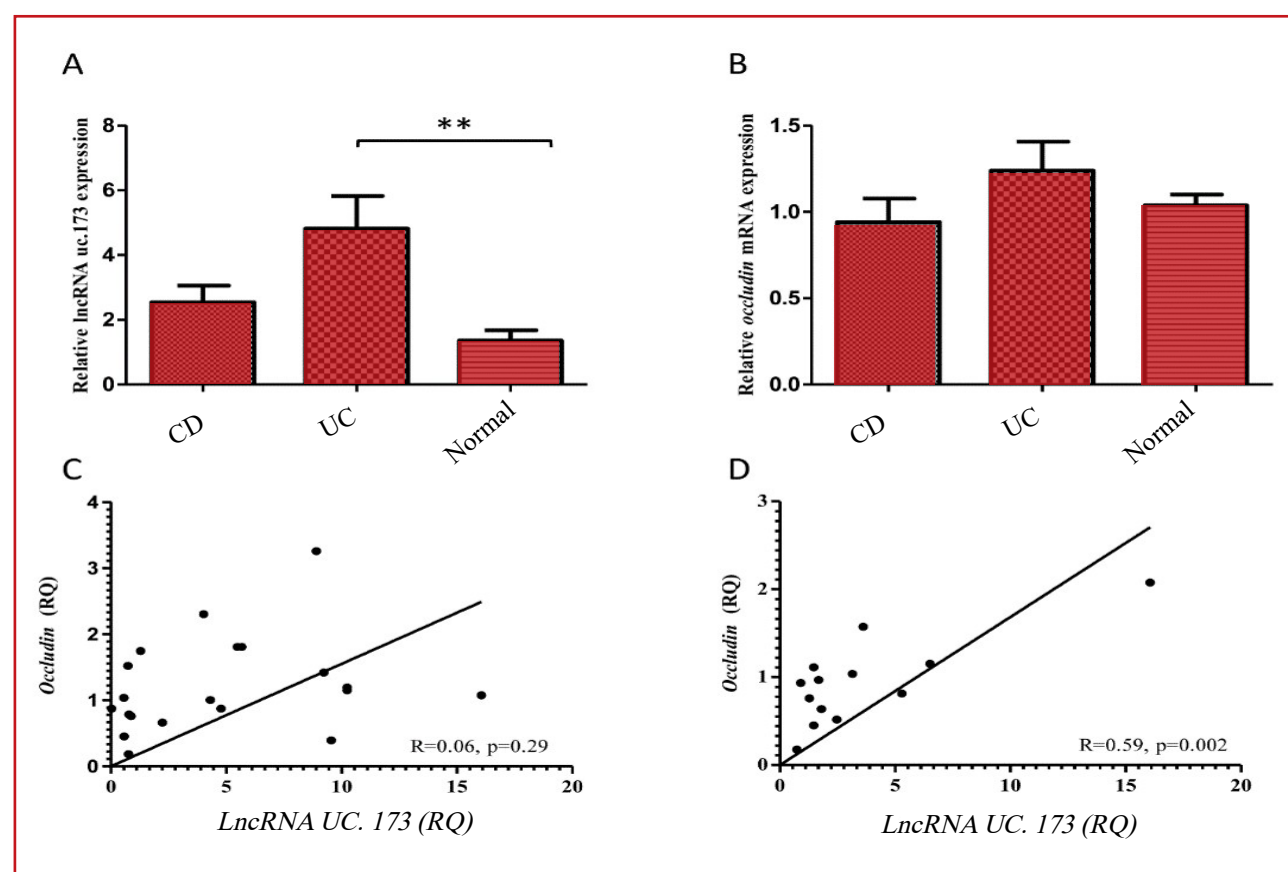


Chart 1. Real-time quantitative PCR analysis of *LncRNA uc.173* and *occludin* expression in human tissue (A) Relative mRNA expression of *LncRNA uc.173* in UC, CD comparing healthy groups (B) Relative mRNA expression of *occludin* in UC, CD comparing healthy groups (C) Association analyses using a linear regression between *LncRNA uc.173* and *occludin* expression in UC tissues compared with healthy tissues. (D) Association analyses using a linear regression between *LncRNA uc.173* and *occludin* expression in CD tissues compared with healthy tissues. CD, Crohn's disease; UC, ulcerative colitis; RQ, relative quantification; (* $P < 0.05$) (** $P < 0.01$) (***) $P < 0.001$)

Characteristics of *LncRNA uc.173* as predictive UC-related biomarker

To investigate the characteristics of *LncRNA uc.173* as the potential biomarker for UC, the receiver operating characteristics (ROC)

curves and the area under the ROC curves (AUC) was performed on 20 UC patients and 20 control samples. The ROC curve showed an area under the curve of 0.730 (95% CI: 0.569–0.891; $P<0.001$) (Chart. 2) (Table 2).

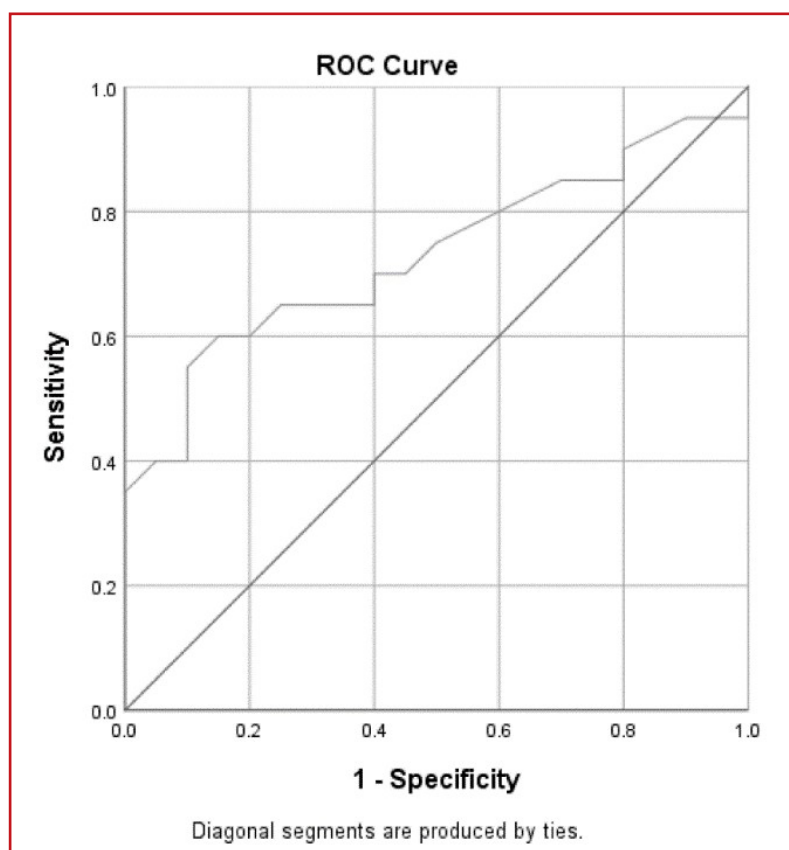


Chart 2. Receiver-operating characteristic (ROC) curves of normalized *LncRNA uc.173* to distinguish UC patients' tissue from the normal. The area under the curve (AUC) was determined for *LncRNA uc.173*

Table 2. The diagnosis value of *LncRNA uc.173* between UC patients and healthy controls tissue

Variable	<i>LncRNA uc.173</i>
Cut-off	0.801
Specificity (%)	55%
Sensitivity (%)	70%
Area	0.730
%95 CI	0.569-0.891

Discussion

Increased permeability plays a biological role in creating intestinal epithelial barrier dysfunction. It has been described in many human diseases such as IBD, IBS, severe acute pancreatitis, alcoholic liver disease, etc. (23-25). The loss of TJ barrier function results in the disruption of the intestinal epithelium as seen in the IBD patients (26). The TJs have a complex molecular composition which are mainly formed by claudins, occludins, and junctional adhesion molecules (13, 27). In the knockdown model of *Occludin* in the intestinal cell line it was shown to increase the macromolecule permeability (28, 29). Although in the present study no significant change was observed in *Occludin* gene expression between UC and CD patients compared to the control group, some studies have shown that the expression level of Occludin protein is reduced in inflammatory bowel disease (30-32). The Occludin protein plays an important role in the process of neutrophil migration from the epithelial cells into the intestinal lumen, which is involved in the activation of inflammatory bowel disease (32). Nikolaus Gassler and colleagues reported that *Occludin* gene expression in the active phase of CD and UC patients reduced significantly (33). In contrast, Yamamoto-Furusho et al., showed in their study that *Occludin* gene expression in the active UC increased significantly compared to the UC in remission (34). However, Yamamoto-Furusho et al., suggested that *Occludin* mRNA level reduced significantly in the UC remission of patients' colonic mucosa as compared to the healthy control group.

According to the studies, UCRs are highly conserved regions and about 481 UCRs have been discovered in the human genome. A category of the UCRs can be transcribed to RNAs and a subgroup of these RNAs which are noncoding RNAs (ncRNAs) (35) have different levels in normal, tumor, IBD and other human disease tissues (13, 36, 37). The role of T-UCRs in the pathogenesis of diseases is not yet fully understood, therefore, studies on whether T-UCRs

contribute to the pathology of IBD are necessary. However, Xiao Xian Qian et al., (2016) pointed out that the expression of *LncRNA uc.261* is significantly increased in the CD patients compared to the controls (colonic samples) (10). Data from Xiao Xian Qian et al., (2016) have identified that the over-expression of *LncRNA uc.261* damages the intestinal epithelial mucosa in the UC patients, by affecting the expression of tight junction proteins including JAMA, Occludin, Claudin-1, and Zo-1 (10).

Our findings suggest that *LncRNA uc.173* had a significant increase in the UC patients compared to the controls. Also, no significant difference was found in the expression of *LncRNA uc.173* between the CD and the control samples. In the present study, decrease in the expression of *LncRNA uc.173* in Crohn's patients' tissues correlated with a decrease in the expression of the Occludin gene. Recently, the studies have shown that *LncRNA uc.173* levels decreased significantly in the patients with Crohn's disease (small intestine samples) (38). The association between *LncRNA uc.173* and claudin-1 proteins has been investigated (Jun-Yao Wang, 2018). Their data showed that depletion in *LncRNA uc.173* expression in cultured Caco-2 epithelial cells decreases *claudin-1* mRNA expression and thereby increasing the permeability (39).

To the best of our knowledge, no association was found between *Occludin* expression and *LncRNA uc.173* in the IBD patients' tissues. Thus, we suggest further functional studies to evaluate the *LncRNA uc.173* study in a larger population in different Iranian ethnicities, including Turk, Kurd, and Lur and replicate on the specimens of Crohn's patients with small bowel involvement.

In addition, the increased expression of *LncRNA uc.173* in the UC patients' tissues may be considered in future studies as diagnostic or prognostic biomarker.

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Contribution

Conception and design; Vahid Chaleshi, Saeideh Oloumi Kalantar, Iman Salahshourifar, Analysis and interpretation of the data; Vahid Chaleshi, Shabnam Shahrokh, Drafting of the article; Vahid Chaleshi, Saeideh Oloumi Kalantar, Iman Salahshourifar, Critical revision of the article for important intellectual content; Hamid Asadzadeh Aghdaei, Mohammad Reza Zali, Final approval of the article; Vahid Chaleshi, Saeideh Oloumi Kalantar, Iman Salahshourifar, Shabnam Shahrokh, Hamid Asadzadeh Aghdaei, Mohammad Reza Zali

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Conflict of Interest

The authors declare no conflict of interest.

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