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Predictive Value of NKILA and lnc13 lnc RNAs for Distinguishing of Celiac Disease and IBD: A Case-Control Study

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Abstract: Long-noncoding RNA 13 (lnc13) and NF-κB-interacting lncRNA (NKILA) are long noncoding RNAs (lncRNAs) that play a role in inflammatory disorders pathogenesis. This study aimed at investigating the expression of these lncRNAs in inflammatory bowel disease (IBD) and celiac disease (CeD) patients compared to controls. We evaluated mRNA expression of lnc13 and NKILA in peripheral blood and intestinal biopsy samples of 50 CeD, 46 IBD patients, and 20 controls using qRT-PCR method. Compared to the controls, CeD and IBD patients had a significantly higher PBMC mRNA level of Lnc13 ($p < 0.0001$ for both of them) and NKILA ($p < 0.0001$ and $p=0.0174$, respectively). NKILA mRNA level was significantly higher in CeD than in IBD patients ($p < 0.0001$). IBD subjects had significantly increased colonic NKILA expression ($p=0.004$). In UC, NKILA, and CD, lnc13 colonic expression was significantly increased compared to others ($p=0.002$ and $p=0.0111$ respectively for UC, $p = 0.0075$ and $p = 0.002$, respectively for CD). Collectively, increased peripheral expression of lnc13 and NKILA along with their decreased duodenal expression demonstrates CeD. Moreover, suppose the increased peripheral expression of lnc13 and NKILA is associated with increased lnc13 colonic expression. In that case, it indicates CD, and if it is associated with increased NKILA colonic expression, it indicates UC.

Keywords: celiac diseases; inflammatory bowel diseases; long noncoding; intestines; blood.

1. Introduction

Inflammatory bowel disease (IBD) is a term for a multifactorial disease that refers to two various inflammatory intestinal disorders, Crohn's disease (CD) and ulcerative colitis (UC) [1-4]. In fact, IBD is an immunologic disturbance that both dysregulated innate and adaptive immune pathways that play an essential role in the disease pathogenesis [5, 6]. IBD is demonstrated in persons with genetic predisposition, but its etiology remains unclear [7].

On the other hand, Celiac disease (CeD) is an autoimmune-mediated disease of the upper small intestine, triggered by the ingestion of gluten-containing grains (including wheat,

rye, and barley) in genetically predisposed persons, that results in small intestinal injury [8,9]. It has been suggested that in CeD, like many autoimmune disorders, the complex interaction between innate and adaptive immunity is the main cause of the disease progression [10-13].

Various studies indicated that IBD and CeD have overlapped, and the coexistence of these two disorders in the same patients has already been described [14-16].

Nuclear factor-kappa B (NF-κB) is an inducible transcription factor that plays a relevant role in modulating the immune and inflammatory responses in the intestinal mucosa [17,18]. Increased NF-κB signaling leads to the development of chronic inflammatory disorders [17,19-22]. Various studies have shown that NF-κB has an increased and persistent expression level in IBD and CeD patients and plays a significant role in the pathogenesis process of these two disorders [19,20,23-28].

Long noncoding RNA 13 (lnc13) and NF-κB-interacting lncRNA (NKILA) are long noncoding RNAs (lncRNAs) that interact with NF-κB and can play a role in inflammatory disorders [29-32].

Lnc13 can maintain intestinal immune homeostasis and keep inflammation down under basal conditions [30]. In response to inflammatory stimuli and after NF-κB activation, Decapping enzyme 2 (DCP2) can degrade lnc13, which leads to dysregulation of lnc13 expression or function [29,30].

Moreover, NKILA is another lncRNA, which is upregulated by NF-κB and found to confine NF-κB-driven inflammation by prevention of the translocation of NF-κB to the nucleus and gene expression. Therefore, it can inhibit inflammatory signaling in this way [31,33].

We hypothesized that lnc13 and NKILA could be used as biomarkers for IBD and CeD assessment and conducted this study to investigate their expression in peripheral blood and intestinal biopsy samples of diagnosed IBD and CeD patients compared to healthy controls by real-time PCR method.

2. Materials and Methods

2.1. Study subjects.

A total of 96 patients had CeD (n=50) and IBD (CD=16, UC=30) and were admitted by the Research Institute for Gastroenterology and Liver Diseases at the Taleghani Hospital (Tehran, Iran) during 2019 were enrolled in this study. Twenty healthy individuals without any clinical evidence of autoimmune disorders were also selected as a control group.

Each CeD patient was untreated and had not received a gluten-free diet before participating in our study. Also, newly IBD diagnosed patients who had not received any treatment participated in this study.

Patients with complete medical records and pathological diagnosis of the disease (endoscopy and duodenal biopsy compatible with serological results for CeD and colonoscopy and biopsy specimens compatible with clinical findings for IBD) and willingness to participate in the research were included in this study. In addition, patients who had other autoimmune diseases, those who used any prohibited medications, and those who failed to cooperate with researchers were excluded.

Healthy individuals who had no current or prior history of gastrointestinal disorders, females who were not pregnant, with willingness of participating enrolled in our study.

This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. As well, this study was approved by the Ethics Committee of the

Research Institute for Gastroenterology and Liver Diseases (RIGLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RIGLD.REC.1395.3). All participants signed an informed written consent form.

Basic information of studied groups is mentioned in Table 1.

Table 1. Basic information of study groups.

variable		IBD		Celiac	Control
		(Blood/Tissue)		(Blood/Tissue)	(Blood/Tissue)
$Age(mean \pm SD)$		UC	33.10±14.63	37.84±13.960	27.95± 4.211
		CD	33.90±12.84		
BMI$(mean \pm SD)$		UC	21.74±14.63	24.453±9.442	25.008±4.859
		CD	24.02±12.84		
Gender	Male	UC	15(68.18%)	19(38.0%)	5(25.0%)
		CD	7(31.81%)		
	Female	UC	15(62.5%)	31(62.0%)	15(75.0%)
		CD	9(37.5%)		

2.2. Sample collection.

10 ml peripheral blood samples and mucosal biopsy specimens (duodenal biopsy for CeD and colon biopsy for IBD assessment) were collected from studied groups.

2.3. RNA Extraction & cDNA synthesis.

In brief, total RNA was extracted from PBMC and biopsy samples by Qiagen RNA extraction kit (AllPrep DNA/RNA/miRNA Universal Kit) according to the manufacturer's protocol. The concentration and purity of extracted RNA were determined by Nanodrop spectrophotometer (Thermo Fisher Scientific). RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was also used for reverse transcription.

2.4. Quantitative real-time PCR (RT-qPCR).

RT-qPCR was performed on a Rotor-Gene Q real-time PCR system (QIAGEN) using SYBR Green Real-time PCR Master Mix (Takara) kit instruction. Expression of lnc13 and NKILA was normalized to β2-Microglobulin (B2M) according to the $2^{-\Delta\Delta Ct}$ method. The gene-specific primers sequences are shown in Table 2.

Table 2. Primers used in qPCR.

Gene Symbol	Primer Sequence	Length	GC(%)	Product Length	TM	Ref
NF-κB interacting lncRNA (NKILA)	F:5'-TGATGATTCCAGCACAGACAG-3' R:5'-CACACACGAAGCCTCCTATG -3	21 20	47.62 55.00	108bp	58.02 58.35	-
LncRNA13	F:5'-CTTTGGCACCAAGCAACATC-3' R:5'-TTCACTGAGACCCTGCAATG-3	20 20	50.00 50.00	123bp	58.21 57.81	-
B2M	F:5'- TGCTGTCTCCATGTTTGATGTATCT -3' R:5'- TCTCTGCTCCCCACCTCTAAGT -3'	25 22	40.00 54.55	86bp	60.34 61.98	[34]

2.5. Statistical analysis.

Statistical analysis was carried out using SPSS (V.21) and GraphPad, Prism (V.6) software. Comparisons between the groups were examined by t-TEST and ANOVA. P-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Evaluation of NKILA mRNA expression.

We measured the expression of NKILA in the duodenal mucosa and PBMC of CeD patients compared to the control group using RT-qPCR. Figure 1A shows that the expression of NKILA was significantly upregulated in PBMC of CeD patients relative to controls ($p < 0.0001$). On the other hand, as illustrated in Figure 1B, duodenal NKILA lncRNA was significantly down-regulated in CeD patients than in healthy controls ($p < 0.0001$).

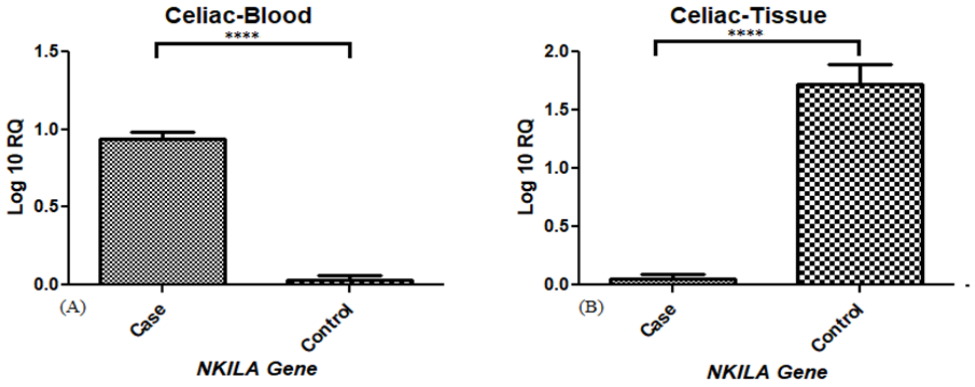

Figure 1. NKILA gene expression analysis in CeD patients compared to the control group. (**A**) mRNA expression in PBMC; (**B**) mRNA expression in duodenal tissue.

In IBD group, PBMC levels of NKILA lncRNA were significantly higher compared to controls (p=0.0174) (Figure 2A). In fact, this significant up-regulation was seen in both UC and CD patients compared to healthy controls ($p < 0.0001$ for both of them). However, this difference was not significant when we compared the NKILA expression between UC and CD patients ($p > 0.05$) (Figure 2B). In addition, NKILA gene expression has a significant up-regulation in the colonic mucosal specimens of IBD patients relative to controls (p=0.004) (Figure 2C). Between IBD patient's groups, UC patients showed significant up-regulation of colonic NKILA gene expression compared to CD patients and controls (p=0.002 and p=0.0111 respectively) (Figure 2D).

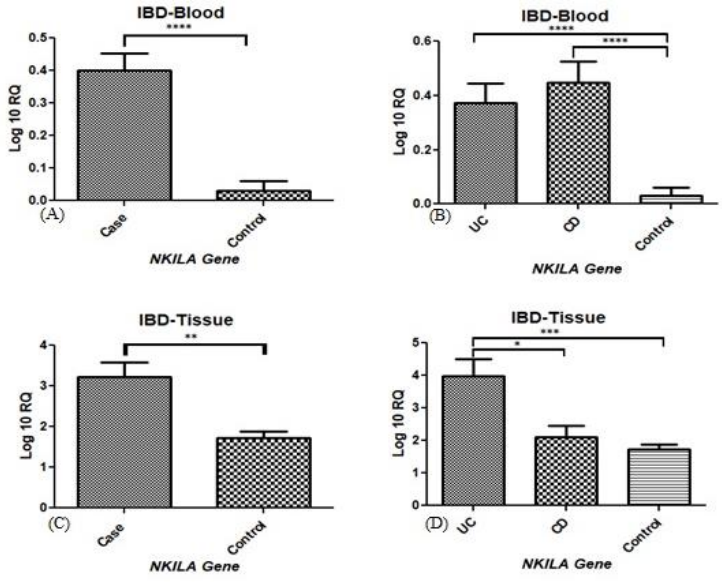

Figure 2. NKILA gene expression analysis in IBD patients compared to the control group. (**A**) and (**B**) mRNA expression in PBMC; (**C**) and (**D**) mRNA expression in colonic tissue.

3.2. Evaluation of lnc13 mRNA expression.

Regarding the mRNA expression of lnc13, the current result revealed that lnc13 was significantly overexpressed in the PBMC of CeD patients compared with healthy controls ($p < 0.0001$) (Figure 3A). In contrast, its expression was significantly lower in duodenal biopsy samples of CeD patients compared with controls ($p < 0.0001$) (Figure 3B).

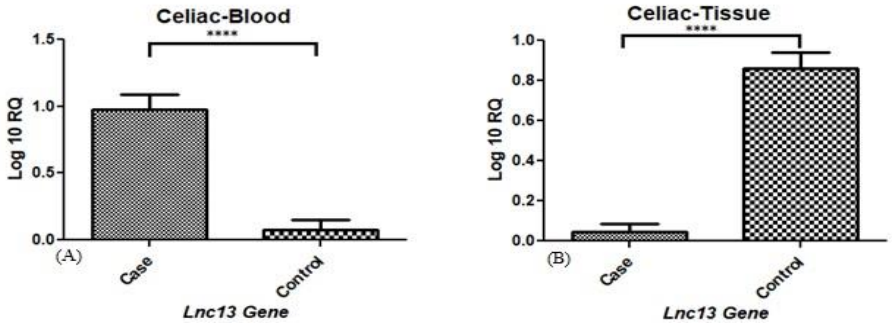

Figure 3. Lnc13 gene expression analysis in CeD patients compared to the control group. (**A**) mRNA expression in PBMC; (**B**) mRNA expression in duodenal tissue.

By comparing relative lnc13 mRNA levels of IBD patients versus healthy controls, we found that lnc13 was significantly upregulated in patients' PBMC specimens ($p < 0.0001$) (Figure 4A). High expression was also observed in both UC and CD patients compared to the control group ($p < 0.0001$ for both of them). Nevertheless, there was not any significant difference between the CD and UC groups ($p > 0.05$) (Figure 4B).

In addition, IBD patients' colonic lnc13 expression was higher than controls; however, this difference was non-significant ($p > 0.05$) (Figure 4C). On the other hand, among the IBD group, CD patients had significantly higher colonic expression levels of lnc13 compared to both UC patients and healthy controls ($p = 0.0075$ and $p = 0.002$, respectively) (Figure 4D).

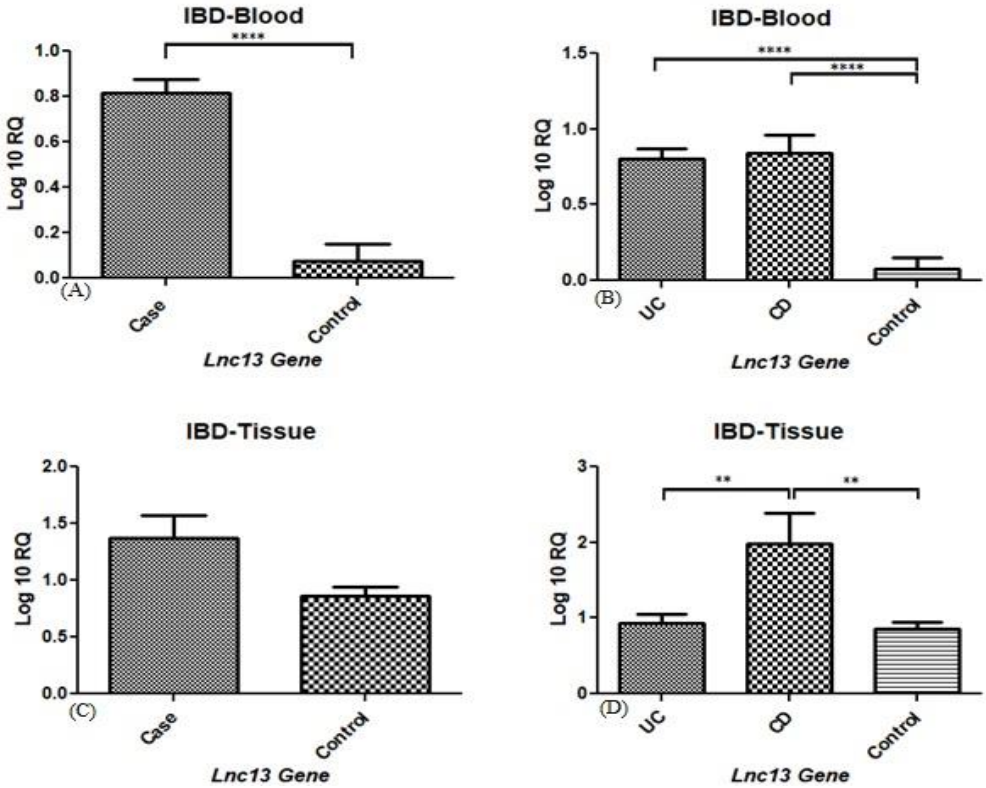

Figure 4. Lnc13 gene expression analysis in IBD patients compared to control group. (**A**) and (**B**) mRNA expression in PBMC; (**C**) and (**D**) mRNA expression in colonic tissue.

A comparison of the expression of these two lncRNAs between patients with CeD and IBD showed that NKILA mRNA expression was significantly down-regulated in the tissue of CeD compared to IBD patients ($p < 0.0001$) (Figure 5A). On the other hand, this mRNA expression was significantly upregulated in the PBMC of CeD patients relative to IBD subjects ($p < 0.0001$) (Figure 5B).

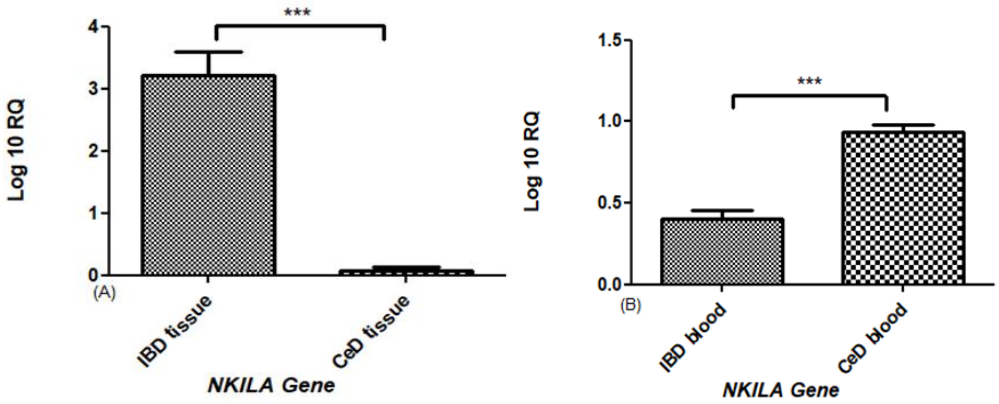

Figure 5. NKILA gene expression comparison between IBD and CeD patients. (**A**) mRNA expression in tissue samples; (**B**) mRNA expression in PBMC.

In addition, a significantly lower intestinal level of lnc13 was observed in the CeD patients' group than in the IBD patients ($p < 0.0001$) (Figure 6A). However, despite a higher expression of lnc13 in CeD patients' PBMC, the difference was non-significant ($p > 0.05$) (Figure 6B).

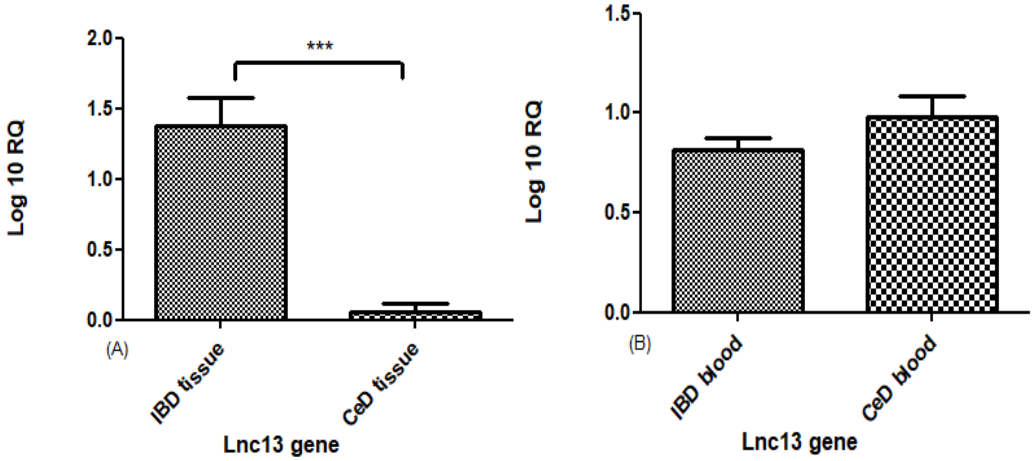

Figure 6. Lnc13 gene expression comparison between IBD and CeD patients. (**A**) mRNA expression in tissue samples; (**B**) mRNA expression in PBMC.

As far as we know, this is the first study to investigate the expression profile of lnc13 and NKILA long noncoding RNAs in diagnosed CeD and IBD patients in comparison to controls. The basis of selecting these two lncRNAs for the current study was their association with the NF-κB transcription factor [30,31,33].

NF-κB plays an important role in modulating inflammatory responses in the intestinal mucosa, and its increased and persistent signaling leads to the CeD and IBD development [17,19,20,23-27,35,36]. Lnc13 is an immune homeostasis mediator that increased activation of NF-κB leads to degradation and function dysregulation of this lncRNA [29-31]. Moreover, NKILA is a suppressor of NF-κB-driven inflammation, which NF-κB upregulates, and its degradation results in increased NF-κB expression and further Lnc13 suppression [31,33,37-39].

The results of this study showed that the expression of both lnc13 and NKILA long noncoding RNAs significantly increased in the PBMC of CeD ($p < 0.0001$ for both of them) and IBD ($p < 0.0001$ and $p=0.0174$, respectively) patients than controls and, also this significant overexpression was seen in both CD and UC patients ($p < 0.0001$ for both of them). It is noteworthy that the expression of NKILA in CeD patients' PBMC was significantly higher than in IBD patients ($p < 0.0001$).

We also found that these two lncRNAs had a significantly lower expression in celiac disease patients' intestinal tissue samples than IBD patients and control subjects ($p < 0.0001$). This finding is consistent with the result of the study by Castellanos-Rubio and his colleagues [30] in 2016, which reported a considerable down-regulation of lnc13 in the CeD patient's small bowel biopsy samples compared to that in healthy controls. To date, the study of Castellanos-Rubio *et al*. [30] is the only investigation that has explored the alteration of lnc13 RNA expression between celiac disease patients and the healthy group. However, they did not investigate the lnc13 expression in the blood [30].

On the other hand, of our two targeted lncRNAs, only the NKILA had significantly increased expression in intestinal tissue specimens of IBD patients compared to the healthy controls ($p=0.004$).

A key original finding from the present study is that, in UC, NKILA, and CD, lnc13 colonic expression was significantly increased compared to the other studied groups ($P<0.05$).

Decreased expression of lnc13 in the duodenal specimens of celiac disease patients can be justified through its degradation by DCP2 enzyme in the pathogenesis process as Castellanos-Rubio *et al*. [30] have pointed out in their study that the expression of DCP2 was increased in CeD patient's biopsy samples compared to controls and DCP2 expression had a converse correlation with the level of lnc13 [29,30]. Since lnc13 keeps inflammation down under basal conditions, this decrease in lnc13 level can lead to the progression of inflammation in CeD patients [30]. On the other hand, lnc13 increased expression in CD patients may be a sign of its effort to suppress inflammation.

Furthermore, studies have shown that intestinal fibrosis is an insoluble and undeniable problem caused by chronic inflammation in IBD patients. Since the TGF-β signaling pathway via Smads has a central role in IBD intestinal fibrosis progression and NKILA can be directly activated by this signaling pathway, it can be concluded that colonic overexpression of NKILA in IBD patients may be a sign of intestinal fibrosis formation in studied patients [40,41].

Studies have also shown that NKILA can function as a tumor suppressor in various cancer types by inhibiting tumor cell proliferation and migration. In fact, NKILA overexpression can inhibit cancer growth, and it is usually downregulated during tumor progression [42-44]. IBD patients are at a significantly increased risk of colorectal cancer (CRC), which is the important cause of global cancer-associated mortality [45,46]. Jian Huang and colleagues [44] reported reduced NKILA expression in patients with colon cancer through the suppressed activity of the PI3K/Akt pathway. Therefore, it can be concluded that colonic overexpression of NKILA in IBD patients, especially in UC group, can be due to its attempt to suppress the growth of colon cancer in these patients.

On the other hand, as mentioned earlier, NKILA is involved in the suppression of NF-κB-induced inflammation, which has been reported to be increased during CeD pathogenesis [19]. Therefore, it can be supposed that a reason for increased NF-κB-triggered inflammation in CeD patients could be a decrease in the NKILA intestinal expression level.

Moreover, it has been reported that lncRNAs are expressed in many different human cell lines and have a PBMC overexpression in diverse autoimmune disorders. For instance, Song *et al*. [47] reported increased PBMC expression of HOTAIR (HOX transcript antisense RNA) lncRNA in patients with rheumatoid arthritis than healthy controls. Likewise, NEAT1 (Nuclear enriched abundant transcript 1) lncRNA upregulation was reported in PBMCs of systemic lupus erythematosus patients compared to healthy subjects by Zhang *et al*. [48]. As a result, it can be considered that NKILA and lnc13 are two lncRNAs that have overexpression in CeD and IBD patients PBMC.

4. Conclusions

In conclusion, our data corroborate and further expand the Rubio *et al*. [30] reports of lnc13 duodenal expression in CeD patients and also provides important and novel basic knowledge in this context. However, there are still important ambiguous points that need to be evaluated. Given the variations in the expression of these lncRNAs between the two groups of patients, it has been hypothesized that these lncRNAs may emerge as novel biomarkers to be evaluated in the IBD and CeD patients' intestinal tissue and PBMC samples for differential diagnostic targets. Accordingly, we considered a hypothetical diagnostic chart for differentiating these diseases, which is shown in Figure 7. Based on this diagnostic chart, it can be considered that increased peripheral expression of lnc13 and NKILA, along with decreased duodenal expression of them, demonstrates celiac disease. On the other hand, if the increased peripheral expression of lnc13 and NKILA is associated with increased lnc13 colonic expression, it indicates Crohn's disease. If it is associated with increased NKILA colonic expression, it indicates ulcerative colitis. But its validation and extension require further studies with the same results.

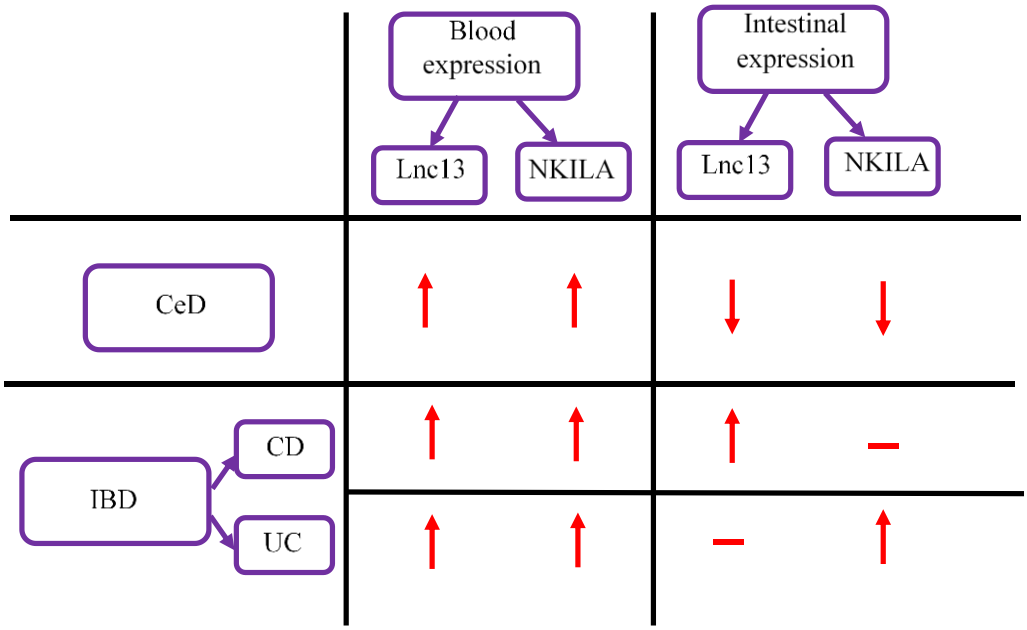

Figure 7. Hypothetical diagnostic chart for differentiating IBD and CeD.

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Conflicts of Interest

The authors declare no conflict of interest.

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