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Original Article

Quantitative Evaluation of NOD2 Promoter Methylation Profiling in Colon Biopsy Samples from Ulcerative Colitis Patients and Non-Colitis Controls

Golshid Sanati[1], Mehrdad Noruzinia[2], Davood Jafari[3], Mohammad Ahmadvand[2], Shahram Teimourian[4], Naser Ebrahimi Daryani[5], Sara Hanaei[6,7], Nima Rezaei[1,6,7*]

[1] *Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[2] *Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[3] *Department of Immunology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran*
[4] *Department of Genetics, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*
[5] *Department of Internal Medicine, Division of Gastroenterology, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran*
[6] *Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran*
[7] *Network of Immunity in Infection, Malignancy and Autoimmunity (NIIMA), Universal Scientific Education and Research Network (USERN), Tehran, Iran*

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Abstract

Background: Ulcerative colitis (UC) is an idiopathic chronic inflammatory disease of the colon with evidence addressing the role of epigenetic changes. The intention of this study was to detect the association of DNA methylation levels of NOD2 gene with UC and to evaluate whether any of these changes might be a useful biomarker for detecting patients with UC.

Methods: The methylation status of the promoter CpG islands (CGIs) of NOD2 gene was examined in the colonic mucosae of 15 cancer-free patients with UC and 15 age- and sex-matched healthy controls by the real-time quantitative multiplex-methylation specific PCR (QM-MSP) assay. Methylation-specific melting curve analysis (MS-MCA) was used to analyze the data.

Results: The median unmethylated DNA index was significantly higher in cases compared to controls, and hypormethylation of NOD2 gene was significantly correlated with UC (Unmethylated DNA in UC vs. HC; 0.102±0.055 vs. 0.025±0.016, $P = 0.000$).

Conclusion: The NOD2 gene that was differentially methylated in UC patients, provides new insights into the pathogenesis of UC, with a view to making steps toward the development of accurate biomarkers for diagnostic tools in UC.

Keywords: DNA Methylation; Ulcerative Colitis; Epigenetics; NOD2; Biomarker

***Corresponding Author:** Nima Rezaei, MD, PhD
Children's Medical Center Hospital, Dr. Gharib St, Keshavarz Blvd, Tehran, Iran
E-mail: rezaei_nima@tums.ac.ir

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Introduction

Inflammatory Bowel Disease (IBD) represents a chronic relapsing and remitting disorder of the gastrointestinal (GI) tract which encompasses two broad clinical subtypes of the disease, ulcerative colitis (UC) and Crohn's disease (CD) (1-3). Histopathologic examination of specimen from CD typically exhibits a non-caseating granulomatous inflammation complicated by fibrostenotic lesions, abscess formation, and fistulas, however, classical UC has a diffuse, continuous, and ulcerating inflammation localized mostly to the bowel (1, 2, 4, 5). The precise etiology of UC is poorly understood, but evidence suggests that several immunological, genetic, and environmental factors contribute to the aberrant innate immune response to the natural flora of the gut, resulting in characteristic inflammatory lesions of the GI tract (4, 6, 7).

There is ample evidence for the genetic influence on the pathophysiology of IBD (8). Many UC candidate loci have been identified through the assumption-free approach of genome-wide association study (GWAS) and subsequent meta-analyses, the majority of them are related to the immune-inflammatory process (7, 9). The concordance rate of UC in monozygotic twins stands at 6–18% compared to 5% for dizygotic twins, highlighting a role for genetic background, however, identified these loci explain only a modest fraction of overall disease variance (7). This evidence indicates the need for better investigation into immunological factors and gene-environment interactions and a putative role for epigenetics (10).

By definition, epigenetics is an umbrella term covering several different mechanisms which are defined as heritable changes in gene expression, without alterations in DNA sequence (7, 11, 12). Of major components involved in the epigenetic mechanism, DNA methylation is the most stable and best-studied epigenetic modification which appears as modifications of CpG dinucleotides through the addition of methyl groups at the 5th position of cytosine (5mC) to alter gene expression (13, 14).

The hope is that epigenetic modifications might be able to explain some aspects of "hidden heritability" in the context of UC development and further our understanding to improve treatments (7, 10). DNA methylation levels have been averted to differ between inflamed and healthy (non-inflamed) controls, suggesting some of the many potential links between epigenetic processes and a higher risk of developing IBD, and UC in particular (15). Many differentially methylated genes have been identified in UC that are involved in a wide range of immune functions, notably those related to immune responses to microbial antigens (7).

Nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) or caspase recruitment domain-containing protein 15 (*CARD15*) gene codes for a protein which is an intracellular pattern recognition receptor that senses the presence of bacterial muramyl dipeptide (MDP) motifs (16-18).

It has been demonstrated that GI tract involvement in active IBD is associated with altered *NOD2* gene expression in both macrophages and intestinal epithelial cells (19). We hypothesized that the epigenetic effect on *NOD2* gene expression can advance our knowledge of the pathophysiology of UC and account for its phenotypical variability. Furthermore, aberrant DNA promoter methylation has previously been introduced to the suitability of epigenetic biomarkers in the early detection of UC disease (7). The present case-control study was designed to detect whether the differences in DNA methylation levels of the *NOD2* gene, between inflamed and non-inflamed mucosa were associated with increased risk of UC and to evaluate whether any of these changes might be a useful biomarker for detecting UC patients.

Materials And Methods

Colon Biopsy Sample Selection

We included fifteen patients with ulcerative colitis (8 females, 7 males) in the study, who were diagnosed according to standard clinical, laboratory, endoscopic, and pathologic findings (20). We excluded patients who were receiving biological treatment and also patients with other immune-mediated diseases. In this retrospective study, all colorectal mucosa specimens analyzed were obtained from colonoscopy mucosal resection-on at the gastroenterology clinics of Kasra and Laleh hospitals in Tehran, Iran, between May 2014 and July 2015. Tissue collection was obtained directly from areas around the disease lesions and immediately stored at -80 C. Histopathological examination was performed based on H&E stained sections blinded to all clinical data.

Studies have shown that DNA methylation pattern near promoter regions for genes differs noticeably depending on cell type (15), thus to investigate the difference in methylation status between inflamed and non-inflamed mucosa,

only DNA sampled from colonic mucosa was included.

Furthermore, a group of fifteen age- and sex-matched healthy, unrelated subjects (8 females, 7 males) were selected as controls. The tissue collection and protocol of this study were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran. Informed consent was obtained from each participant.

DNA Isolation and DNA Conversion system

DNA was extracted directly from fresh-frozen tissue samples using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions, checked by absorbance and gel electrophoresis, and stored at −20 °C until analysis. In all cases, DNA was chemically modified to convert all unmethylated cytosine to uracil by the commercially available MethylE-dge™ Bisulfite Conversion System (Promega, Madison, WI) according to the manufacturer's protocol.

Real-Time Quantitative methylation-specific polymerase chain reaction (qMSP)

We characterized the methylation status of the inflammatory colonic mucosa in UC using a SYBR green dye-based DNA methylation assay known as the real-time quantitative multiplex methylation-specific PCR (QM-MSP) method (21). The MethySYBR procedure consists of two sequential steps of PCR reactions. Step 1 (pre-amplification or multiplex step), was carried out to enable the simultaneous amplification of many discrete target alleles in a single reaction with MethySYBR primers including external forward primer (EXT-F;5'-GGGGTTTTTATTTATTTGTGG-3′) and external reverse primer (EXT-R; 5'-CCAAAATTAACCAACCAACC-3'). The PCR reaction was performed in a volume of 25 µl containing 1 µl of converted genomic DNA. The thermal cycling conditions included an initial denaturing step at 95C for 5 min, followed by 30 cycles at 94C for 30 s, 56C for 30 s, and 72C for 30 s, with a final extension at 72C for 5 min.

In the 2nd round, the specific methylated target was quantified from multiplex step products using both nested methylation-independent and methylation-specific primer sets including nested methylation-specific forward(FM;5'-TTATTTATTTGTGGTTTGTTTTGTC -3') and reverse primer (RM; 5'- ACCAACCTTCCAAAACTAAACA -3').

The methylation profile of the promoter CpG islands was defined based on the UCSC database. Methylation-specific primers were adopted from the group of Li *et al.* (22). Also, CpG island prediction and primer blasting were performed using the MethBlast tool. The qMSP was carried out using a 10 µl reaction volume including 0.25 µl each of forward and reverse primers, 1 µl bisulfite-treated template (tissue samples), 3.5 µl DDW, and 5 µl SYBR® Green Master Mix. Fragments were amplified at 95°C for 1 min, then 30 cycles of 94°C for 30 sec, 60°C for 1 min, followed by 30 sec at 72°C using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

The median value was used for data analysis. No unmodified template controls were included in each run as negative controls. Additionally, bisulfite-converted completely methylated human plasmid DNA served as a positive control for the qMSP reaction.

The ratio of unmethylated to total amplifiable bisulfite-treated DNA was determined using the $\Delta\Delta C_q$ method. Also, the cycle of quantification (C_q) was calculated for each reaction with methylation-specific primers (MSP) and bisulfite-specific primers (BSP) (23, 24). Using a reference sample for standardization indicates the relative difference between the template of interest and a control template:

$$\text{Unmethylated DNA level} = 2^{-\Delta\Delta CT}$$
$$\Delta\Delta C_q = \Delta C_{q\ \text{sample}} - \Delta C_{q\ \text{plasmid}}$$
$$\Delta C_{q\ \text{sample}} = C_{q\ \text{MCP}} - C_{q\ \text{BSP}}$$
$$\Delta C_{q\ \text{plasmid}} = C_{q\ \text{MCP}} - C_{q\ \text{BSP}}$$

To determine the relative levels of methylated promoter DNA in each sample, the cycle threshold (CT) values of the gene of interest were compared with the values of the internal reference gene to obtain a ratio. The fold change in target gene samples, after normalization with the expression of PCR products amplified by external nested primers as an internal control (BSP), was calculated using the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = \Delta CT$ (samples) - ΔCT (controls) and ΔCT was calculated by transforming the difference in CT values of target gene vs. the BSP products.

Statistical analysis

The non-parametric Mann-Whitney U-test was used to examine the potential statistical significance in CpG island methylation levels between colon mucosa of patients with UC and healthy controls. A Receiver-Operating Characteristic (ROC) curve analysis was performed to determine the optimal methylation level cutoff value for discriminating patients with UC from healthy individuals. The area under the ROC (AUC) as well as the best cutoff to evaluate the performance

of specific patterns of DNA methylation at CG dinucleotides (CpGs) as a biomarker were calculated. *P* values less than 0.05 were considered statistically significant. For statistical analyses, SPSS 21.0 (SPSS, Chicago, IL, USA) was used.

Results

There have been several studies in the literature reporting pattern of methylation of some genes is probably modulated by both sex (25) and age (26, 27), therefore, sex- and age-matched healthy controls were included. Fifteen UC patients, with a mean age of 45±15.42 years (range: 25–82 years) were enrolled in this prospective study and included 7 (46.7%) males and 8 (53.3%) females. Furthermore, fifteen were healthy controls (8 females, 7 males), with a mean age of 46±15.38 years (range: 28–78 years).

Melting curve analysis of target amplicons using SYBR green dye during quantitative PCR in methylation studies allows the determination of whether the PCR products originate from methylated or unmethylated templates. The resulting melting curve for the *NOD2* gene is shown in **Figure 1.**

Methylation assay data profiling evidence points to differential promoter methylation status between patients with active UC and healthy controls (**Figure 2**) in which the median methylated DNA levels of the *NOD2* gene were significantly lower in the mucosa of UC compared to the mucosa of healthy controls (Unmethylated DNA in UC vs. HC; 0.102±0.055 vs. 0.025±0.016) and CpG hypermethylation of promoter region significantly correlates with active UC ($P = 0.000$). Evaluation of the performance and the utility of biomarkers is often evaluated by generating a receiver operating characteristic (ROC) curve. For epigenetic biomarkers, ROC curves will be generated based on methylation values. The result of ROC analysis showed that the differences in DNA methylation levels of the *NOD2* gene, between the two groups allowed optimal detection of UC with 93.5% sensitivity and 93.2% specificity (CI: 0.9-1.00, *P*-value = 0.000). The area under the curve (AUC), an indicator of marker performance, was 0.962 for the *NOD2* gene (**Figure 3**).

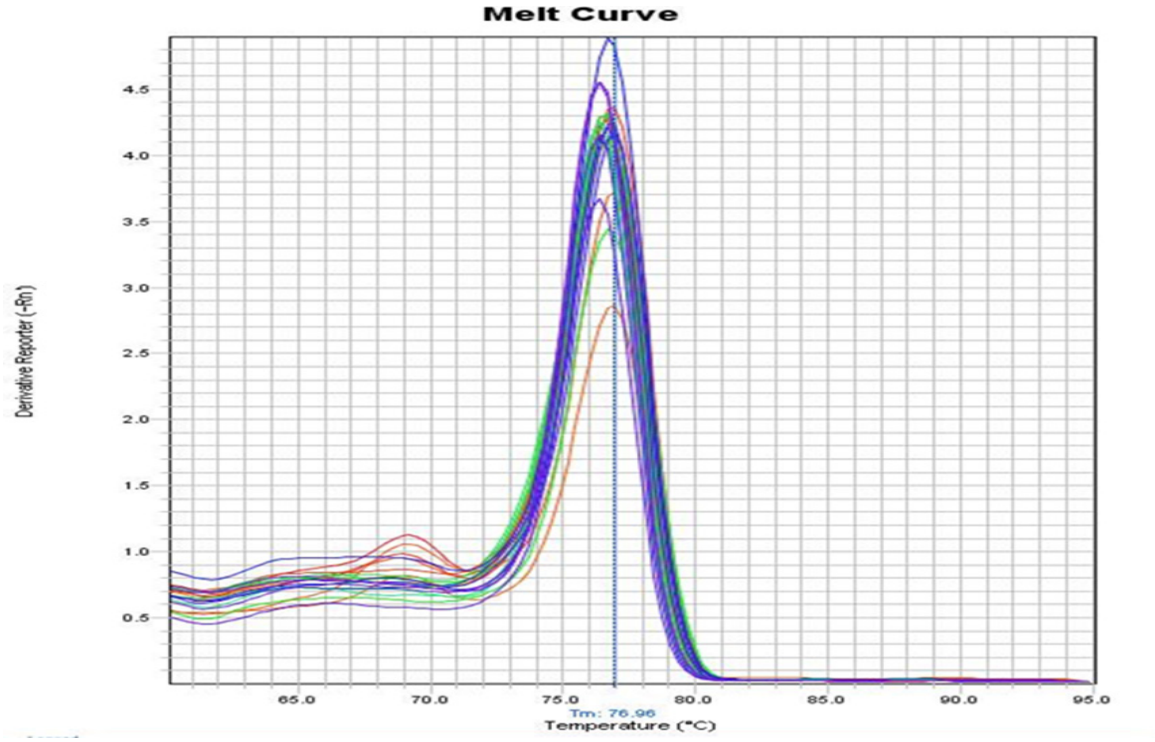

Figure 1. Fluorescence melting peak analysis for the NOD2 gene promoter showing changes in CpG. DNA melt curve analysis of changes in CpG methylation status was examined based on the amplification of unmethylated chemically modified DNA from colorectal mucosal biopsies of UC cases.

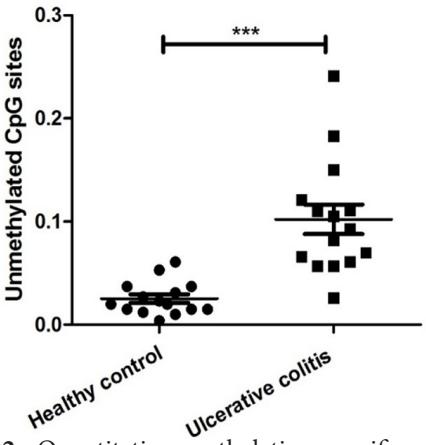

Figure 2. Quantitative methylation-specific polymerase chain reaction data of the NOD2 gene promoter region is shown. Comparison of changes in the methylation pattern of CpG islands (CGIs) in the promoter region of NOD2 gene in colonic mucosa specimens of UC patients (n=15) and paired healthy controls (n=15). P-value was obtained via the Mann-Whitney U-test. Error bars mean ± SD ($^*P < 0.05$).

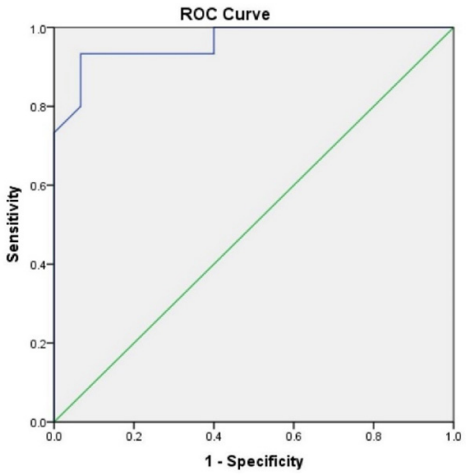

Figure 3. Receiver operating characteristic (ROC) curve analysis. Evaluation of the performance of the unmethylation value of CpG islands (CGIs) in the promoter region of the NOD2 gene as epigenetic biomarkers using ROC curve analysis in which sensitivity versus 1-specificity at all possible cutoff values is plotted.

Discussion

The etiology of UC is poorly understood but is thought that a combination of genetic background, abnormal immune interaction with the natural flora of the gut, and environmental triggers play a role in the development of UC (7). Recent advances in genome-wide DNA methylation analyses of the colonic tissues of patients with UC have demonstrated widespread epigenetic drifting, particularly DNA methylation (12, 28). In recent years, there has been an increasing amount of literature on the association between epigenetic changes and IBD states, including UC (7). Indeed, according to the current literature, epigenetic alterations are considered as promising candidates for explaining processes of pathophysiology and development of disease beyond the identified risk loci (15).

DNA methylation status has been shown to differ between UC patient tissue samples and healthy groups, making UC itself an environment in which epigenetic changes may occur within the course of the disease (7). The first evidence that DNA methylation is related to UC pathogenesis was reported in 1996 by Gloria et al, who found a significantly higher degree of intrinsic DNA hypomethylation in patients with histologically active disease compared with those with resolving or inactive colitis (29). Recent systematic reviews report several genes implicated in either innate or adaptive immune response were found to be differentially methylated in UC patients compared to control samples (7, 15).

In the present study, to define the effect of the differences in DNA methylation levels, between UC patients and healthy control group, we looked for a measure of *NOD2*. The identification of missense and frameshift mutations in the *NOD2* gene in patients with Crohn's disease was a significant development in our understanding of the pathogenesis of inflammatory bowel diseases (18, 30). However, there are several controversial data about the role of the variations of the *NOD2* gene in the pathogenesis of UC.

It has been described that GI track involvement in active IBD is associated with increased *NOD2* gene expression in both intestinal epithelial cells and macrophages. There were more NOD2-expressing cells in lesions of IBD disease than in unaffected areas (19). We hypothesized that epigenetic impact on gene expression could improve our understanding of the pathophysiology behind UC and account for its phenotypical variability. We showed a decreased level of the methylation status of the *NOD2* gene between inflamed UC colon biopsies and healthy colon. The difference in methylation status between UC mucosa and healthy mucosa could implicate hypermethylation in the pathophysiology of UC.

Hypermethylation is a term referring to the addition of methyl groups in the promoter region of a gene which is associated with silencing or inactivation of that gene. The opposite methylation change, hypermethylation defines as lower levels of methylation in the promoter region of a gene

which makes it transcriptionally more active (15). The result is in the lines of earlier literature (19) that found the CpG hypermethylation at the promoter region of the *NOD2* gene might be associated with increased *NOD2* gene expression and subsequent GI track involvement.

Whether DNA methylation changes are the cause or a response to pathological processes, from a clinical perspective, IBD-associated DNA methylation is the most promising epigenetic mechanism as a putative biomarker for the diagnosis of disease (7, 15). It has been postulated that genetic methylation status could be considered a surrogate biomarker for the diagnosis of UC. Our findings suggest UC-specific DNA methylation patterns for the promoter region of *NOD2* in epithelial colorectal cells to be suitable epigenetic biomarkers with high sensitivity and specificity for UC diagnosis.

In conclusion, we have shown a decreased level of methylation of the *NOD2* gene in inflamed UC colon biopsies compared to a healthy colon. Although the etiological role of methylation remains controversial, we proposed data for differential methylation alterations in the *NOD2* gene involved in the inflammatory process in UC. The evidence from this study suggests that DNA methylation patterns for the promoter region of *NOD2* could be suitable epigenetic biomarkers with high sensitivity and specificity for UC diagnosis. This study provided new sight into the methylation status of the *NOD2* gene that is differentially methylated in UC patients, as well as a view to taking steps toward the development of accurate biomarkers for diagnostic tools in UC.

Conflict of interest

The authors declare that they have no conflict of interest.

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