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Original Article

Abnormal Promoter Methylation of Nucleotide-Binding Oligomerization Domain Containing 2 (*NOD2*) Gene in the Pathogenesis of Crohn's Disease

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Abstract

Background: Changes in the expression of nucleotide-binding oligomerization domain containing 2 (*NOD2*) play an important role in the pathogenesis of a variety of autoimmune diseases including inflammatory bowel diseases (IBDs). Epigenetic modifications, including DNA methylation, are considered an important mechanism in the suppression of gene activity. In this study, we investigated the relationship between DNA methylation patterns of the promoter region of the *NOD2* gene and the pathogenesis of Crohn's disease (CD).

Methods: Colonic mucosa samples were obtained from 15 Iranian patients with IBD and 15 age- and sex-matched healthy controls with no history of autoimmune disease. After the bisulfite conversion of genomic DNA, the DNA methylation status of three CpG sites in the promoter region of the *NOD2* gene was determined by the real-time quantitative multiplex methylation-specific PCR (QM-MSP) assay.

Results: Using this approach, we identified that IBD patients showed a decreased level of methylation of the *NOD2* promoter in the colonic mucosa than did the healthy controls. (Unmethylated DNA in Crohn's disease vs. healthy controls; 0.128±0.093 vs. 0.025±0.016, $P<0.000$).

Conclusion: According to our findings, promoter hypomethylation of the *NOD2* gene in the colonic mucosa might contribute to the development and severity of CD. Furthermore, aberrant DNA methylation levels are expected to serve as a clinically useful risk marker.

Keywords: NOD2; Inflammatory Bowel Disease; Crohn's Disease; DNA Methylation

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Introduction

Inflammatory bowel disease (IBD) is characterized by chronically relapsing intestinal inflammatory conditions which Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms (1-3). Understanding the underlying mechanisms that cause IBD including Crohn's disease remains a clinical challenge in gastroenterology research (4). IBD is most likely caused by a complex interplay of genetic, environmental, and epigenetic factors (2, 5-7). A considerable number of studies have been performed investigating possible genetic associations of IBD with various susceptibility genes, but to our knowledge, epigenetic aspects of these genes do not fully explain the pathogenesis of IBD (2, 6, 8).

Epigenetic changes have been current hot topic of recent reviews and investigate the DNA methylation and histone modification that modify the structure of chromatin without changing the nucleotide sequence of the DNA. It also investigates the role of short non-coding RNAs (miRNAs) molecules in RNA silencing and post-transcriptional regulation of gene expression (1, 5, 9). There is emerging evidence that epigenetic changes are important in chronic inflammation, and are considered to be a vital factor affecting the pathogenesis of disease (1, 10). DNA methylation is one of the most stable and most commonly studied epigenetic modifications and therefore DNA methylation levels could be assumed to serve as a clinically useful risk marker (1). Nevertheless, DNA methylation temporal profiling in IBD has yet to be clearly elucidated (2).

Overwhelming evidence supports the theory that dysregulated mucosal immune response toward commensal bacterial flora in genetically susceptible individuals is thought to initiate the IBDs (11). Genes involved in the innate immune handling of intracellular bacteria, such as the nucleotide-binding oligomerization domain containing 2 (*NOD2*), are considered to be Crohn's disease-specific (11, 12). The main biological action of *NOD2* (CARD15), as the first susceptibility gene for CD, is the discrimination between normal intestinal flora and enteric pathogens (1, 3). It is a pattern recognition receptor of the innate immune system that functions as an intracellular sensor for bacterial peptidoglycan, an evolutionarily conserved pathogen-associated molecular pattern (12). Continuous stimulation of NOD2 activates the signal transduction that leads to the translocation of NF-κB to the nucleus, expression of particular genes, and induction of proper innate and adaptive immune responses. The well-replicated IBD genetic association is the *NOD2* gene association with illeal CD (12-14). The association of susceptibility variants in the *NOD2* gene with IBD highlights that if this gene is not functioning appropriately, an increased risk of disease can result. Epigenetic patterns may be one crucial mechanism by which the expression of the *NOD2* gene could be regulated, and may therefore be important in IBD (1).

Although it is evident that cell-type specific gene expressions in epithelial barrier integrity are supported through genetic and epigenetic factors, the role of DNA methylation in IBD, particularly the *NOD2* gene, remains largely unknown. A high-accuracy measurement of the degree of DNA methylation is therefore fundamental to better understanding chronic inflammatory conditions such as IBD (1). Accordingly, in this study, the DNA methylation status of three CpG sites in the promoter region of the *NOD2* gene as an important mechanism in regulating the gene expression in colon mucosa of CD patients was evaluated to elucidate its association with CD development.

Materials and Methods

Patients and Intestinal Mucosal Samples

Human intestinal mucosal samples of 15 patients with CD (8 females, 7 males) were obtained following informed consent from colonoscopic mucosal resection at the gastroenterology clinics of Kasra and Laleh hospitals in Tehran, Iran, between May 2014 and July 2015. The diagnosis of CD was performed using standard clinical, radiological, endoscopic, and pathological criteria. 15 age- and sex-matched volunteers (8 females, 7 males) were healthy individuals, free of medication, and with no known personal or family history of autoimmune disease. Protocols of this study were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran.

DNA Preparation and Bisulfite Conversion System

The DNA in colonic tissue was extracted using

the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's protocol. Genomic DNA was modified by sodium bisulfite to convert unmethylated cytosines to uracil using the MethylEdge™ Bisulfite Conversion System (Promega, Madison, WI) according to the manufacturer's protocol. Bisulfite-modified DNA specimens were aliquoted and stored at -20°C.

Methylation Analysis

Distinctive methylation status at three successively located CpG sites within the *NOD2* promoter region was evaluated using a SYBR green dye-based DNA methylation assay named the real-time quantitative multiplex methylation-specific PCR (QM-MSP) method (15). Two sequential steps of PCR reactions are needed in the MethySYBR method. The multiplex step as the first pre-amplification PCR reaction was done 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 DNA was denatured 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.

For the second round of PCR, primers were specifically designed to bind to bisulfite-converted DNA of gene loci 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'). Methylated CpG islands of the *NOD2* promoter region were defined based on the UCSC database. Methylation-specific primer design for the *NOD2* gene was adopted from the group of Li *et al.* (16). For PCR-based methylation primer blasting analysis and CpG island prediction, the MethBlast tool was used. The bisulfite-treated DNA was amplified in a volume 10 µl reaction containing 5 µl SYBR® Green Master Mix, 0.25 µl of each of the methylated primers, 3.5 µl DDW, and 1 µl of bisulfite-treated DNA. The DNA was denatured at 95C for 1 min, followed by 30 cycles of 30 s at 94°C, 1 min at 60°C, 30 s at 72°C subsequently followed by 5 min at 72°C.

Fully converted methylated human plasmid DNA was used as a positive control for MSP in each run to serve as the 100% methylated reference for calculating the relative methylation percentages of DNA samples. No untreated template controls were included in each run as negative controls. Quantitative MSP was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

In other to calculate the ratio of unmethylated versus total amplifiable bisulfite-treated DNA, the ΔΔCq method was used and also the cycle of quantification (Cq) for the reaction between methylation-specific primers (MSP) and bisulfite-specific primers (BSP) was obtained (17, 18). The fold change in target gene samples, after normalization with the expression of PCR products amplified by external nested primers as internal control (BSP), was calculated using the $2^{-\Delta\Delta CT}$ method, where ΔΔCT=ΔCT (samples) - ΔCT (controls) and ΔCT was calculated by transforming the difference in CT values of target gene vs. the BSP products.

Statistical Analysis

The differences in CpG island methylation levels between the colon mucosa of patients with CD and healthy controls were calculated using the Mann-Whitney U-test. To find a relationship between two categorical scales, the differences in promoter methylation levels between the two groups were studied using the Chi-square statistic. To calculate the measure of association, the odds ratio (OR) and 95% confidence interval were considered. For all calculations, SPSS version 21.0 (SPSS, Chicago, IL, USA) was used. A two-tailed test was used for all analyses, and two-sided p-values of 0.05 were considered significant.

Results

Because methylation of some genes is probably modulated by both sex and age, sex- and age-matched healthy controls were included. The methylation status of 15 CD patients with a mean age of 46±17.2 years (range: 17–71 years), including 7 (46.7%) males and 8 (53.3%) females who underwent colectomy was assessed. Furthermore, 15 volunteers were healthy controls (8 females, 7 males), with a mean age of 46±15.38 years (range:

28–78 years).

Quantitative PCR protocol using SYBR Green reagents allows melting curve analysis of target DNA to determine the methylation status of CpG sites on the *NOD2* gene. A typical result from expected melting curves for amplified *NOD2* gene product is shown in **Figure 1**.

Analysis of methylation data showed evidence of differential promoter methylation status of the *NOD2* gene in colonic mucosa specimens of all 15 CD patients and 15 paired healthy controls. Methylation assay data profiling exhibits the mean methylation levels of the *NOD2* gene were significantly lower in mucosa of CD (Unmethylated DNA: 0.128±0.093) than in mucosa of healthy controls (Unmethylated DNA: 0.025±0.016) (P<0.000, **Figure 2**).

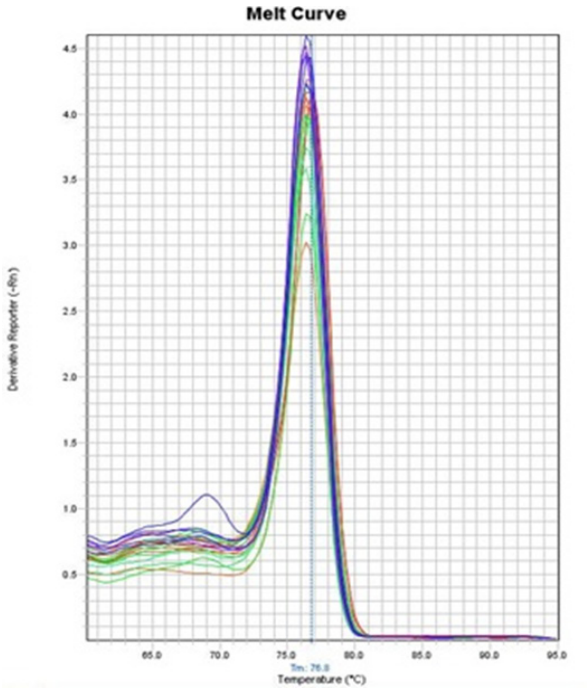

Figure 1. Fluorescence melting peak analysis for the *NOD2* gene promoter showing changes in CpG methylation status based on amplification of unmethylated bisulfite-treated DNA from colorectal biopsies of CD cases.

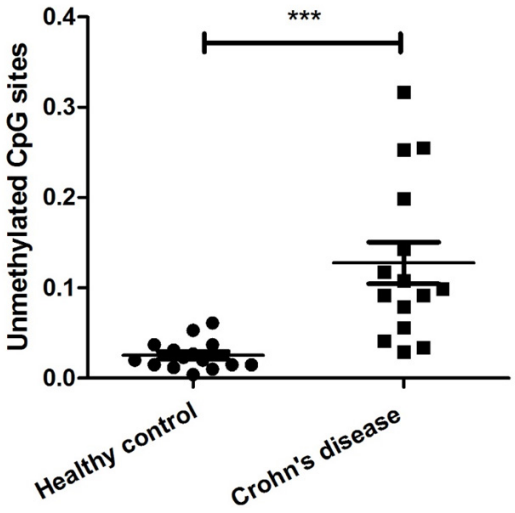

Figure 2. Comparison of the methylation patterns of CpG islands (CGIs) in the promoter region of *NOD2* gene in colorectal tissue specimens of patients with Crohn's disease (n=15) and healthy controls (n=15). *P*-value was obtained via the Mann-Whitney U-test. Error bars mean ± SD (*P < 0.05).

Discussion

Research on epigenetic changes, including DNA methylation, is emerging as a field with huge potential for developing new diagnostic and treatment pathways for human diseases (7). DNA methylation is a well-established epigenetic mechanism for the suppression of gene activity (11, 19). There is additional evidence demonstrating a strong association between chronic inflammation and altered DNA methylation events (7, 19, 20). Currently, genome-wide association studies have identified more than 32 susceptibility loci for IBD. However, all these genetic risk factors only represent approximately 20% of the disease risk suggesting that other factors, including epigenetic mechanisms, may have an important role in the pathogenesis of IBD (7, 9).

While the effects of epigenetic factors on the development of complex diseases such as type 2 diabetes, cardiovascular disease, and in the context of colon cancer have been elucidated, the gene regulation via DNA methylation in IBD is largely unknown, although DNA methylation profiles in IBD colorectal tissue specimens have recently been described (1, 2, 21).

A wide variety of recent studies, including human gene association studies and those using transgenic mice, have mainly elucidated the potential role of more than 30 genes in the development of IBD (1). Chronic intestinal inflammation associated with IBD may be a secondary consequence of innate immune deficiency or dysfunction (20). Genes involved in the innate immune handling of intracellular bacteria, such as *NOD2* have been linked with CD risk (1, 11, 20).

We reported an aberrant hypomethylation pattern of the CpG islands within promoter regions of *NOD2* gene loci in the colonic mucosa of CD patients. There is a well-established reciprocal relationship between the degree of methylated cytosine residues and the transcriptional activity of a gene (19). Gene hypomethylation of the promoter region, especially, results in increased transcript expression of genes (22).

Abnormal innate immune responses to the gut microbiota are a major cause of IBD and are thought to result from the loss of immune tolerance to the microbial flora, although limited data exist for this in CD (20). There is great interest in the finding that certain polymorphisms in the gene encoding the *NOD2* cytoplasmic innate immune sensor increase the risk for an inflammatory disease of the bowel called Crohn's disease, probably because of abnormalities in innate immunity to commensal and pathogenic organisms in the intestine (12, 23). It was also shown that the disease-associated polymorphism impairs the function of NOD2, which cannot provide optimal responses against gut microbes (12). As a consequence, these microbes are capable to pass the epithelium and trigger a chronic inflammatory process in the intestinal wall, which is the hallmark of inflammatory bowel disease (12, 20).

However, the situation is different in CD lesions. It has been reported that increased CARD15 expression was detected in CD lesions. Indeed, intestinal involvement in active CD is linked with increased CARD15 gene expression in intestinal epithelial cells. Our finding is consistent with this concept. We reported an aberrant hypomethylation pattern of the CpG islands within the promoter region of *NOD2* gene loci in the colonic mucosa of CD patients.

There is a consensus among scientists that normal NOD2 function is required for optimal innate and adaptive immune responses. However, dysregulation/overexpression of the *NOD2* gene through the presence of an aberrant hypomethylation pattern of the CpG islands within the promoter region may contribute to the increased CD susceptibility (12). The overexpression of NOD2 significantly altered the expression of genes, including those related to inflammatory response (24). NOD2 may thus play a crucial role during and/or following granuloma formation in chronic inflammatory disorders. This process is thought to fuel the inflammation and consequently may refer to an increase in the development and severity of CD disease (14).

Conclusion

In summary, the present study identified that the colonic mucosa of CD patients showed a lower methylation level of the *NOD2* promoter region than healthy controls which was associated with the susceptibility to UC development. This study is an initial example investigating the association between *NOD2* methylation and CD development. Collectively, our findings suggest that the CpG hypermethylation at the promoter region of

the *NOD2* gene might impact gene expression. The data provide a key insight into the underlying mechanisms that cause CD by representing that epigenetic modifications in the *NOD2* gene regulation are the basis for hyperactivity of the inflammatory responses in CD patients. However, future studies are required to confirm this finding and even propose that epigenetic aberrances could be considered as precise targets for potential application in diagnosis and therapy.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of Interest

All the authors approved that they have no conflict of interest.

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