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

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Abstract- Changes in the expression of nucleotide-binding oligomerization domain containing 2 (NOD2) play an important role in the pathogenesis of several autoimmune diseases, such as inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC). It seems that epigenetic modifications, including DNA methylation, have an important role in the suppression of gene activity. In this study, the relationship between DNA methylation patterns of the promoter region of the NOD2 gene and the pathogenesis of CD was assessed. Colonic mucosal samples were obtained from 15 Iranian patients with CD and 15 matched healthy controls with no history of autoimmune diseases. After 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 assay. Using this approach, we identified a decreased level of methylation of the NOD2 promoter in the colonic mucosa of patients with CD (0.128±0.093 vs. 0.025±0.016, unmethylated DNA in CD *vs.* healthy controls, respectively, $P<0.000$). According to our findings, promoter hypomethylation of the NOD2 gene in the colonic mucosa might contribute to the development and severity of CD.

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Keywords: Nucleotide-binding oligomerization domain containing 2 (NOD2); Inflammatory bowel disease; Crohn's disease; DNA methylation

Introduction

Inflammatory bowel disease (IBD) is a chronic and relapsing intestinal inflammatory condition, which can be categorized into Crohn's disease (CD) and ulcerative colitis (UC) (1-3). Understanding the underlying mechanisms that cause IBD remains a clinical challenge in gastroenterology research (4). Genetics, environmental, and epigenetic factors seem to have a role in predisposition to IBD (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 have not been explained well in the pathogenesis of

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the disease so far (2,6,8).

Epigenetic changes are still hot topics for studies on the DNA methylation and histone modification. Studies on the role of short non-coding RNAs (miRNAs) molecules are also of interest (1,5,9). Emerging evidence shows that epigenetic changes are important in chronic inflammation, and are considered to be a vital factor affecting the pathogenesis of the disease (1,10). Studies on DNA methylation seem to be one of the most commonly used epigenetic modifications; 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 those susceptible to IBD (11). Genes involved in the innate immune handling of intracellular bacteria, including nucleotide-binding oligomerization domain containing 2 (NOD2), are considered to be CD-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 (PRR) of the innate immune system that functions as an intracellular sensor for bacterial peptidoglycan, an evolutionarily conserved pathogen-associated molecular pattern (PAMP) (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 ileal 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 that the expression of the NOD2 gene could be regulated and may have a role in the pathogenesis of IBD (1).

A high-accuracy measurement of the degree of DNA methylation is fundamental to have a better understanding of 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 studied to check its association with predisposition to CD.

Materials and Methods

Patients and intestinal mucosal samples

Human intestinal mucosal samples of 15 patients with CD (8 females and 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 diagnoses of CD was performed using standard criteria. Fifteen age- and sex-matched healthy volunteers (8 females and 7 males) were included as the control group. This study was 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 an 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 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 95° C for 1 min, followed by 30 cycles of 30 s at 94° C, 1 min at 60° C, 30 s at 72° C, followed by 5 min at 72° C. 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 $\Delta\Delta C_q$ method was used and also the cycle of quantification (C_q) 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 the 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 differences in CpG island methylation levels between the colonic 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 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* 0.05 were considered significant.

Results

Quantitative PCR protocol using SYBR Green reagents allows melting curve analysis of target. 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 patients with CD and healthy individuals. 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).

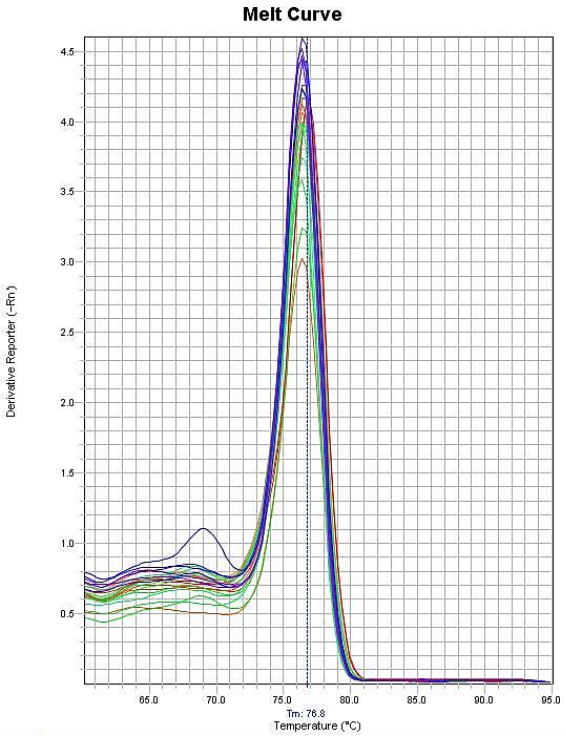

Figure 1. Fluorescence melting peak analysis for the NOD2 gene promoter showing changes in CpG methylation status

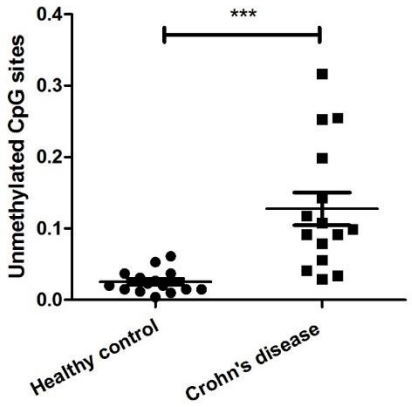

Figure 2. Comparison of the methylation patterns of CpG islands (CGIs) in the promoter region of the NOD2 gene in colorectal tissue specimens of patients with Crohn's disease (*n*=15) and healthy controls (*n*=15). *P* was obtained via the Mann-Whitney U-test. Error bars mean±SD (*P<0.05).

Discussion

Research on epigenetic changes, including DNA methylation, is an emerging field that can be helpful in designing new diagnostic and treatment protocols (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).

The role of epigenetic factors on the development of a number of immune-mediated diseases has been described. Although DNA methylation profiles in IBD colorectal tissue specimens have recently been described, gene regulation is still under debate (1,2,21).

Several human gene association studies showed the potential role of several genes in the pathogenesis of IBD (1). It seems that chronic intestinal inflammation could be a secondary consequence of innate immune dysfunction (20). Therefore the 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 response to gut microbiota due to immune intolerance to the microbial flora could be one of the major causes of IBD (20). NOD2 polymorphisms increase the risk of CD, probably due to abnormal innate immune response to commensal and pathogenic organisms in the intestine (12,23). It was also shown that the polymorphism impairs the function of NOD2, which leads to low responses against gut microbes (12). As a consequence, microbes can pass the epithelium and initiate the inflammatory process of the intestine, which is the main mechanism in CD (12,20).

However, the situation is different in CD lesions. It has been reported that CARD15 expression is high CD lesions. Indeed, intestinal involvement in active CD is linked with high expression of CARD15i n intestinal epithelial cells. Our finding is consistent with this concept. We reported an aberrant hypomethylation pattern of the CpG islands within promoter region of the 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 an aberrant hypomethylation pattern of the CpG islands within the promoter region may contribute to the increased CD susceptibility (12). Overexpression of NOD2 can modify the gene expression, particularly those related to inflammatory response (24). Therefore, NOD2 could play an important role during granuloma formation in chronic inflammation. This process is thought to fuel the inflammation and consequently may refer to an increase in the development and severity of CD disease

(14).

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. This study is an initial example investigating the association between NOD2 methylation and CD development. Collectively, our findings suggest that the CpG hypomethylation at the promoter region of 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 precise targets for potential application in diagnosis and therapy.

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