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Original Article

Evaluation of *ABCB1* Gene Promoter Methylation in Patients with Ulcerative Colitis

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Abstract

Background: The pathogenesis of inflammatory bowel disease may be associated with the disruption in interactions between the immune system and gut flora. Epigenetic mechanisms especially, DNA methylation appear to be significant regarding the interaction between the environment and genome. *ABCB1* is the encoding gene for multi-drug resistance protein 1 (MDR1) (P-glycoprotein), which is an important transmembrane protein responsible for the efflux of cellular molecules from the intestinal wall to the lumen.

Method: In this study, we compared the methylation status of the promoter of *ABCB1* in rectal mucosa of patients with ulcerative colitis (UC) and healthy controls by using the bisulfite conversion system and real-time quantitative multiplex methylation-specific PCR (QM-MSP).

Results: We demonstrated that the mucosal specimen of 26 UC patients had significantly higher levels of promoter methylation in comparison to 26 controls.

Conclusion: As the first investigation of Iranian patients with UC, we showed that patients had higher levels of *ABCB1* promoter methylation in their inflammatory rectal mucosa compared to controls. However, this altered state of methylation did not associate with the characteristics of the patients such as age and sex. Our findings are a basis for further studies on concurrent assessment of promoter methylation and expression of ABCB1 in UC.

Keywords: *ABCB1*; Epigenetics; Gene Methylation; MDR1; Ulcerative Colitis

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Introduction

Inflammatory bowel disease (IBD) is a chronic heterogeneous gastrointestinal inflammation that has two subtypes including Crohn's disease (CD) and ulcerative colitis (UC) (1). Over the past years, IBD has become more prevalent in developing countries. Notwithstanding, IBD incidence has barely changed in developed countries such as in European and North American countries (2, 3). In contrast to Europe and the US, IBD has been an infrequent disorder in Asia (4). However, the incidence and prevalence of IBD is now growing in Asia as well (5).

To date, related studies have suggested that the triggering incident in the pathogenesis of IBD may be the disruption in interactions between the immune system and flora within the intestine, though, the accurate etiology of this disease is still ambiguous (1, 6). Several studies have demonstrated the onset and progression of IBD are associated with approximately 32 predisposing loci (7-10). It is estimated that merely 20 percent of genetic susceptibility is due to these predisposing loci (11, 12) implying that the pathogenesis of IBD is probably correlated with other distinct changes such as epigenetic alterations (13). More specifically, there are several models of epigenetic alterations, from which DNA methylation is one of the most investigated. For instance, it has been reported that P14ARF, E-cadherin, and estrogen receptor (ER) genes undergo inappropriate DNA methylation in UC individuals (14). Moreover, an increasing number of studies support this notion that epigenetic changes are of the essence in the modulation of gene expression in patients with IBD (14, 15).

ABCB1 is the encoding gene for MDR1 protein (P-glycoprotein), which is an important transmembrane protein responsible for the efflux of cellular molecules from the intestinal wall to the lumen. It has the highest expression in the apical epithelium of the intestine (16). It is a member of the gene family encoding ATP-binding cassette (ABC) transporters and showed altered methylation and expression in different types of cancer (17). Patients with IBD are also at increased risk of developing CRC (18). Recently, it has been demonstrated that gastrointestinal inflammatory diseases e.g. IBD, are associated with alterations in ABCB1 expression (19). In this regard, a bulk of animal studies indicated that inflammatory conditions in the intestine are correlated with decreased expression and activity of ABCB1 (20-22). Furthermore, it was revealed there is an association between ABCB1 single nucleotide polymorphisms (SNPs) and IBD pathogenesis (23-25). Overall, data shows a decreased level of ABCB1 expression and/or function in inflammatory conditions of the intestine. As it is documented that DNA methylation is an essential epigenetic factor involved in altered protein expression and it gets exacerbated in chronic inflammatory conditions; in this study, we assessed the level of DNA methylation in the *ABCB1* gene in the inflammatory mucosa of UC patients.

Methods

Participants

The participants of the study consisted of 26 UC patients along with a group of 26 healthy controls (HCs) enrolled at the gastroenterology clinics of Shahid Sadoughi Hospital in Yazd, Iran, between May 2016 and July 2017. The diagnosis of UC was made based on clinical impression and confirmed pathology. Individuals with normal biopsy, which has been taken to rule out microscopic colitis, were included as healthy controls. Besides, written informed consents were obtained from all patients and healthy controls before inclusion in the study.

DNA isolation and bisulfite modification

Rectal mucosal specimens were taken from the patients and HCs. Specimens were taken during a colonoscopic biopsy and preserved at -80˚C until use.

The genomic DNA was extracted from the specimens by using the High Pure PCR Template Preparation Kit (Roche). DNA bisulfite modification was performed by using the MethylEdge™ Bisulfite Conversion System (Promega Co) Based on the protocol, 20µl of each genomic DNA sample with optimal concentration (200–500ng) and high quality (optical density: 1.8-2) were applied. Through DNA bisulfite modification, the unmethylated cytosine residues are converted to uracil, which in turn will be converted to thiamine following the PCR. On the contrary, the methylated cytosine (5-methylcytosine) of the genomic DNA remains intact.

Quantitative Multiplex Methylation-Specific PCR

The promoter DNA methylation of the ABCB1 gene was assessed by the real-time quantitative multiplex methylation-specific PCR (QM-MSP) method. First, bisulfite-specific primers (BSP) or external primers were used for amplification of the 271 bp amplicon from the converted genomic DNA. The forward and reverse external primers are listed in **Table 1**. In each reaction well, 1µl of the converted DNA along with 19 µl of other reaction materials was applied. The sequence of reactions included one cycle of 95°C for 5min, then 30 cycles of 94°C for 30s, 58°C for 30s, 72°C for 30s, and finally 72°C for 5min.

Then the real-time PCR was performed on the product of the previous PCR to amplify a 75 bp amplicon by using methylation-specific primers (MSP) or internal primers **(Table 1)**. The UCSC database was utilized to examine the methylation status of CpG islands in the promoter of ABCB1 and the Meth Blast tool was utilized for primer blasting. In each reaction well, 1µl of the BSP product as a template along with 5µl SYBR® Green Master Mix, 3 µl DDW, and 1µl of the MSP were applied. The sequence of reactions included one cycle of 95°C for 5min, then 30 cycles of 94°C for 30s, 60°C for 30s, 72°C for 30s, and finally 72°C for 5min.

The ΔΔCt method was used to calculate the unmethylated DNA level in each sample. The BSP was used to normalize data and a converted methylated human control DNA (Promega, N1221) was used as a reference. The following formula, which is used for relative gene expression (26, 27), was applied:

Unmethylated DNA Level = $2^{-\Delta\Delta Ct}$

ΔΔCt = ΔCt sample – ΔCt reference

ΔCt sample = CtMSP – CtBSP

ΔCt reference = CtMSP – CtBSP

Table 1. External (bisulfite-specific) and internal (methylation-specific) primers used in conventional and real time PCR

Primer Name	Primer Sequence (5' → 3')
EF	TTGTGGTGAGGTTGATTGGTT
ER	CCCAACTTTACGTACCCCTACC
IF	CGTGGGTTGAGTATAGTCGTTT
IR	ACGTACCCCTACCTCGCG

EF, External Forward; ER, External Reverse; IF, Internal Forward; IR, Internal Reverse

Statistical analysis

Statistical analyses were performed by using SPSS 20. The Chi-squared test was used for the comparison of sex between the two study groups. Student t-test was used to compare age between UC patients and HCs. The Mann-Whitney U test was used to compare the levels of promoter methylation between the patients and HCs. A *P-value* < 0.05 was considered statistically significant.

Results

The promoter methylation of the *ABCB1* gene was assessed in the rectal inflammatory mucosal samples of 26 patients with UC and 26 paired HCs. The characteristics of patients and HCs are illustrated in **Table 2**. The patient and control group were matched based on age. We demonstrated that the mucosal specimen of the UC patient had significantly higher levels of promoter methylation compared to HCs [median (IQR) for relative unmethyaltion level, 3.7*10-6 (2.4*10-6) vs 4.2*10-6 (2.6*10-6), *P-value* = 0.010]. However, there was no association between age and the level of promoter methylation in UC patients (*P-value*=0.444). Furthermore, sex was not associated with promoter methylation in UC patients (*P-value*=0.778).

Table 2. Clinical and methylation characteristics of UC patients and healthy controls

Variables	UC patients (n=26)	HCs (n=26)	*P-value*
Age[#]	34.4 ± 13.5	37 ± 4.5	0.554[a]
Sex (male %)	19 (73%)	12 (48%)	0.089[b]
Unmethylated ABCB1 Promoter (median [IQR])	$3.7*10^{-6}$[$2.4*10^{-6}$]	$4.2*10^{-6}$[$2.6*10^{-6}$]	0.010[c]

 # for UC n=17 and HC n=3, a student t-test, b Chi-squared test, c Mann-Whitney U test

Discussion

Epigenetic mechanisms appear to be of significant value regarding the interaction between the environment and genome (28). Further, than germline DNA alterations, epigenetic alterations, for instance, DNA methylation and histone modifications can influence gene function leading to disease-associated manifestations (29). In another words, epigenetic alterations are potential role players in disease development when the susceptible genetic loci are not responsible. In line with other autoimmune diseases, the exact pathological mechanisms behind IBD, and specifically UC are unknown. However, it has been speculated that the interactions between the host and the environment are involved in the pathogenesis of UC (1, 30). The significant factors in these interactions include the microbiota, immune responses, genetic susceptibility of the host, and particular environmental factors such as smoking, breastfeeding, drugs, and dietary products (1, 31, 32). Growing evidence revealed the hypermethylation of many gene promoters in UC. In this regard, it was demonstrated the incorporation of the 3H-methyl group into DNA was enhanced in patients with UC compared to HCs. The pattern was also observed in patients with histologically active compared to inactive disease (33). Moreover, hypermethylation of gene promoters of ABCB1, E-cadherin, CDH1, GDNF, and p16 were reported in UC patients (15, 34). Overall, DNA methylation has been considered a player in the pathogenesis of UC, which has especially been reported in specimens from inflamed tissue. Also, DNA methylation in UC is associated with several determinants including disease severity, duration, active inflammation, and dysplasia (15, 35-37).

The *ABCB1* gene is located on chromosome 7 at q21.1 (38) and its altered methylation status has been reported in many types of disorders including malignancy and autoimmunity (17, 39). It is considered one of the susceptibility loci to IBD in genome-wide analysis in a UK cohort (40) and was further confirmed by genome scan meta-analysis (41). Lots of polymorphism studies have reported the association between *ABCB1* and UC. Independent studies in Europe demonstrated that a C3435T polymorphism in exon 26 of ABCB1 is associated with susceptibility to UC but not to CD (25, 42). A Japanese study reported an association between the ABCB1 C3435T polymorphism and late-onset UC (43). ABCB1 C1236T polymorphisms are reported to be associated with susceptibility to UC (44). Moreover, a number of studies showed decreased expression of ABCB1 in UC patients (45, 46).

Considering the role of ABCB1 protein as an energy-dependent efflux pump to transport different types of substances such as ions and peptides (47), through the efflux of toxins, it prevents mitochondrial damage. Decreased expression of ABCB1 associated with mitochondrial damage (48). A previous study also showed that mice with polymorphism in the mitochondrial gene which generates high ATP are protected against colitis (49). The mitochondria are a key player in energy production. It has been suggested that there is an important connection between colonic epithelial cells' energy deficiency through mitochondrial damage and the pathophysiology of IBD (50). Based on the significant role of ABCB1 in the pathogenesis of UC, we investigated the methylation status of ABCB1 promoter in patients with UC and HCs in this experiment. In line with previous studies, we demonstrated that patients with UC have higher levels of methylation in the promoter of the *ABCB1* gene.

Conclusion

This is the first investigation into the methylation status in the promoter of the *ABCB1* gene in Iranian patients with UC. We showed that patients with UC have higher levels of ABCB1 promoter methylation in their rectal inflammatory mucosa. However, this altered state of methylation did not associate with characteristics of the patients such as age and sex. Our findings highlight a need for further studies on concurrent assessment of promoter methylation and expression of ABCB1 in UC.

Ethics approval and consent to participate

The protocol of this study was approved by the Ethics Committee of Tehran University of Medical Sciences and written informed consent was obtained from all participants before inclusion in the study.

Conflict of interests

The authors have no conflict of interest.

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