

Impact of Cyclooxygenase-2 Polymorphism -765G>C (rs20417) on the Development of Ulcerative Colitis

Atika M. Alahmed^{1*}, Eaman A. Al-Rubae²

¹Department of Oral Medicine, University of Baghdad, College of Dentistry, Baghdad, Iraq

²Department of Basic Science, University of Baghdad, College of Dentistry, Baghdad, Iraq

***Corresponding author:**

Atika Alahmed, M.D.

Baghdad, Bab-almoatham street, Iraq

Phone number: +9647710818950

E-mail: alig24624@gmail.com

ORCID: 0000-0001-7069-2266

ORCID: 0000-0001-6230-098X

ABSTRACT

Background: Ulcerative Colitis is chronic, progressive disease affecting large intestine. Cyclooxygenase-2 enzyme is an inducible enzyme that has important role in the process of inflammation. Ulcerative Colitis pathogenesis is comprising chronic inflammation; any alteration in the gene responsible for cyclooxygenase-2 expression such as -765G>C (rs20417) polymorphism may affect risk of having the disease. Objectives: To record and evaluate the prevalence of -765G>C (rs20417) polymorphism in group of Ulcerative Colitis Iraqi patients and correlation it with disease clinical severity.

Method: A case-control, single-center study included ninety participants collected from Gastroenterology and Hepatology Hospital/Basrah/Iraq, divided in to 30 newly diagnosed patients, 30 previously diagnosed patients and 30 healthy controls. Genetic analysis done through dual color discrimination assay.

Results: To our knowledge the present study is the first report in Iraqi population that investigates the correlation between COX-2 polymorphism, rs20417 with risk of developing UC. The prevalence of the -765G and -765C alleles was 85.8% and 14.1% in the patient group and 86.6% and 13.3% in the control group respectively, the -765CC variant in Basrah city was infrequent in population of UC patients (14.1%) and in controls (13.3%). There was no statistical difference in the disease association and in the relationship to the disease severity but GG genotypes showed more severe clinical disease than those with other variants.

Conclusion: In disparity to other reports on the role of COX-2 polymorphisms at position -765 in the term of modifying the risk for UC in a group of Arab Iraqi patients, we did not find significant results for this variation's association but in term of association with the level of disease clinical severity, carriers of GG wild type had more active clinical disease than those with mutant variants.

Key words: cyclooxygenase-2, ulcerative colitis, clinical disease activity, polymorphism.

INTRODUCTION

Inflammatory bowel disease (IBD) includes both Crohn's disease (CD) and ulcerative colitis (UC). UC is a progressive disease of the gastrointestinal tract (GIT) that has a chronic nature and mainly affects the large intestine (1,2). In UC, the stimulation of white blood cells arises after bacterial imbalance and infection in the gut, along with an impaired mucosal barrier. Even if the infection

Received: 16.12.2023

Accepted: 10.02.2024

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subsides, the response of the immune system continues, leading to chronicity (3,4). One of the scoring systems that clinicians use to estimate the clinical severity of UC is the Simple Clinical Colitis Activity Index (SCCAI), which offers an easy way of detecting possible relapse and decreases the need for invasive and uncomfortable examinations or laboratory tests (5,6).

The cyclooxygenase-2 (COX-2) enzyme is an important enzyme that converts arachidonic acid to prostaglandins and thromboxane. Its two isoforms are COX-1 and COX-2, among which COX-2 is the inducible form. Together, bacterial endotoxins, pro-inflammatory cytokines, and hormones may stimulate COX-2, and its metabolites are deeply involved in the initiation of pain and inflammation in peripheral body tissues (7,8).

Single nucleotide polymorphisms (SNPs) are the most frequent type of human genetic variation. Each SNP represents an alteration in a single DNA nucleotide, and they can be used as biological markers to locate genes that are related to disease (9,10). The gene encoding COX-2 is on chromosomes 1q25.2-25.3 and participates in the immune response to inflammation (11). SNPs in COX-2 may change its activity or its expression (and amount in the cell), which affects the inflammatory response and the risk of developing immune-mediated inflammatory diseases. It may even increase the risk cancer as the up-regulated COX-2 may enhance tumorigenesis (12).

In one polymorphism in the promoter region of COX-2, a transition from guanine (G) to cytosine (C) occurs at position -765 (-765G > C), which appears to interrupt a binding site of stimulatory protein 1 (Sp1), a positive stimulator of COX-2 transcription. The resulting variant leads to reduction of COX-2 promoter activity by approximately 30% in vitro (13). This SNP can also establish E2 promoter binding factor 1 (E2F1), which can enhance COX-2 transcription. COX-2 transcription is a complicated process that depends on the type of cell and type of stimulus (cytokine, growth factor, or bacterial endotoxins) (14,15). The objective of this study was to evaluate the prevalence of -765G>C (rs20417) polymorphism in a group of Iraqi patients with UC and its correlation with the clinical severity of the disease.

METHODS

Study subjects

This study examined 90 participants, who were divided into three groups: 30 patients previously diagnosed with UC and receiving treatment (PD), 30

patients who were newly diagnosed with UC and not receiving any treatment (ND), and a control group of 30 healthy participants, who were and matched in terms of age and sex and had no signs or symptoms of any systemic diseases. Approval for this study was obtained from the research ethics committee of the College of Dentistry of the University of Baghdad, as well as the Ministry of Health of Iraq. Samples were collected at the Gastroenterology and Hepatology Hospital in Basrah, Iraq, from May 2022 to December 2022.

All the participants were of Arabic ethnicity and were from the same geographical region. They were clearly informed about the aims of the study and were freely permitted to decline to participate. Informed consent was obtained from all participants. Patients were excluded if they were under 18 years old, pregnant, or also diagnosed with other autoimmune diseases.

Simple Clinical Colitis Activity Index (SCCAI) obtaining

The SCCAI was used with the aid of clinicians to quantify the disease activity of UC. This index may have several advantages as it reduces the need for uncomfortable and invasive examinations and laboratory tests, while it also provides the possibility of being filled out by a patient or employee. The index includes questions about the frequency of bowel movements during the day and at night, the feeling of any urgency, the presence of blood in the stool, general wellbeing as determined by the patients, and any extracolonic manifestations. The SCCAI categorizes patients into 3 levels: a score < 5 represents mild disease, 5-9 represents moderate disease, and >9 represents severe disease, while those in a state of remission have a score ≤ 2 (5).

Blood sample collection

Around 2 mL of venous blood were drawn from the anti-cubital vein of each participant. The blood was drawn into EDTA containing tubes and used for genetic analysis. DNA was extracted using a DNA extraction kit (ReliaPrep™ Blood gDNA Miniprep System/ USA), and the DNA concentration of all samples was assessed using a spectrophotometer (Nanodrop). The purified DNA was stored at -20°C until use.

Primers design and preparation

Primers were designed to amplify the DNA region

located around the -765G>C SNP along with probes for a dual-color discrimination assay. The sequences were based on a study by Peter et al. (16) and supplied by the Macrogen company (South Korea) in a lyophilized state (reverse primer: 5' AAA TAC TGT TCT CCG TAC CTT C 3'; forward primer: 5' GCT TAG GAC CAG TAT TAT GAG G-3') (Fam probe 5'Fam-tac CTT TCC CGC CTC TC-BHQ1-3', Hex probe 5'Hex-tac CTTTCC CCC CTC TC-BHQ1-3'). The lyophilized primer tubes were dissolved in nuclease-free H₂O to generate a final concentration of 100 pmol/ul (stock solution).

Whenever a PCR reaction was started, 10 µl of the stock solution were added to 90 µl of nuclease-free H₂O to make a working solution with a concentration of 10 pmol/µl, and the rest was stored at -20°C for use when needed. The probes were prepared using the same steps and then incubated at room temperature for at least 20-30 minutes to guarantee that the product had totally dissolved. During the incubation period, the vials were kept away from light by covering them with aluminum foil.

PCR reaction

The PCR analysis was done with 10 µL of Taqman master mix (GoTaq™ RT-qPCR System, Promega/USA) containing Taq DNA polymerase, MgCl₂, dNTPs, and reaction buffers at optimal concentrations for effective amplification of DNA templates by PCR, as well as 1 µL of F-primer, 1 µL of R- primer, 1 µL of Fam-probe, 1 µL of Hex-probe (which were added in a dark environment because they are light sensitive), 3 µL of the sample, and 3 µL of nuclease-free H₂O. The cycle conditions were enzyme activation (initial denaturation) at 95°C

from 60 s to 10 min (1 cycle), denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The software used was QuantStudio™ Design and Analysis Software v. 1.5.2.

Statistical analysis

SPSS ver. 22.0 was used to analyze the results. Baseline characteristics were examined with standard descriptive statistics. The observed genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium. Differences in genotype distributions between the control and patient groups were tested by chi-squared analysis. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated for normal versus altered genotypes between patient group and the control group.

RESULTS

General characteristics of the participants are given in *table 1*. There was no significant difference in age and sex distribution between groups. The clinical severity of UC was estimated via the SCCAI. The results in *table 2* demonstrate that a larger percentage of the patient group showed moderate clinical activity (73.3% vs. 43.3%). The mean activity score was 7.3 for the ND group and 6 for the PD group. The ND group had more active disease according to the score (7.3), and only 6.7% of them showed a mild score, whereas the PD group had a larger percentage of the mild disease status (36.7%). Both groups showed equal percentages for the severe score (20%).

Table 3 shows the distribution of genotype between

Table 1 - General characteristics of participants involved in this study

Variable	Groups	PD		ND		Control		P-value
	Classes	No.	%	No.	%	No.	%	
Gender	Male	15	50	16	53.3	17	56.7	CC= 0.054
	Female	15	50	14	46.7	13	43.3	P=0.875
	Total	30	100	30	100	30	100	NS
Age Groups Yrs.	< 20	1	3.3	5	16.7	4	13.3	CC= 0.334
	20 _ 29	11	36.7	9	30	7	23.3	P=0.332
	30 _ 39	5	16.7	3	10	10	33.3	NS
	40 _ 49	7	23.3	8	26.7	3	10	
	50 _ 59	4	13.3	4	13.3	3	10	
	≥ 60	2	6.7	1	3.3	3	10	
	Mean ± SD	36.37 ± 13.38		34.40 ± 14.19		35.93 ± 14.47		
	Total	30	100	30	100	30	100	

NS: Non Sig. at P>0.05; SD: standard deviation, Testing is based on a Contingency Coefficient test, PD: previously diagnosed, ND: newly diagnosed.

Table 2 - The distribution of the patients according to the scoring system of SCCAI

Variable	Groups Classes	PD		ND		P-value
		No.	%	No.	%	
SCCA Index	< 5	11	36.7	2	6.70	CC= 0.353
	5 _ 9	13	43.3	22	73.3	P=0.014
	> 9	6	20.0	6	20.0	S
	Mean $\pm$ SD	6.00 $\pm$ 3.77	7.33 $\pm$ 2.35			
	Total	30	100	30	100	

S: significant difference (P<0.05), SCCAI: simple clinical colitis activity index, PD: previously diagnosed, ND: newly diagnosed.

Table 3 - Distribution of Genotypes and alleles frequency between control and diseased groups with an Odd Ratio and Comparison's significant

Variable	SNP	Patients N (%)	Control N (%)	OR	95%CI	p-value
Genotype	GG	45 75.0%	23 76.7%	1 ref.	-	-
	GC	13 21.7%	6 20.0%	1.107	0.372-3.294	0.854 (NS)
	CC	2 3.3%	1 3.3%	1.022	0.088-11.875	0.986 (NS)
Alleles	G	103 85.8%	52 86.7%	1 ref.	-	-
	C	17 14.2%	8 13.3%	1.073	0.434-2.649	0.879 (NS)

NS: Non Sig. at P>0.05; OR: Odds Ratio; CI: confidence interval. The null hypothesis that the population is at H-W equilibrium. Testing based on multinomial logistic regression

the patient and control groups, as well as the significance of comparisons. The results showed that GG (wild type) was the most commonly detected genotype in both the patient group (75%) and control group (76.7%). The frequency of GC/CG (mutant heterozygote type) was found at lower percentages in the patients (21.7%) and in the control group (20%). The results also showed that the homozygote mutant genotype (CC) was rare in our samples with a percentage of 3.3% in the patient group and 3.3% in the control group. There was no statistical difference in the distribution of all genotypes (P-value > 0.05). The OR results between the patient group and control group showed that GC/CG and CC genotypes were not

significant predictors of disease development [OR (CI)= 1.1 (0.372-3.294) and 1.02 (0.088-11.875) for CG/GC and CC, respectively].

The allele frequencies were calculated, and the G allele was the most common (85.8% and 86.7% for the patient group and control group, respectively). The C allele also showed close frequencies between groups (14.2% and 13.3% for patient group and control group, respectively). *Table 4* shows a comparison between the observed and expected frequencies of both genotype and alleles. Genotype frequencies in both were not different from that was expected from the Hardy-Weinberg equilibrium.

The observed genotype frequencies were compared

Table 4 - Comparison between the observed and expected frequencies of both genotype and alleles according to HWE

Gene Symbol SNP	Genotype	Diseased Group			Control Group		
		Obs.	Exp.	H-W Freq.	Obs.	Exp.	H-W Freq
rs20417	Mutant GG	45	44.20	73.67%	23	22.53	75.11%
	Mutant CG/GC	13	14.59	24.32%	6	6.93	23.11%
	Wild CC	2	1.20	2.01%	1	0.53	1.78%
H-W Equilibrium		P=0.3981			P=0.4609		
χ^2 value at (α = 0.05/2)		NS			NS		
Alleles frequency							
Allele Frequency		G : 103 (85.83%)			G : 52 (86.67%)		
		C : 17 (14.17%)			C : 8 (13.33%)		

Obs: observed; Exp: expected; H-W: Hardy-Weinberg; NS: non-significant.

Table 5 - Distribution of Genotypes on the SCCAI scores in the patients with testing the significance.

SNP(rs20417)	SCCAI			Total (60 patients)	Statistical
	Mild (13 patients)	Moderate (35 patients)	Severe (12 patients)		
GG	10 76.9%	23 65.7%	12 100.0%	45 75.0%	Chi-Square= 6.95 p-value=0.139 NS
GC	2 15.4%	11 31.4%	0 0.0%	13 21.7%	
CC	1 7.7%	1 2.9%	0 0.0%	2 3.3%	

SCCAI: simple clinical colitis activity index; NS: Non Significant at P>0.05; (Testing based on chi-square test).

with the severity levels of the disease according to the SCCAI. *Table 5* shows that among those with the mild disease score, 76.9% of patients with UC had the wild GG genotype, and 15.4% had CC/CG, and with only 7.7% had the CC variant. Concerning moderate scores, a larger percentage was observed for patients with the wild GG variant at 65.7% than the heterozygote and homozygote mutant variants at 31.4% and 2.9%, respectively. The severe disease scores were only reported by the 12 patients with the wild variant (100% of them), and no patient with the mutant variant showed clinically severe disease. The results not only show that the mutant variant is not significantly related to the risk of developing the disease but also that those with the mutant variants had less clinically severe disease than those with the wild GG genotype.

As shown in *table 6*, multinomial logistic regression was used as a prediction test for the 60 patients to explore the OR and determine the effect size of genotype variables (representing a risk factor) for the outcome (the clinical disease activity of UC). The GG wild type was used as a reference category to which the other two mutant variants were compared. In terms of the possibility of genotypes predicting the clinical activity of UC, the results show that the heterozygote (GC) genotype represents a significant predictor for mild and moderate clinical courses of the disease (OR

(95%CI): 0.200 (0.044-0.913), 0.478 (0.233-0.981), respectively). The homozygote (CC) genotype could also be a significant predictor for the mild and moderate clinical courses of the disease (OR (95%CI): 0.100 (0.013-0.781), 0.043 (0.006-0.322) for mild and moderate disease, respectively). The results for severe disease were nonapplicable because no patients with these variants were recorded, so they could not be compared with the GG type.

DISCUSSION

To our knowledge, the present study is the first to investigate the correlation between COX-2 polymorphism rs20417 and the risk of developing UC in an Iraqi population, as well as the first to estimate its frequency in this context. The genomic material of the study samples was extracted using the ReliaPrep™ Blood gDNA Miniprep System, which provided high-quality DNA. It eliminates the usage of ethanol, which may damage the DNA molecules, it uses a binding matrix made up of cellulose, which easily captures and preserves the large molecules of nucleic acid through the washing step, and it uses microtubes with a geometric design that facilitates the washing procedure (17).

The COX-2 enzyme shows a significant cytoprotective role in the GIT and represents an important

Table 6 - Multinomial logistic regression between genotypes and SCCAI

Genotype		B	p-value	OR	95% Confidence Interval for OR	
					Lower Bound	Upper Bound
GC	Mild	-1.609	0.038* S	0.200	0.044	0.913
	Moderate	-0.738	0.044* S	0.478	0.233	0.981
	Sever	-21.151	NA	0.000	0.000	0.000
CC	Mild	-2.303	0.028* S	0.100	0.013	0.781
	Moderate	-3.135	0.002* S	0.043	0.006	0.322
	Sever	-21.151	NA	0.000	0.000	0.000

The reference category is GG. * significant at p-value <0.05. OR: odds ratio. B: effect size.

NA: non- applicable.

determinant of GIT homeostasis through the healing of ulcers and lesions. Prostaglandins derived from COX-2 show two roles. The first is a constitutive role that is highlighted by the conservation of colonic integrity, as shown in studies that included models of colitis and the ability of selective COX-2 inhibitors to considerably worsen colonic injury. On the other hand, whenever COX-2 levels are upregulated, prostaglandin E2 PGE2 production is enhanced, and susceptibility to disease increases. One example is active IBD, which shows increased expression of COX-2 in the colonic epithelium and in the walls of arteries.

Colorectal neoplasia and colorectal cancer are also interrelated with high levels of COX-2 due to its ability to produce phenotypic cell changes, which augment resistance to apoptosis and promote angiogenesis. These functions all represent the inducible role of COX-2 (18,19,20). Studying polymorphisms in the COX-2 gene is important as they may affect the level of this enzyme's expression in the gut, which was the main concept of the present study.

The results showed that carrying the polymorphic genotype was not significant predictor for development of the disease, which was in line with what was stated by De Varies et al. They reported that those carrying the polymorphism showed no increased risk of developing UC or CD, which suggests that altered enzyme expression caused by the mutant genotype changed the enzyme activity toward potential anti-inflammatory states (21). However, our results differ from those of a Danish study conducted by Østergaard et al., who found that carriers of rs20417, especially the CC variant, have much higher risk for developing CD and UC (22).

The association that we observed between the mutant genotype and lower clinical disease activity was similar to the observations of Orbe (23), who observed reduced systemic inflammation CC genotype carriers when compared with GG genotype carriers. This finding is also similar to those reported by Cipolone, who also stated that the rs20417 polymorphism (GC genotype) is related to a decreased level of inflammation and lower risk of myocardial infarction and stroke. This could be related to the ability of PGE2 to promote T cell differentiation to T helper-17 (Th17) cells, which have been recognized as potent effector cells in IBD in terms of reducing the inflammatory response in the colonic tissues (24).

Our study is the first to report the CC-variant frequency in Basrah, which had a low rate among patients with UC (14.1%) and the control group (13.3%). No other Iraqi studies about this SNP were found for comparison, but the results were higher than those

reported in Egypt for Arabic patients with colorectal cancer (8%) and a control group (2%) (25). They were also higher than the frequency reported by a study on Jordanian adult patients evaluating aspirin resistance (6.5% in the aspirin responders and 3.3% in the non-responders) (26). These comparisons with the same ethnic background (Arabic) are based on the fact that COX-2 SNPs have ethnic differences between different populations and modulate disease phenotypes, which might explain differing results when compared to studies involving other ethnicities.

Even though the current results shows that carrying the polymorphism has no effect on developing UC, the polymorphisms in COX-2 (including rs20417) and their ability to change its activity or expression will eventually alter the amount of prostaglandins produced and then modulate the inflammatory response. All of these events have a well-known role in several types of GIT carcinogenesis and in UC-associated neoplasia. This is especially important when the effects of these polymorphisms are amplified by other dietary, metabolic, and environmental risk factors, as well as a chronic inflammatory status, as that seen in UC. Therefore, patients with UC who have the G/C and C/C variants need more evaluation and screening to detect any dysplastic or neoplastic changes (26-30).

Limitations

One limitation of our study is the small study sample. Furthermore, there is a need to determine whether the change in COX-2 promoter activity is associated with lower prostanoid production in the cells that produce this isozyme in reaction to patho-physiologic stimuli. This might be established by exploring COX-2 and PGE2 production in vitro. The small sample size may be related to the small numbers of patients with UC and those who not receiving any treatment and attended the medical care center.

Limitation of our study is the small study sample and the need to know whether this change in COX-2 promoter activity is going to be associated with lower prostanoid production in the cells that producing this isozyme in reaction to patho-physiologic stimuli. This might be established by exploring COX-2 and PGE2 production in vitro. Small sample size may be related to small number of patients with UC as well as those without any treatment attended the medical care center.

CONCLUSION

We did not find significant results in terms of

modifying the risk for UC in a group of Arab Iraqi patients. This contrasts with other reports on the role of COX-2 polymorphisms at position 765. However, in terms of the association with the clinical severity of the disease, carriers of the GG wild type had more active clinical disease than those with mutant variants.

Authors contribution

AA did the data collection, analysis and initial draft writing; while EA put the study design and concept, edit the final draft.

Conflicts of interest

The authors declare that there are no conflicts of interest in connection with this paper.

Funding

The study is self-funded

Ethical statement

The researched was approved by the Ethical Committee in the University of Baghdad/ College of Dentistry (project No. 431721 at 27/12/2021) as well as that of Ministry of Health, Basrah/Iraq (226 in 10/May/2022).

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