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Original Research Article

Association of Cyclooxygenase 2 Gene Polymorphism in Irritable Bowel Syndrome

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Abstract: The COX2 gene encodes for the cyclooxygenase-2 enzymes in prostaglandin synthesis. There is a polymorphism (T to C substitution) in the 3' untranslated region of COX2 gene, providing an increased rate of transcription of COX2 mRNA, which in turn increases enzyme cyclooxygenase-2 synthesis, increases prostaglandin production. 5-HT_{1A} receptor responsiveness is reduced by arachidonic acid metabolite. Activation of serotonin 5-HT_{1A} receptor in the enteric nervous system suppresses gut motility. Genotype analysis was done on 50 patients with IBS and 50 healthy controls by polymerase chain reaction followed by restriction digestion. Patients with C/C COX2 genotype had a risk of IBS with an odds ratio (OR), of 0.84; 95% confidence interval (CI) =0.23-3.08] compared with those with the T/T genotype. The trend of an increasing risk of IBS with an increasing number of C allele was not statistically significant. The C allele of the COX2 gene polymorphism is not associated with increased risk of IBS.
Keywords: COX2 gene polymorphism, Irritable Bowel Syndrome, restriction digestion

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional disorder that affects 10% to 20% of the population worldwide [1-3]. IBS is characterized by altered bowel habits and abdominal discomfort in the absence of organic disease. No clear diagnostic markers exist for IBS and thus diagnoses are based on the clinical presentation. A relationship between irritable bowel syndrome (IBS) and broncho-pulmonary disease was initially suspected in 1991, when White and co-workers [4] reported bronchial hyper-responsiveness to be more frequent in patients with IBS. Further, Kennedi and colleagues showed an association between symptoms of IBS and those of bronchial hyper-responsiveness [5]. Amra *et al.* reported that patients with IBS have increased airway resistance as compared to healthy subjects, as measured by impulse oscillometry [6]. Two independent results showed bronchial asthma may be more prevalent in IBS patients than in otherwise healthy subjects [7,8]. Chan *et al.* reported PTGS2.8473 polymorphism is associated with asthma, atopy and lung function [9]. Sanak *et al.* reported increased production of prostaglandins in bronchial asthma in association with T→C transition within the 3′-untranslated region (COX2.8473) [10].

Serotonin (5-HT) is secreted in copious amounts from gut enteroendocrine cells and serves as a critical messenger for gastrointestinal fluid secretion and gut motility [11,12]. There are seven subclasses of serotonergic receptors, differentiated on the basis of structure, molecular mechanism, and function [13]. The 5-HT_{1A} receptor is a G protein-coupled receptor that is coupled to G_i/G_o and mediates inhibitory neurotransmission. Activation of serotonin 5-HT_{1A} receptor in the enteric nervous system suppresses gut motility [14,15]. Clarke *et al.* reported marked elevations in proinflammatory polyunsaturated fatty acid metabolites like PGE2 in females with Irritable Bowel Syndrome [16]. Kenda *et al.* showed the responsiveness of the 5-HT1A receptor system was reduced by a cyclooxygenase-dependent metabolite of AA [17].

In this study, the association of COX2.8473 T→C single nucleotide polymorphism (SNP) with IBS was investigated. Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder characterized by abdominal pain or discomfort and altered bowel habits [18,19]. As such, IBS occurs in the absence of identifiable physical, radiologic, or laboratory indications of organic disease [19,20]. Characterized as a "brain–gut disorder," [21] IBS is associated with such altered physiologic processes as changes in gut motility [22, 23] visceral hypersensitivity [24] and altered immune activation of the gut mucosa and intestinal microflora [25]. IBS has a reported prevalence of 5%-10% in most of Asia [26]. The IBS group was subdivide into diarrhea predominant (IBS-D), constipation predominant (IBS-C), mixed with diarrhea and constipation (IBS-M), or undetermined categories (IBS-U) according to the bowel movement frequency and stool consistency.

Generally, race [27], gender [28-31], age [2,32,33], marital status [34,35], stress [28-30,34-37], food [1,32,35,38], or alcohol and tobacco [32] use have been considered as risk factors to IBS. IBS accounts for significant health care resource utilization and economic burden. Direct costs are often high in the initial diagnostic phase as historically IBS has been considered a diagnosis of exclusion, prompting sequential testing and invasive procedures in an attempt to identify organic GI disease [19,20,39]. Dean *et al.* [40] found that IBS-related symptoms reduced work productivity by an estimated 21% per week thus increasing costs to employers. Because IBS is not a mortal illness, the impact on patients is often underestimated. In reality, however, IBS patients have substantially poorer health-related quality of life (HRQoL) than the general population and HRQoL that is on par with that seen in diabetes, depression, and gastroesophageal reflux disease [41,42].

The COX2.8473 SNP is located downstream of the stop codon, in the 3'-UTR region. Binding of proteins to the 3'-UTR can control mRNA stability and degradation, and this may be affected by polymorphisms [44,45]. This region is characterized by multiple repeats of AU-rich elements, which are also found in several other genes encoding inflammatory mediators (cytokines and protooncogenes), whose mRNA is very unstable. It may be possible that the T→C substitution at COX2.8473 stabilizes the mRNA of COX2, thus resulting in a larger amount of protein produced and therefore an increased pro-inflammatory stimulus. Mild inflammation is a component in the pathogenesis of Irritable Bowel Syndrome.

COX2 gene polymorphism is associated with increased production of prostaglandins. Activation of serotonin 5-HT_{1A} receptor in the enteric nervous system suppresses gut motility. 5-HT_{1A} receptor responsiveness is reduced by arachidonic acid metabolite.

As there is increased incidence of bronchial asthma in patients with Irritable Bowel Syndrome, the polymorphism associated with increased production of prostaglandin *PTGS2*.8473T→C SNP is investigated in this study to know whether the same polymorphism is associated with patients of Irritable Bowel Syndrome.
PTGS2.8473 T→C SNP → ↑PGE2 → ↓ 5-HT_{1A} responsiveness → ↑ Gut motility

MATERIALS AND METHODS

STUDY POPULATION

CASES

The study sample comprised 50 unrelated Irritable Bowel Syndrome patients (30 male, 20 female) of Mean age of 40.56 ± 11.36 years. Inclusion criteria were individuals aged between 18 and 65 years who satisfied Rome II criteria for IBS**.** Organic gastrointestinal diseases and clinically significant systemic diseases, individuals with known lactose intolerance or immunodeficiency or who had any recent transient illness (i.e. within 2 weeks or participation in the study) such as viral illnesses or chest infections were excluded.

CONTROL SUBJECTS

Controls were recruited from outpatient department during their visit for non gastric illness. Age, Sex were matched.

METHODS

2 mL of blood was obtained by Venipuncture & collected in EDTA tub & was centrifuged at 2000 rpm for twenty minutes to get the buffy coat for DNA extraction.

COX2 Gene Polymorphism Screening

DNA was extracted from buffy coat by high salt method [13] and was used to amplify the 177 bp target region in the COX2 gene by PCR using forward 5′-GAAATTTTAAAGTACTTTTGAT–3' and reverse 5'-CTTTTACAGGTGATTCTACCC – 3' primers.

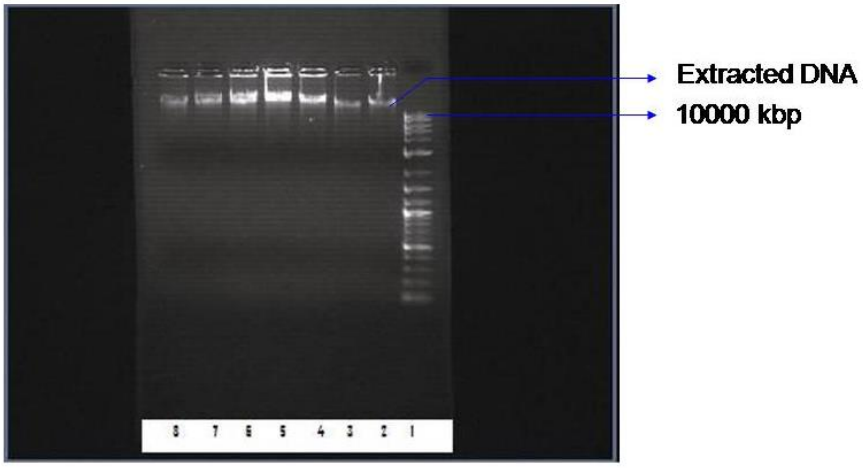
Genomic DNA (1µg) was amplified in 50µl (PCR master mix 25 µL, Forward primer 1 µL, Reverse primer 1µL, DNA 1.0µL, Distilled water 22 µL) reaction mixture containing 10 µmol/L of each primer and red dye master mix containing 250µmol/L of each dNTP, 2.5µL of 10x reaction buffer and 1.5 unit of Taq DNA polymerase. After the DNA was denatured for 3 minutes at 94ºC, the reaction mixture was subjected to 30 cycles of denaturation for one minute at 94ºC, 1 minute of annealing at 50 ºC and 1 minute of extension at 72 ºC. Final extension was carried over at 72 ºC for 5 minutes. COX2 gene polymorphism was detected by digestion of the PCR amplified product with 1 units of Bcl1 restriction enzyme for 1 hour followed by size fractionation in 3% Agarose Gel Electrophoresis. T allele does not have the restriction site hence will yield a 177bp fragment, C allele has the restriction site, hence gets cleaved to give 156bp and 21bp fragment. Analysis was done using a low molecular weight DNA ladder (25 bp).

Statistical Analysis

- Allele frequencies were calculated by allele counting.
- Age, Sex, Comorbidity with mental disorders, H/O Asthma, H/O Fibromyalgic symptoms, H/O Sexual and physical abuse, H/O Major life stress were compared between control subjects and patients by students t test.
- Genotype frequency distribution between cases and controls were compared with a χ^2 test for 2*2 contingency table.

RESULTS

- Table 1 shows Age , Sex ,Comorbitity with mental disorders , Fibromyalgia, Asthma, Sexual and physical abuse, Major life stress, risk factor distribution among patients and control subjects. We obtained a nonsignificant p value with respect to all the confounding variables like Age, Sex, Comorbitity with mental disorders, Fibromyalgia, Asthma, Sexual and physical abuse, Major life stress.
- Table 2 & 3 shows Genotype distribution and Allele frequencies of COX2 gene in patients with IBS and control subjects. The Allele frequencies were TT = 30, TC =14 and CC = 6. This was found to be in Hardy Weinberg equilibrium. χ^2 value is 3.84, P value is 0.37.
- TT genotype was frequent among cases (60%) when compared to controls (50%). TC genotype was common among controls (36%) when compared to cases (28%). CC genotype was common among cases (14%) when compared to controls (12%).
- There is no difference in T+ genotype among controls (88%) & cases (86 %). P value =0.766. There was no significant difference in the distribution of CC genotype between cases (14%) and controls (12%). P value = 0.597.



Fig–1: Extracted DNA (lane 2 to 8) was tested on 1% agarose gel using 1kb ladder (lane 1) Ladder shows 10000, 8000, 7000,6000, 5000, 4000, 3000, 2000,1000 bp fragments

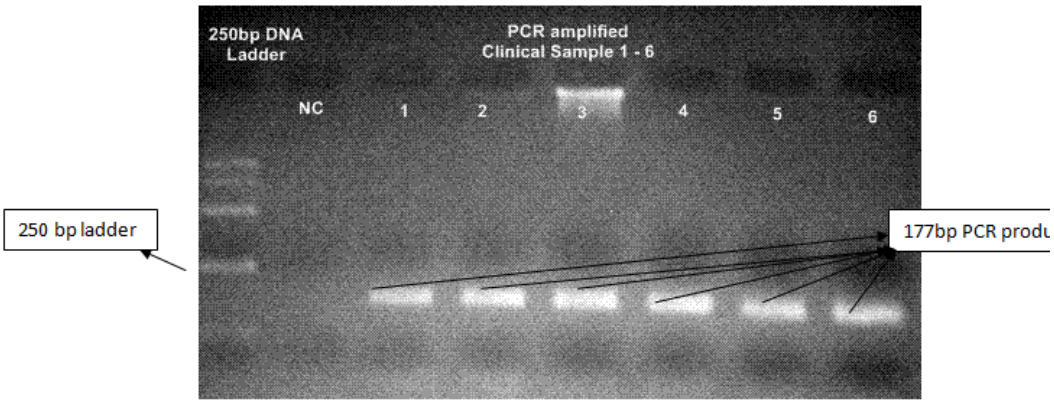


Fig-2: Shows 177bp PCR product (clinical samples 1 to 6) on 2.5% agarose gel. First lane shows 250 bp ladder [Second lane - Negative Control (NC)]

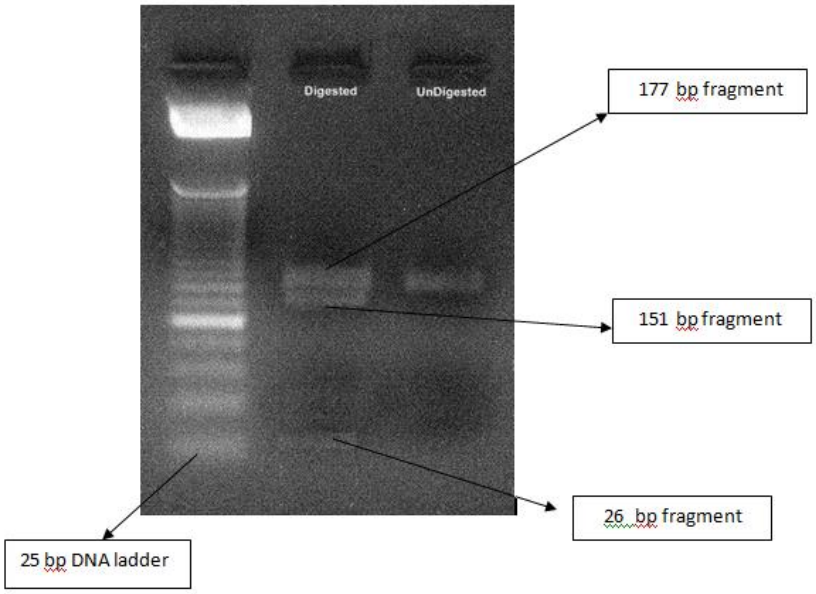


Fig-3: Shows TC GENOTYPE RFLP products (177 bp fragment, 151 bp fragment, & 26 bp fragment)

Table-1.characteristics of patients with ibs and of control subjects (students t-test)

Variables	Case	Control	p Value
Age	39.84 ± 11.99	40.56 ± 11.37	**0.79**
Sex Male	30(60%)	30(60%)	**1.00**
Female	20(40%)	20(40%)	**1.0**
Comorbidity with mental disorders	28(56%)	26(52%)	**0.68**
Fibromyalgia	26(52%)	28(56%)	**0.68**
Asthma	3(6%)	2(4%)	**1.00**
Sexual and physical abuse	10(20%)	6(12%)	**0.28**
Major life stress	2(4%)	1(2%)	**1.00**

Table-2: Allele frequencies of cox gene

Genotype	Control	Case	P value
T-*	6	7	Chi sq=0.09
T+*	44	43	P = 0.766 Odds ratio = 0.84 95% CI for OR=(0.23-3.08)

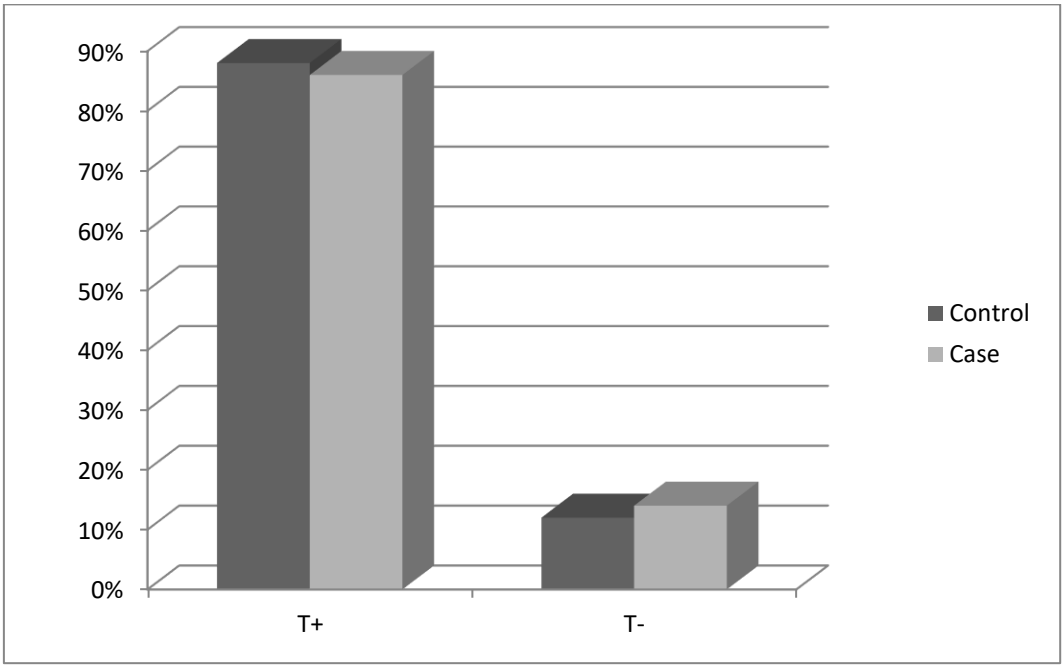


Fig-3: Allele frequencies of cox gene

Table-3: Genotype distribution of cox2 gene

Genotype	Control	Case	p value
TT	30	25	Chi sq = 1.03 p value= 0.597
TC	14	18	
CC	6	7	

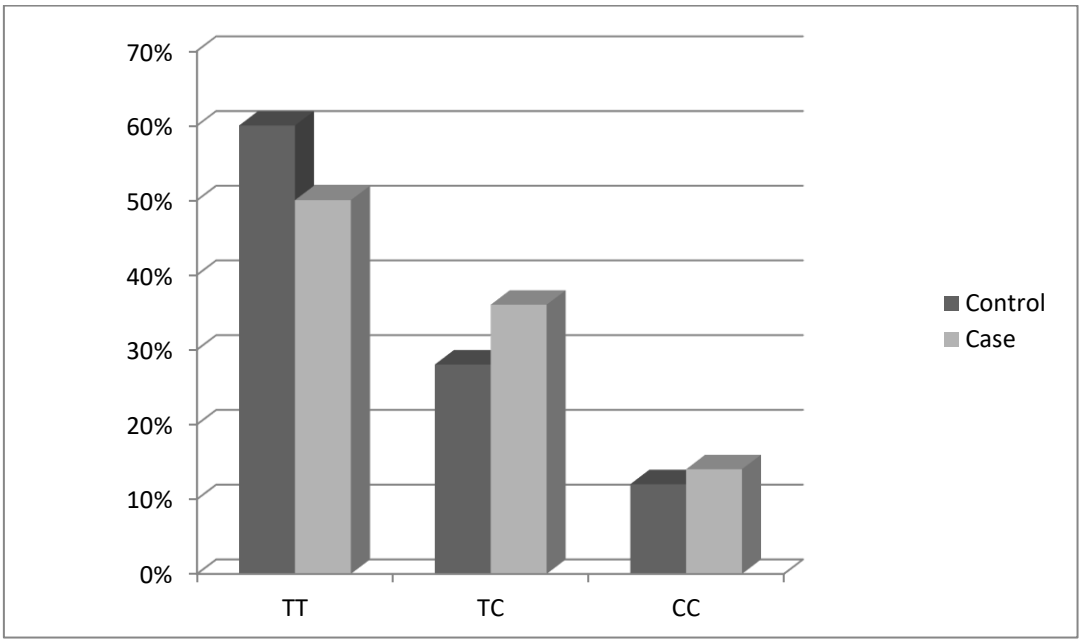


Fig-4: Genotype distribution of cox2 gene

DISCUSSION

Genetic factors in combination with a number of environmental risk factors are involved in predisposition to Irritable Bowel Syndrome. Recently, interest has focused on the presence of mucosal inflammation in the pathogenesis that may be particularly valid for diarrhoea-predominant IBS [46]. $5HT1_A$ receptor decreases gut motility. As the arachidonic acid metabolite reduces the responsiveness of the 5HT1A receptor system which may consequently increase gut motility, any polymorphism associated with arachidonic acid metabolism is being looked for. As studies showed increased amount of PGE2 in IBS, polymorphism in the COX pathway, if any is being studied. As studies showed increased prevalence of bronchial asthma in IBS patients, the COX2 (8473 T → C) gene polymorphism, which is associated with bronchial asthma, was analyzed in this study. In this study, the effect of polymorphism in the 3-UTR region of the COX2 gene (8473 T → C) is assessed in the occurrence of IBS. CC genotype which is associated with increased amount of PGE2 levels in previous studies involving asthma population, does not significantly associate with our IBS population.

As it has been described before, stress activates CRF-CRF1 signaling pathways in the brain linked with hypothalamic and pontine nuclei which stimulate the sacral parasympathetic nucleus and subsequently the enteric nervous system. The increased CRF/urocortin activates mast cells [43] & other immune cells which subsequently increases motility, mucus secretion, PGE2 production [47]. The biopsychosocial model of IBS integrates a number of psychosocial, motility, sensory abnormalities and abnormalities in central nervous system processing of visceral pain as the causes of abdominal pain and altered bowel habits. As IBS has multifactorial causes, factors other than COX2 gene polymorphism may be involved in this population like the above mentioned stress induced inflammatory pathway or Gene polymorphisms involving IL-10; 5-HTTLPR; α2-Adrenergic Receptors; Cathechol-o-methyl transferase; G-proteins; Mitochondrial DNA SCN5A (Na+ channel); Cannabinoid (CB) receptor; Neuropeptide S receptor 1. Further studies may be undertaken in future to investigate the occurrence of the polymorphisms in this population.

IBS is the most common disorder encountered by gastroenterologists, and is responsible for reduced quality of life and considerable economic burden on society. Presently there is no known biochemical or structural markers for identifying patients with IBS**.**Attention has recently been focused on increased perception of visceral stimuli arising from the

gastrointestinal tract wall, a phenomenon referred to as visceral hypersensitivity. Among the sensitising factors acting on nerve terminals at the peripheral level, altered interaction between the mucosal immune system and the afferent nerve terminals which project to the intestine is now receiving increasing attention. Low grade inflammation in the intestinal mucosa has been found in subgroups of patients and may be involved in the pathophysiology of visceral hypersensitivity, in at least some cases of IBS.

As COX2 gene polymorphism is associated with inflammation, this study was designed to investigate polymorphism in the 3-UTR region of the COX2 gene with occurrence of IBS.

In this study homozygous COX2 TT genotype was more frequent than TC or CC.

CONCLUSION

This study showed that CC polymorphism in the 3'-UTR region of the COX2 gene (8473) which is associated with inflammation was not significantly associated with increased risk for IBS. This study may be further explored in diverse and larger population and newer techniques such as new generation sequencing as this COX2 gene polymorphism is one of the main polymorphism involving inflammation.

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