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• *BRIEF REPORTS* •

A haplotype of prostaglandin synthase 2/cyclooxygenase 2 is involved in the susceptibility to inflammatory bowel disease

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Abstract

AIM: Prostaglandin G/H synthase 2 (PTGS2 or COX2) is one of the key factors in the cellular response to inflammation. PTGS2 is expressed in the affected intestinal segments of patients with inflammatory bowel diseases (IBD). In IBD patients, non-steroidal anti-inflammatory drugs, which have been shown to reduce both the production and activity of PTGS2, may activate IBD and aggravate the symptoms. We aimed at examining genetic variants of *PTGS2* that may be risk factors for IBD.

METHODS: We genotyped 291 individuals diagnosed with IBD and 367 controls from the Dutch population for the five most frequent polymorphisms of the *PTGS2* gene. Clinical data were collected on all patients. DNA was extracted via normal laboratory methods. Genotyping was carried out using multiplex PCR followed by the Invader Assay and the 5' exonuclease assay (TaqMan). New polymorphism screening was performed by pre-screening with denaturing high-performance liquid chromatography, followed by fluorescent sequencing.

RESULTS: Allele 5209G was weakly associated with Crohn's disease (odds ratio [OR] 1.63, 95% confidence interval [CI] 1.03-2.57), and allele 8473T with ulcerative colitis (OR 1.50, 95%CI 1.00-2.27). The haplotype including both alleles showed a strong association with IBD (OR 13.15, 95%CI 3.17-116.15). This haplotype, while rare (-0.3%) in the general population, is found more frequently in patients (3.5%).

CONCLUSION: Our data suggest that this haplotype of *PTGS2* contributes to the susceptibility of IBD.

Key words: Inflammatory bowel disease; Prostaglandin G/H synthase; Cyclooxygenase; SNP; Haplotype

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INTRODUCTION

Risk factors for inflammatory bowel diseases (IBD) include several environmental and genetic exposures. Diet, tobacco smoking, and childhood diseases or poor hygiene have been identified as risk factors for IBD, with some important differences between ulcerative colitis (UC) and Crohn's disease (CD)[1-3]. Caucasians have been shown to be at higher risk than non-Caucasians, with North-South gradients seen in Europe[4,5].

Familial aggregation of IBD suggests a genetic component in the susceptibility to IBD. One major locus of susceptibility to IBD has been mapped by linkage analysis to chromosome 16, at the *CARD15/NOD2* gene[6-8], which has a role in inflammatory responses, through activation of nuclear factor NF-κB. Two more susceptibility genes have recently been identified[9,10]. Population-based association studies focused on polymorphisms of inflammatory genes, such as genes of the interleukin-1β pathway. Few showed significant associations with some degree of contradiction between studies[11-15].

Key genes involved in the regulation of the inflammatory processes, such as prostaglandin G/H synthase/cyclooxygenase (PTGS/COX), are obvious candidates to look for variants predisposing to IBD. One of the two PTGS isoforms, PTGS2/COX2, is expressed in epithelial cells and mononuclear cells in IBD[4], and it is induced in response to pro-inflammatory cytokines, including interleukin-1β[16-18]. PTGS2 is the rate-

limiting enzyme in the production of prostaglandins. Prostaglandins are thought to be essential in the process of wound healing in the gastrointestinal tract[19]. The use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit both the transcription and activity of PTGS2, exacerbates the symptoms in UC[20] and may even activate quiescent IBD[21]. Thus, the expression of PTGS2 in the inflamed intestine might be a protective response within the wound-healing process[22]. Consequently, polymorphisms that change the amount of prostaglandins produced in inflamed cells could cause susceptibility to IBD.

To address this hypothesis, we have studied 145 UC patients, 146 CD patients, and 367 controls from the Dutch Caucasian population, which has a high incidence of IBD[23]. The present study was to search for associations between IBD and common single nucleotide polymorphisms (SNPs) in the *PTGS2* gene.

MATERIALS AND METHODS

Samples

The recruitment of cases took place at the Department of Gastroenterology of the VU University Medical Center (VUmc), a referral center for IBD, between April 1992 and September 2000. Patients with indeterminate colitis were excluded from this study. Seventy-five UC patients were females and seventy males, with a median age of 46 years (range 19-89 years). The gender repartition of CD patients was 101 females and 45 males, with a median age of 40 years (range 15-80 years). The control population consisted of 175 healthy individuals who were students or staff at the VUmc, with a median age of 51 years (range 24-88 years). All subjects were unrelated Dutch Caucasians. Genomic DNA was extracted using the DNAzol procedure (Invitrogen).

In order to ensure that the control group was representative of the general population, a second control group was also used, consisting of 192 Dutch subjects (96 males and 96 females). These samples were selected from the European Prospective Investigation on Cancer (EPIC) cohort[24] to provide information on the genetic background in the overall Dutch population. These samples were selected to provide a similar age distribution and sex ratio as the original control group. DNA was extracted from buffy coat using Puregene chemistry (Gentra Systems, Minneapolis, MN, USA). This population-based sample was indistinguishable from the VUmc controls from the point of view of frequencies of *PTGS2* alleles and genotypes (data not shown). We take this as confirmation that both groups were drawn from the general Dutch population, at least from the genetic point of view.

Patient classification

Diagnosis and assessment of maximal extent of IBD were based on endoscopic, histopathological, and radiological criteria[25]. Patients with indeterminate colitis were not included in this study, as they can be categorized as UC or CD only at a later stage. UC patients were subdivided into three groups: proctitis, limited to the rectum (12 cases), left-sided colitis with disease activity up to the splenic flexure (72 cases), and pancolitis (61 cases), extending beyond the splenic flexure. In addition, the patients with UC were subdivided into one group with their colon *in situ* (116 cases), and a second group in which (procto)colectomy had to be performed during the course of disease (29 cases), indicative of severe or intractable disease (i.e., unresponsive to medical therapy). No patient was operated on prophylactic indication. In CD, patients were subdivided according to the Vienna classification[26]. This classification subdivides patients with CD according to age at onset (<40 years [125 cases] or ¡Ý40 years [21 cases]), disease behavior (non-stricturing, non-penetrating [51 cases], stricturing [63 cases], penetrating [32 cases]) and location of disease (terminal ileum [50 cases], colon [32 cases], ileocolonic [62 cases], or upper gastrointestinal tract [2 cases]). Anatomical classification is defined as the maximal extent of the disease prior to the first surgical procedure, while behavior is assessed at any time during the course of the disease.

SNP selection and genotyping

SNPs were selected from our previously collected data[27] to include those SNPs with a prevalence of greater than 5% in Caucasians. Among the SNPs we studied, the only one with a proven functional role is a SNP located in the promoter of *PTGS2*, at position 926 of GenBank entry D28235 (dbSNP rs20417), which has been shown to affect the expression of *PTGS2* mRNA[28].

The order of samples of patients and controls was randomized in the PCR plates, so that a uniform number of patients and controls could be analyzed simultaneously in each run. EPIC samples were genotyped separately.

Three SNPs were genotyped with the Invader Assay[29]. PCR for the Invader assay (Third Wave Technologies, Madison, WI, USA) was carried out in 25 μL reactions, using the following concentrations: 20 ng DNA, 20 μmol/L dNTPs, 1×Taq Platinum buffer, 1.5 mmol/L $MgCl_2$, 1.25 U Taq Platinum polymerase (Life Technologies, Inc., Gaithersburg, MD, USA), 0.5 μmol/L of each primer. The primers used were as follows (nomenclature of SNPs refers to base numbers in GenBank entry D28235):

PTGS2.401 (rs689465)
401F: AAG GAC TTA GGA CAT AAC TGA ATT TTC
401R: ATG GGT AGT GCT CAG GGA GGA G
PTGS2.3050 (rs5277)
3050F: CGT TGT GAA TAA CAT TCC CTT
3050R: ATT TTT CTT TGA GAA GGC TAA AA
PTGS2.5209 (rs20432)
5209F: ATG ATG TAT GCC ACA ATC TGG CTG
5209R: TTG TCT GGA ACA ACT GCT CAT CAC.

This multiplex reaction was then carried out in a Tetrad DNA Engine PCR machine (MJ Research, Waltham, MA, USA), with the following cycling conditions: 96 ¡æfor 5 min, then 30 cycles of 96 ¡æfor 30 s, 50 ¡æfor 60 s, and 72 ¡æ for 10 s, with a final extension of 72 ¡æfor 5 min. PCR volumes were then brought up to 150 μL, and Invader reactions were carried out as per manufacturer's instructions. Plates were read in a fluorometer with excitation and emission spectra as recommended by Third Wave.

The SNPs *PTGS2*.926 and *PTGS2*.8473 were tested using the TaqMan assay with MGB chemistry (Applied Biosystems,

Foster City, CA, USA). The assay was carried out in a 10 μL reaction, with 20 ng DNA, 1×TaqMan master mix (Applied Biosystems), 0.1 μmol/L of each primer, and 0.2 μmol/L of each probe. Primer and probe sequences are as follows:
PTGS2.926 (rs20417)
926F: TTA ACT ATT TAC AGG GTA ACT GCT TAG G
926R: CTT CAC CCC CTC CTT GTT TC
926VIC: CCT TTC CCG CCT CT
926FAM: CTT TCC CCC CTC TC
PTGS2.8473 (rs5275)
8473F: ATG CAC TGA TAC CTG TTT TTG TTT G
8473R: GTT TCC AAT GCA TCT TCC ATG A
8473VIC: TGA CAG AAA AAT AAC CAA AA
8473FAM: TGA CAG AAA AAT GAC CAA A

The cycling conditions for these PCR reactions were as follows: 95 ¡æfor 10 min, then 35 cycles of 95 ¡æfor 15 s, and 60 ¡æ(58 ¡æfor *PTGS2*.926) for 60 s. Plates were then read in the ABI 7900HT sequence detection system.

Data analysis

For genotype data from the Invader assay, raw intensity counts were corrected with a negative control, and the ratio between the two signals (one color for each allele) was used to call each genotype. For the TaqMan assay, groups of genotypes (homozygote common, heterozygote, and homozygote rare) were determined manually within the SDS software (Applied Biosystems). Each SNP was tested in the control group to ensure that it does not deviate from Hardy-Weinberg equilibrium. Linkage disequilibrium tests were performed using macros developed by the authors in conjunction with the PHASE program[30] to reconstruct haplotype frequencies. Odds ratios (OR) and 95% confidence interval (CI) were calculated in Stata 7.0, using logistic regressions correcting for sex and age in analyzing genotypes at single SNPs. Simple OR were calculated when data consisted of frequencies of alleles or haplotypes in the population. All *P* values reported are two sided. SNPs were tested for effects on splicing using the Delila Server (http://www.lecb.ncifcrf.gov/-toms/delilaserver.html) at the Laboratory of Experimental and Computational Biology of the National Cancer Institute[31].

New polymorphism screening

Individuals termed "at risk" based on association testing (i.e., the 19 cases carrying the haplotype, which includes the two alleles showing increase in risk at SNPs 5 209 and 8 473) were screened for novel polymorphisms using denaturing high-performance liquid chromatography (DHPLC) via the WAVE system (Transgenomic, Omaha, NE, USA). The areas studied were the promoter region, exons 6 and 10, and the 3' UTR. The protocols followed were as published previously[27].

RESULTS

Figure 1 shows a graphical representation of the structure of the *PTGS2* gene. The five major SNPs as well as the polymorphisms discovered as part of this study are depicted. The allele frequencies of the five major SNPs in the control group were 11.4% (*PTGS2*.401), 13.4% (*PTGS2*.926), 14.6% (*PTGS2*.3050), 13.5% (*PTGS2*.5209), and 33.9% (*PTGS2*.8473). All SNPs, except *PTGS2*.3050, were in Hardy-Weinberg equilibrium in the control group. Table 1 shows the genotype counts of the SNPs studied, among cases and controls. The SNP at position 5 209 shows an increase in the carriers of the G allele in cases as compared to controls, with a weak but significant association was found only in CD (OR = 1.63, 95%CI 1.03-2.57, $P = 0.04$). The SNP at position 8 473 shows an increase in the frequency of the T/T homozygote genotype in cases as compared to controls, with an association of borderline significance found only in UC (OR = 1.50, 95%CI 1.00-2.27, $P = 0.05$). No association was seen with the SNPs at position 401, 926, or 3 050, although it should be noted that there is a lack of homozygous rare genotypes at bp 3 050 in the control group, which is the reason for its departure from Hardy-Weinberg equilibrium.

We analyzed differences in genotype counts among the different clinical variables for CD and UC. No statistically significant differences between classification levels were observed (data not shown).

Significant levels of linkage disequilibrium were seen among the most common SNPs in the *PTGS2* gene (data not shown). Table 2 shows the frequencies of haplotypes of the *PTGS2* gene in both IBD patients and controls. The SNP at bp 3 050 was not included in haplotype analysis, as it was not in Hardy-Weinberg equilibrium in the control population, and this has been shown to affect the accuracy of haplotype reconstruction methods[32]. All individuals were genotyped for the SNP at bp 3 050, eliminating the risk that its lack of Hardy-Weinberg equilibrium is caused by bias due to the genotyping technique missing preferentially one genotype. Haplotype frequencies were calculated using all four valid SNPs, and all data are shown. There were no differences in haplotype frequency between the two groups of IBD patients and the controls, except for haplotype AGGT, which shows an OR of 11.9 (95%CI 2.83-105.76, $P<0.00005$, for UC and CD combined). Interestingly, this haplotype includes the two alleles showing an increase in risk at SNPs 5 209 and 8 473. If we consider the haplotype including the 5209.G and 8473.T alleles (by lumping haplotypes

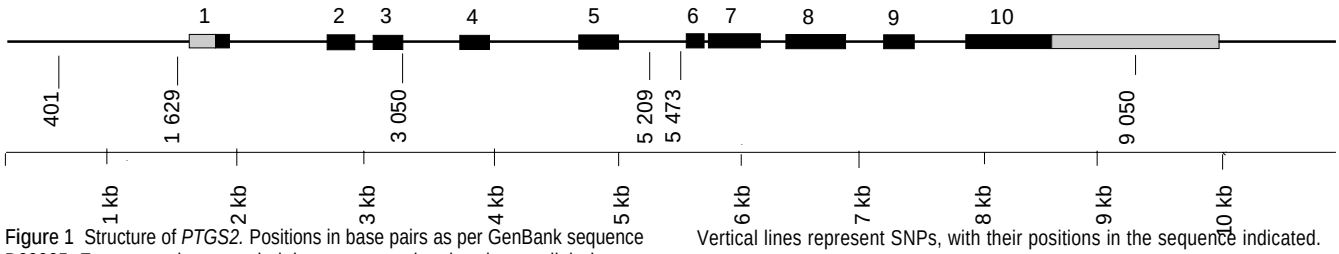

Figure 1 Structure of *PTGS2*. Positions in base pairs as per GenBank sequence D28235. Exons are shown as dark boxes, untranslated regions as light boxes. Vertical lines represent SNPs, with their positions in the sequence indicated.

Table 1 Genotype frequencies and OR for UC and CD of *PTGS2* SNPs

Genotype	Controls		UC			CD		
	n[1]	%	*n*[1]	%	OR (95%CI)[2]	*n*[1]	%	OR (95%CI)[2]
PTGS2.401								
A/A	277	79	115	82	1.00	107	77	1.00
A/G	66	19	24	17	0.82 (0.48-1.39)[3]	30	22	1.17 (0.70-1.94)[3]
G/G	7	2	1	1		1	1	
Total	350	100	140	100		138	100	
PTGS2.926								
G/G	256	75	112	81	1.00	106	76	1.00
G/C	75	22	23	17	0.77 (0.47-1.28)[3]	31	22	1.06 (0.65-1.73)[3]
C/C	8	2	4	3		3	2	
Total	339	100	139	100		140	100	
PTGS2.3050								
G/G	250	71	109	75	1.00	102	71	1.00
G/C	99	28	32	22	0.83 (0.52-1.32)[3]	38	26	0.97 (0.62-1.53)[3]
C/C	1	1	4	3		4	3	
Total	350	100	145	100		144	100	
PTGS2.5209								
T/T	265	75	110	76	1.00	95	66	1.00
T/G	81	23	30	21	1.00 (0.62-1.61)[3]	46	33	1.63 (1.03-2.57)[3]
G/G	7	2	5	3		2	1	
Total	353	100	145	100		143	100	
PTGS2.8473								
C/C	44	13	18	14	1.00[3]	21	15	1.00[3]
T/C	147	42	49	34		54	38	
T/T	155	45	78	54	1.50 (1.00-2.27)	68	47	1.10 (0.72-1.68)
Total	346	100	145	100		143	100	

[1]Numbers may not sum up to the totals of controls or cases due to genotyping failure. All samples that did not give a reliable result in the first round of genotyping were resubmitted to up to three additional rounds of genotyping. Data points that were still not filled up after this procedure were left blank. [2]All ORs are adjusted for sex and age. [3]Heterozygotes and homozygotes for the rare allele have been grouped in order to improve statistical power.

Table 2 *PTGS2* haplotype frequencies and OR for IBD

Haplotype[1]	Controls	UC cases		CD cases		UC+CD cases[2]	
	n (%)	*n* (%)	OR (95%CI)	*n* (%)	OR (95%CI)	*n* (%)	OR (95%CI)[3]
AGTT	471 (64.7)	193 (65.6)	1.00 (ref.)	183 (62.7)	1.00 (ref.)	376 (64.2)	1.00 (ref.)
AGTC	147 (20.2)	55 (18.7)	0.91 (0.64-1.30)	54 (18.5)	0.95 (0.66-1.35)	109 (18.6)	0.93 (0.69-1.24)
GCGC	64 (8.8)	22 (7.5)	0.84 (0.50-1.39)	27 (9.2)	1.09 (0.67-1.75)	49 (8.4)	0.96 (0.63-1.45)
ACGC	25 (3.4)	8 (2.7)	0.78 (0.35-1.73)	7 (2.4)	0.72 (0.31-1.66)	15 (2.6)	0.75 (0.36-1.51)
AGGT	2 (0.3)	10 (3.4)	12.2 (2.97-inf.)	9 (3.1)	11.58 (2.79-inf.)	19 (3.2)	11.9 (2.83-105.76)
Rare[4]	19 (2.6)	6 (2.0)	0.77 (0.31-1.90)	12 (4.1)	1.63 (0.78-3.37)	18 (3.1)	1.19 (0.61-2.29)

[1]The order of SNPs in the haplotypes is *PTGS2*.401, *PTGS2*.926, *PTGS2*.5209, and *PTGS2*.8473. [2]UC and CD combined. [3]ORs are not corrected as they are based on population level haplotype frequencies and are relative to the most common haplotype (AGTT). [4]Rare haplotypes (frequency ¡Ù1% in both cases and controls).

AGGT and GCGT), we observe an increase in its frequency in patients with IBD (0.035, UC and CD combined) as compared to controls (0.003). This increase in frequency yields an OR of 13.15 (95%CI 3.17-116.15, $P<0.00005$, for UC and CD combined).

In order to test the possibility that these positive associations are in reality indicative of linkage disequilibrium with yet undiscovered nearby polymorphisms, we studied by DHPLC the 19 cases who could carry the risk haplotype. Regions of *PTGS2* surrounding the three SNPs, and in addition the promoter region, exon 1 and the UTRs were analyzed. No novel polymorphisms were discovered in the coding sequence of the gene. One SNP was discovered at bp 9 850 (A-G), with a minor allele frequency of lower than 2%. Therefore, we considered it unlikely that polymorphisms other than 5 209 or 8 473 could explain the observed associations.

DISCUSSION

PTGS2 is a critical enzyme involved in the production of prostaglandins, which are essential in the process of healing bowel wounds. The use of NSAIDs, which inhibit both the translation and activity of PTGS2, can induce a flare-up[21] and actually exacerbate the symptoms of IBD[20]. We hypothesized that polymorphisms in *PTGS2* might influence prostaglandin production in inflamed cells, thus affecting susceptibility to IBD. In this study, we have found a slightly increased risk of IBD associated to *PTGS2*.5209 and *PTGS2*.8473, and a strong association with a haplotype including alleles of these two SNPs.

PTGS2.5209 is in intron 5 of *PTGS2*. *In silico* analysis

of this SNP for splice site mutations[31] reveals that the T-G substitution creates a new splicing acceptor sequence with nearly the same strength as that found in normal splice sites. While this is most probably not enough of a change to exclude completely the normal splicing of exons 5-6, it could cause some "leaking", reducing the amount of normal *PTGS2* mRNA. *PTGS2*.8473, being in the 3' untranslated region (UTR), could affect the stability of *PTGS2* mRNA[33]. In fact, the unusually long 3' UTR plays an important role in determining the half-life of the mRNA. Various proteins bind to it and cause either acceleration or protection of the degradation of the mRNA[34,35].

The associations we have found between CD and *PTGS2*.5209 G/G, and UC and *PTGS2*.8473 T/T are statistically significant, but of moderate importance. We propose that it is the combination of the G allele at *PTGS2*.5209 with the T allele at *PTGS2*.8473, on the same chromosome (GT haplotype), that would have the most influence on disease status.

The association between IBD and the GT haplotype could be due to linkage disequilibrium with another polymorphism that lies nearby. To test this hypothesis, we have analyzed subjects who carry the GT haplotype for the presence of novel functional polymorphisms of *PTGS2*, but this search has not yielded any additional candidates. Linkage disequilibrium with polymorphisms in neighboring genes is not a likely explanation either, as there is no gene mapping of at less than 100 kbp on either side of *PTGS2*.

We are inclined to think that this association is not a finding by chance. First of all, we have found a higher level of linkage disequilibrium between alleles at bp 5 209 and 8 473 in controls than in cases. This could mean that natural selection has exerted pressure toward the disappearance of the GT haplotype, i.e. this haplotype might negatively affect the fitness of individuals carrying it. Additionally, we have observed that this haplotype is virtually absent not only in the Dutch population, but in other Northern European populations as well (Cox *et al*., unpublished data).

These results are the first linking of *PTGS2* to IBD. While the GT haplotype of *PTGS2* is rare, the two alleles that compose it are much more frequent, they both show a modest association with the disease, and could therefore be important for a large proportion of the population. Future steps are to replicate these findings by independent studies, to determine experimentally the extent to which *PTGS2* polymorphisms alter the expression or function of the gene, and to discover genetic or environmental factors that may alter the risk of IBD interacting with *PTGS2* alleles.

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