

A functional polymorphism in the promoter region of the cyclooxygenase-2 gene is not associated with asthma and atopy in an Australian population

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Summary

Background Cyclooxygenase (COX)-2 is a key inducible enzyme that regulates the production of anti-inflammatory prostaglandin E₂. A single-nucleotide polymorphism, –765G>C, located within a stimulatory protein-1 binding site in the COX-2 promoter region, has been shown to have significantly lower promoter activity *in vitro* compared with the wild-type and was associated with decreased plasma levels of C-reactive protein after coronary artery bypass surgery. We hypothesized that this polymorphism, which may result in decreased COX-2 transcription, could be associated with more severe asthma, and/or aspirin-intolerant asthma (AIA).

Objective To determine the association between the –765G>C COX-2 polymorphism and asthma, disease severity and AIA in a large, well-phenotyped Australian population.

Methods PCR and restriction fragment length polymorphism analysis was used to characterize the polymorphism in an Australian Caucasian population of patients with mild ($n = 322$), moderate ($n = 254$) or severe ($n = 88$) asthma and in non-asthmatic control subjects ($n = 512$), as well as in patients with AIA ($n = 58$). Genotype and allele association analyses were performed using χ^2 tests.

Results The polymorphic –765C allele was present in approximately 30% of asthmatic patients and non-asthmatic controls. There was no association between the –765G>C polymorphism and asthma ($P = 0.920$), disease severity ($P = 0.840$), atopy ($P = 0.655$) or AIA ($P = 0.841$) in this population.

Conclusion Although the –765G>C polymorphism may have lower promoter activity and result in decreased COX-2 expression, it is not associated with asthma, disease severity, AIA or atopy in this Australian population.

Keywords aspirin-intolerant asthma, asthma, atopy, Australian Caucasian population, cyclooxygenase-2 (COX-2), promoter polymorphism

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Introduction

Asthma is a common chronic respiratory disease, characterized by the occurrence of intermittent and reversible bronchoconstriction, airway hyper-responsiveness, inflammation and/or excessive sensitivity of the airway to allergens and other trigger factors [1]. Although environmental factors are important in asthma, it is increasingly apparent that asthma is a complex genetic disorder [2, 3], with family studies showing strong evidence that polymorphisms in specific genes are relevant to the pathogenesis of the disease [1].

Prostaglandin (PG)D₂ is an important mediator of bronchoconstriction and airway inflammation in asthma [4, 5], whereas PGE₂ relaxes airway smooth muscle and has been hypothesized to have anti-inflammatory effects [6–8]. Cyclooxygenase (COX) is the key enzyme regulating the

synthesis of these prostanoids, with COX-2 being the inducible isoform of the enzyme. Although COX-2 expression is increased in the airways of asthmatic patients [9], it is possible that this represents an anti-inflammatory response to increase the production of bronchoprotective PGE₂ [10].

Aspirin-intolerant asthma (AIA) is a specific subtype of asthma characterized by rhinitis, nasal polyposis and severe attacks of asthma precipitated by the ingestion of aspirin and several other non-steroidal anti-inflammatory drugs (NSAIDs) [11]. NSAID-mediated inhibition of COX-1 may be an important factor triggering asthma attacks in AIA patients, whereas specific COX-2 inhibitors appear to be well tolerated [11]. Although there is some evidence that COX-2 expression is increased in the airways of AIA patients [12], more recent work indicates reduced COX-2 expression in nasal polyps of AIA patients and that this is associated with a down-regulation of nuclear factor- κ B activity [13, 14]. Therefore the transcriptional regulation of COX-2 in AIA may be tissue specific and complex [15] but, as in the case of aspirin-tolerant asthma, will influence the production of

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anti-inflammatory PGE₂. Indeed, current concepts regarding the involvement of COX in the pathogenesis of AIA suggest that inhibition of COX-1 as well as decreased COX-2 expression [13, 14] reduces the production of PGE₂ that might otherwise counter the effects of increased cysteinyl leukotriene synthesis in AIA [11].

Polymorphisms in the promoter region of the COX-2 gene (prostaglandin-endoperoxide synthase 2, PTGS2) are likely to regulate its transcription. Although a number of polymorphisms have been described in the promoter region of the COX-2 gene, only one, located at a putative stimulatory protein-1 (Sp1) binding site, has been shown to be functional. Furthermore, Papafili et al. [16] have shown that this polymorphism, 765 bases upstream of the transcription start site (–765G>C), resulted in a reduction of approximately 30% in *in vitro* promoter activity and was associated with lower plasma levels of the inflammatory marker, C-reactive protein (CRP), following coronary artery bypass graft surgery. Given its potential functional importance and the increasing evidence that expression of COX-2 may have anti-inflammatory consequences [17], we hypothesized that the –765G>C polymorphism might be associated with a more severe inflammatory asthma phenotype and/or possibly with AIA in an Australian Caucasian population.

Materials and methods

Subjects

In order to establish the frequency of the –765G>C polymorphism in the general population, blood samples were collected from 168 Australian Caucasian donors at the Red Cross Blood Bank in Perth, Western Australia. Association studies were performed using DNA isolated from 512 healthy control individuals with no evidence of asthma, and a total of 664 patients with asthma, including 322 individuals with mild, 254 with moderate and 88 with severe asthma as well as 58 patients with AIA. All subjects were unrelated, and aged between 20 and 89 years. The study protocol was approved by the Human Research Ethics Committee of Sir Charles Gairdner Hospital, Perth, the Research Ethics Committee at The Prince Charles Hospital, Brisbane and the Research Ethics Committee at the Monash Medical Centre, Melbourne.

Assessment of asthma severity and aspirin intolerance

A detailed questionnaire was used to categorize patients as having mild, moderate or severe asthma using criteria specified by the Australian National Asthma Council [18] and the NIH National Asthma Education and Prevention Program [19]. These criteria, which assessed lung function, symptoms, medication use and recent asthma-related use of medical services have been described in detail elsewhere [20].

If patients indicated aspirin or NSAIDs as possible triggers for their asthma, they completed a second questionnaire, which we have used previously to assess the prevalence of aspirin intolerance in asthmatic patients [21]. Information was obtained on the incidence of nasal polyps, the specific drug(s) causing adverse reactions, the number of occasions on which adverse reactions had been experienced, the time to

onset of symptoms, incidence of hospitalization following ingestion of these drugs and specific symptoms. Individuals reporting intolerance to these drugs were categorized into (1) those reporting minimal respiratory response or undefined responses, (2) those who had experienced more than one episode with characteristic symptoms of aspirin intolerance or (3) those who had undergone a formal aspirin challenge or who reported multiple characteristic episodes, resulting in hospitalization. For this association study, we included 58 patients from categories 2 or 3 only, of whom 21 were classified as mild, 25 as moderate and 12 as severe asthmatics.

Atopy testing

Assessment of atopic status was based on a skin prick reaction (weal ≥ 3 mm) to at least one of five common aeroallergens: cat, dog, house dust mite, mould mix (*Alternaria tenuis*, *Aspergillus* mix, *Cladosporium*, *Penicillium* mix) and grass pollen mix (Kentucky Blue, Orchard, Red Top, Timothy, Sweet Vernal, Meadow Fescue, Perennial Rye).

The prostaglandin-endoperoxide synthase 2 –765G>C polymorphism

The polymorphism was identified by searching the National Center for Biotechnology Information (NCBI) and SNPper databases [22]. The positions of the polymorphisms in the gene were confirmed using the BLAST program [23] to align the dbSNP cluster (SNP accession no. rs20417) sequences with the COX-2 DNA sequence (GenBank accession no. D28235).

Molecular methods

DNA was extracted from buffy coats using a commercially available DNA extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR and restriction fragment length polymorphism (PCR-RFLP) analysis, and automated sequence analysis (ABI PRISM, Australian Neuromuscular Research Institute, Perth, Australia) were performed to determine –765G>C genotypes. The proximal promoter region of the COX-2 gene was amplified using primers: F: –5'-AGG CAG GAA ACT TTA TAT TGG-3' and R: –5'-ATG TTT TAG TGA CGA CGC TTA-3'. The PCR reaction (10 µL final volume) contained: 20 ng DNA, 1 × PCR buffer (Qiagen), 1 × Q solution (Qiagen), 2.5 mM MgCl₂, 200 µM each of dNTP (Promega, Madison, WI, USA), 10 pmol of each primer and 0.8 U of Taq DNA polymerase (Qiagen). PCR amplification was optimized with an initial denaturation step at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 65 °C for 30 s (decreasing by 0.3 °C per cycle) and 72 °C for 30 s. This was followed by 20 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final extension step was continued at 72 °C for another 5 min.

The –765G>C variant was genotyped using the *AciI* restriction endonuclease (New England Biolabs, Beverly, MA, USA), which recognizes the CCGC sequence. PCR products (10 µL) were incubated with *AciI* (2.5 U) in 1 × buffer (total volume 20 µL) at 37 °C for 4 h. Restriction products were separated in 2.5% agarose gels (Amresco, Solon, OH, USA) in 1 × TBE buffer. Gels were run at 60 V/cm for 1 h, stained with ethidium bromide and visualized

under UV light. Fragment sizes of 209+100 bp indicated a wild-type homozygous –765GG genotype, and an uncut fragment of 309 bp indicated the homozygous –765CC genotype. The presence of all three bands (309, 209, 100 bp) indicated a heterozygous GC genotype and repeat digestions were performed when there was any doubt regarding the visualization of all three bands, to ensure that these represented true heterozygotes rather than random partial digestions.

Statistical methods

Genotype and allele frequencies were calculated for the random Australian Caucasian group and for each of the patient groups. Univariate comparisons of allele and genotype distribution were performed using χ^2 tests and logistic regression analysis, using the R statistics program [24]. Hardy–Weinberg equilibrium analysis for each group was evaluated by the exact test, implemented in the R statistics program.

Results

Subjects

In total, 664 patients with asthma and 512 non-asthmatic control subjects participated in this study and Table 1 summarizes the population demographics of the different

asthma severity groups. All groups had similar demographic characteristics, except that the asthma group as a whole had a significantly higher percentage of atopic subjects (83%) compared with the non-asthmatic control group (51%, $\chi^2 = 134.14$, $P < 0.0001$).

Cyclooxygenase-2 –765G>C promoter polymorphism

The –765G>C promoter polymorphism was investigated in 168 random Caucasian individuals and the frequency of the –765C allele was found to be 0.17 in this population, suggesting that the polymorphism was likely to be common. The frequency of the polymorphism was then determined in 664 asthmatics, including 58 individuals with AIA, and in 512 non-asthmatic control subjects. All groups were in Hardy–Weinberg equilibrium ($P = 0.106$) and Table 2 summarizes the allele and genotype frequencies.

Association analyses

Thirty percent of the patients with asthma and 30% of the non-asthmatic control subjects carried the –765C allele, and the allele frequencies did not differ significantly between these groups ($P = 0.92$). There was no significant difference in allele frequencies among the mild, moderate and severe asthma groups ($P = 0.84$), nor was there an association with AIA ($P = 0.841$) or atopy ($P = 0.655$).

The polymorphic homozygous CC genotype occurred in 1.6% of the non-asthmatic control subjects and in 2.3% of

Table 1. Demographics of the study population

Phenotype	<i>n</i>	Females (%)	Age (years, mean $\pm$ SD)	Atopy (%)	% Predicted FEV ₁ (mean $\pm$ SD)*	ICS use (%)†	Mean ICS dose (μ g/day $\pm$ SEM)	OCS use (%)‡	Frequency of OCS use (mean times/year)
Asthma	664	61.1	48.9 $\pm$ 15.4	82.7	–	–	–	–	–
Mild	322	59.3	47.5 $\pm$ 15.1	83.9	92.0 $\pm$ 16.8	61.4	579.8 $\pm$ 39.0	5.4	1.2
Moderate	254	63.4	50.4 $\pm$ 16.1	81.9	74.5 $\pm$ 20.9	94.0	1414.6 $\pm$ 65.7	38.0	1.4
Severe	88	61.4	49.9 $\pm$ 14.3	80.7	60.0 $\pm$ 23.2	97.7	2682.2 $\pm$ 154.4	79.3	2.9
AIA	58	63.8	46.9 $\pm$ 13.7	81.0	79.6 $\pm$ 21.5	89.6	1589.6 $\pm$ 207.2	48.9	2.2
Non-asthma	512	55.1	51.0 $\pm$ 14.1	50.8	100.6 $\pm$ 15.8	Nil	Nil	Nil	Nil

FEV₁, forced expiratory volume in 1 s; ICS, inhaled corticosteroid; OCS, oral corticosteroid; AIA, aspirin-intolerant asthma; SD, standard deviation; SEM, standard error of the mean.

*FEV₁ measurements were available for 506 non-asthma, 316 mild asthma, 250 moderate asthma, 85 severe asthma and 48 AIA patients.

†Data for ICS use and dose was available for 509 non-asthma, 316 mild asthma, 250 moderate asthma, 87 severe asthma and 48 AIA patients.

‡Data for OCS use and frequency was available for 510 non-asthma, 315 mild asthma, 250 moderate asthma, 87 severe asthma and 47 AIA patients.

Table 2. Allelic and genotype frequencies of the –765G>C polymorphism

Subjects	<i>n</i>	Genotype (%)			Frequency of C allele	Distribution of C allele (%)
		GG	GC	CC		
Random Caucasians	168	116 (69)	48 (28.6)	4 (2.4)	0.17	31
Non-asthmatic controls	512	355 (69.3)	149 (29.1)	8 (1.6)	0.16	30.6
All asthmatics	664	463 (69.7)	186 (28.0)	15 (2.3)	0.16	30.3
Mild asthma	322	229 (71.1)	84 (26.1)	9 (2.8)	0.16	28.9
Moderate asthma	254	171 (67.3)	78 (30.7)	5 (2)	0.17	32.7
Severe asthma	88	63 (71.6)	24 (27.3)	1 (1.1)	0.15	28.4
Non-atopic	366	251 (68.6)	108 (29.5)	7 (1.9)	0.17	31.4
Atopic	810	567 (70.0)	227 (28.0)	16 (2.0)	0.16	30.0
AIA	58	42 (72.4)	14 (24.0)	2 (3.5)	0.16	27.6
ATA	606	421 (69.5)	172 (28.4)	13 (2.1)	0.16	30.5

AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma.

the asthmatic patients, but this difference was not significant ($P = 0.525$). It was interesting to note that the CC genotype occurred in 2.8% of mild and 2% of moderate asthmatic patients, but was present in only 1.1% of patients with severe asthma and in 3.5% of patients with AIA. However, these differences were also not significant ($P \geq 0.315$). Allelic odds ratios (ORs) (assuming a multiplicative penetrance model) and profile likelihood-based 95% confidence intervals (CIs) were calculated. For all asthma vs. no asthma, the OR was 0.988 (95% CI, 0.787–1.240), while for severe asthma vs. no asthma, the OR was 1.117 (95% CI, 0.711–1.812) and for AIA vs. no asthma, the OR was 1.049 (95% CI, 0.620–1.861). The study is reasonably powerful, as measured by the half-width of the confidence regions and we can exclude any large effect of the polymorphism on the total risk of asthma. Even for AIA (with 58 cases), we can strongly exclude a twofold increase in risk associated with this polymorphism, although a true 1.5-fold relative risk could be consistent with our data.

Discussion

COX-2 is an essential regulatory enzyme in the prostanoid biosynthetic pathway and a functional G>C polymorphism has been described in the promoter region of the COX-2 gene, 765 bases upstream of the transcription start site [16]. The –765C allele has been shown to be associated with a 30% reduction in promoter activity *in vitro* compared with the wild-type –765G allele [16]. The study reported here is the first comprehensive investigation of the potential association of this polymorphism with asthma. Our results show that in a large, well-phenotyped population there is no association between this polymorphism and asthma, disease severity or AIA.

The COX-2 gene is localized to chromosome 1q25.2–25.3 and a number of polymorphisms have been described in the promoter region of this gene. To date, only one has been shown to have functional significance. Papafili et al. [16] have described and characterized a polymorphism (–765G>C) at a putative Sp1 binding site in the COX-2 promoter, which resulted in significantly decreased transcription of a reporter gene in transient transfection experiments. Multiple putative Sp1 binding sites are present in the COX-2 promoter and the –765G>C polymorphism could interfere with the mechanism by which Sp1 increases transcription by binding at one of these sites. Alternatively, the polymorphism may alter the binding of other transcription factors such as Sp3 that compete for Sp1 binding sites [25]. The fact that we have been unable to show an association between asthma severity and the –765G>C polymorphism does not exclude the possibility that other putative Sp1 or transcription factor binding sites are important in the regulation of COX-2 transcription in asthma.

To date, only the study of Papafili et al. [16] has reported an association between the –765G>C COX-2 promoter polymorphism and a specific disease phenotype. That study showed that patients carrying the –765C allele, who underwent coronary artery bypass graft surgery, had significantly lower plasma CRP levels after surgery compared with patients homozygous for the –765G allele, suggesting that

the COX-2 –765G>C polymorphism might influence inflammatory responses. CRP is an acute-phase protein and its concentration increases rapidly during systemic inflammation [26]. If it can be assumed that there is a link between reduced CRP levels and decreased COX-2 transcription, then the observation that plasma CRP levels were lower after bypass surgery in patients carrying a –765C allele is consistent with *in vitro* data from the same study showing lower promoter activity for the –765C allele [16]. However, the absence of an association between the –765G>C polymorphism and asthma severity in our study suggests that the hypothesis, that decreased COX-2 transcription because of this polymorphism has anti-inflammatory consequences, may not necessarily hold true in chronic inflammatory conditions such as asthma.

The present study is the first detailed investigation of the possible association of asthma with the COX-2 –765G>C polymorphism. However, a recent conference abstract [27] reported no differences in –765C allele frequencies among control subjects (0.16, $n = 113$), aspirin-tolerant asthmatics (0.19, $n = 93$) and AIA patients (0.17, $n = 118$), which is consistent with the findings from our study. Nevertheless, a significant association was reported between the homozygous CC genotype and asthma in that Polish population. In the present study, we did not find a significant association between the CC genotype and asthma severity. However, it is interesting that in our population 2.8% of mild asthmatics, 2.0% of moderate asthmatics, and 1.1% of severe asthmatic patients were CC homozygotes. Thus, contrary to the results of Szczeklik et al. [27], our data, if anything, indicates an under-representation of the CC genotype in more severe asthma. However, these findings were not statistically significant and should be interpreted with caution as the number of patients in the severe asthma group was relatively small.

Neither in our population nor in that of Szczeklik et al. [27] was there an association between AIA and the frequency of the –765C allele. In our study, the frequency of the CC genotype was also not different in AIA patients compared with control subjects. However in the Polish study, which may have had greater power because of the larger number of AIA patients studied, a significantly greater proportion of AIA patients (7.3%) than control subjects (0.9%) were CC homozygotes [27]. Given that the CC genotype appears to be associated with decreased COX-2 transcription [16], over-representation of the CC genotype among AIA patients would be consistent with the hypothesis that decreased COX-2 expression and PGE₂ production contributes to the pathogenesis of AIA [14, 15]. However, the fact that we were unable to confirm the observations of Szczeklik et al. [27] in our group of AIA patients suggests that further studies are required to assess the potential importance of the –765G>C polymorphism in the context of AIA. Studies on the leukotriene C₄ synthase promoter –444A>C polymorphism suggest an association between the –444C allele and a more severe AIA phenotype [28], but no such association has been found in studies that have included mild, moderate and severe AIA patients [20, 29]. Similarly, genetic variation in the COX-2 gene may be more closely associated with a severe AIA phenotype. Nevertheless, a recent study from Korea also found no associations between AIA and other polymorphisms

in the COX-2 gene ($-162C>G$, $10T>G$), but did identify the possible involvement of a specific 5-lipoxygenase (ALOX5) haplotype in AIA [30].

There is increasing evidence that the products of COX-2 have numerous inflammatory, anti-inflammatory, anti-thrombotic and anti-fibrotic functions [17, 31] and may be associated with onset of the inflammatory process and then later aid in its resolution [17]. COX-2 is known to be differentially expressed in specific inflammatory phenotypes, including asthma and AIA [9, 13]. In this study, we investigated the $-765G>C$ polymorphism in the COX-2 gene in isolation because of its location in a putative Sp1 binding site in the promoter region and its potential functional importance. However, our results indicate that irrespective of its possible effects on gene expression, this polymorphism is not associated with asthma, asthma severity, AIA, or atopy in an Australian Caucasian population. In future work we will investigate other COX-2 promoter and exonic polymorphisms and perform haplotype analyses, which will have greater power to detect associations between asthma and variation in the COX-2 gene.

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