85-kDa cytosolic phospholipase A₂ group IVα gene promoter polymorphisms in patients with severe asthma: a gene expression and case–control study

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Summary

Cytosolic phospholipase A₂ (cPLA₂) group IVα is a critical enzyme involved in the liberation of arachidonic acid from cellular membranes. $cPLA_2^{-/-}$ mice have reduced allergen-induced bronchoconstriction and bronchial hyperresponsiveness. The goal of this study was to investigate polymorphisms of the $(CA)_n$ and $(T)_n$ microsatellites and surrounding regions in the $cPLA_2\alpha$ gene promoter. We analysed the $cPLA_2$ promoter regions containing $(CA)_n$ and $(T)_n$ repeats in 87 patients with severe asthma and in 48 control subjects by bidirectional sequencing. Functional studies were performed utilizing reporter genes derived from subjects with varying numbers of these repeats, and on constructs with a series of deletions. We found that the $(CA)_n$ and $(T)_n$ regions are polymorphic and that constructs with CA or T repeats or CA and T repeats deleted revealed, respectively, a 41·8 ± 7%, 22·3 ± 5% and 100 ± 20% increase in reporter gene activity. A lower number of CA or T repeats caused higher $cPLA_2$ promoter luciferase activity. The group of shorter alleles of the $(CA)_n$ microsatellite region ($n = 12$–18) ($P_{cor} = 0.00006$), and the group of shorter alleles of $(T)_n$ repeats region ($n = 17$–38) ($P_{cor} = 0.0039$) occurred significantly more often in patients with severe asthma. We also found novel SNPs in positions −292 C > G, −185 A > C, −180 T > C and −165 A > C. Two of them were associated with the severe asthma phenotype: −180T allele ($P_{cor} = 0.03996$) and −185 A allele ($P_{cor} = 0.03966$). These results demonstrate that $(CA)_n$ and $(T)_n$ repeats may have an influence on cPLA₂ transcription which might play a role in severe asthma pathogenesis.

Keywords: airway inflammation, cytosolic phospholipase A₂, gene expression, microsatellites, severe asthma

Introduction

The group IV cytoplasmic 85-kDa phospholipase A₂α (cPLA₂α) is the major intracellular form of PLA₂, expressed constitutively in many tissues, which preferentially hydrolyses membrane phospholipids at the *sn-2* position to release arachidonic acid and lysophospholipids. Thus, it represents the rate-limiting enzyme in eicosanoid production [1]. This arachidonic acid-selective event occurs after translocation of cPLA₂ from the cytosol to the perinuclear membrane in response to cell activation [2]. The cPLA₂-induced release of arachidonic acid from glycerol phospholipids provides a precursor for the synthesis of prostaglandins (PGs), leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and

platelet-activating factor (PAF). These eicosanoids may work as intracellular lipid second messengers or potent inflammatory mediators [3].

It has been shown that eicosanoids play a role in airway inflammation in bronchial asthma [4]. Patients with severe asthma had persistent airway inflammation despite chronic long-term treatment with anti-inflammatory agents including oral glucocorticoids [5]. In patients with severe asthma, urinary cysteinyl leukotrienes levels were increased significantly compared to moderate asthma patients, suggesting up-regulation of arachidonic acid pathways in severe asthma [6].

cPLA₂α might play a role in airway inflammation, although this gene has not been well studied in asthma.

Table 1. Characteristics of groups studied.

	Patients	Controls
n	87	48
Median age (range, years)	41·5 (18–75)	37 (18–68)
Female/male (%)	55/45	56/44
Median FEV_1 (range, %)	71 (36–113)	n.a.
Median ICS (range, μg per day)[a]	1600 (800–4800)*	0
Oral GCS (range, mg per day)[b]	5 (0–25)	0
Aspirin intolerance (%)	13·8	0
Chronic rhinosinusitis (%)	33	0
Median eosinophilic count (range, cell/mm^3)	200 (0–1326)	n.a.

[a]Calculated as budesonide equivalent; [b]calculated as prednisone equivalent. *Some patients received inhaled corticosteroids (ICS) in nebulization. FEV_1: forced expiratory volume in 1 second; GCS: glucocorticosteroids.

Critical evidence for the role of $cPLA_2$ in asthma and other inflammatory diseases was delivered by studies on $cPLA_2$ knock-out mice. $cPLA_2^{-/-}$ mice had a more rapid recovery from allergen-induced bronchoconstriction and had no airway hyperresponsiveness [7]; as well, they lacked the ability to generate leukotrienes, prostaglandins and PAF from a variety of cell types and stimuli [8–10]. $cPLA_2$ is essential for both the immediate and the delayed phases of eicosanoid generation in mast cells [10]. Other studies have revealed that ovalbumin challenge increased the expression of $cPLA_2$ in lung tissue [11].

The human $cPLA_2$ promoter has been cloned [12,13]. It is TATA-box-less but does contain an initiator element at the transcription start site. It is GC poor and lacks Sp-1 consensus sites. In addition, some regulatory sequences such as Oct (octamer binding sites) were described within its promoter region, raising the possibility of its regulation at the level of transcription [12]. In addition, studies on the functional activity of the $cPLA_2$ promoter revealed that within the 5′-flanking region there are $(CA)_n$ and $(T)_n$ simple sequence repeats which might act as potential negative regulatory elements [12,14].

Therefore, in order to understand further the transcriptional regulation of the $cPLA_2$ gene, we performed studies on these two possible regulatory regions within the $cPLA_2$ promoter. The aim of our study was, first, to investigate whether $(CA)_n$ and $(T)_n$ simple sequence repeats are conservative or polymorphic in the $cPLA_2$ gene in humans in order to analyse whether the possible inhibitory effect of these regions depends on the number of $(CA)_n$ or $(T)_n$ repeats. The final goal of the study was to examine the association of polymorphisms in the analysed regions with the severe asthma phenotype.

Materials and methods

Patients

A group of 135 unrelated subjects living in the central area of Poland was studied. The study group involved 87 patients with severe bronchial asthma selected randomly from a severe asthmatic group treated in the out-patient unit of the department of immunology, rheumatology and allergy in Lodz. Asthma was defined according to criteria consistent with the Global Initiative for Asthma (GINA) 2005 report [15]. The severity of the disease was assessed according to the above guidelines. A patient was included in the severe asthma group if, before the beginning of treatment, any of the following features were observed: daily symptoms, frequent exacerbations, frequent nocturnal asthma symptoms, limitation of physical activities, forced expiratory volume in 1 second (FEV_1) or peak expiratory flow (PEF) $\leq$ 60%, PEF or FEV_1 variability > 30%. When the patient had already been on treatment, the classification of asthma severity was performed by daily medication regimen and response to treatment. The sex- and age-matched control group consisted of 48 unrelated subjects with a negative history of atopic diseases and no history of allergy and asthma in parents and siblings. Characteristics of the studied groups are presented in Table 1. The study was approved by the Ethics Committee of the Medical University of Lodz and informed consent was obtained from every subject prior to the study.

Tissue culture

A549 cells, a human adenocarcinoma cell line, were obtained from the American Type Culture Collection (ATTC, Manassas, VA, USA) and cultured as described previously [16]. Briefly, cells were grown in Ham's 12K medium (Biosource, Carlsbad, CA, USA) with 10% fetal bovine serum (Biosource) and 2 mM of L-glutamine (Biosource). All experiments were performed when cells were 80–90% confluent.

Genomic DNA preparation

Genomic DNA was extracted from peripheral blood mononuclear cells of the studied subjects using Genomic Maxi AX (A&A, Gdansk, Poland) according to the manufacturer's protocol.

(CA)$_n$ and (T)$_n$ repeat regions in cPLA$_2$ promoter genotyping

A 349-base pairs (bp) fragment containing the (T)$_n$ repeat region was obtained with the following primers: 5′-TTGTGTCTGGGGGAAAGAGATG-3′ (forward) and 5′-GAGACTATCCTGGCTAACACGGTG-3′ (reverse) (Tib MolBiol, Poznan, Poland).

A 422-bp fragment containing the (CA)$_n$ repeat region was amplified with the following primers: 5′-CAACACAGGTGTTCTGAGTCTGGAG-3′(forward) and 5′-AGAGGGAGACGCTCTCTTCTCATAG-3′(reverse) (Tib MolBiol). PCR fragments were purified with the Qiaquick Purification Kit (Qiagen, Valencia, CA) and Clean-up (A&A), according to the manufacturers' protocols. These fragments were then labelled using a fluorescently labelled dye terminators technique (Big Dye Terminator Cycle Sequencing version 1·1, Applied Biosystems, Foster City, CA, USA) with specific primers for each direction. To analyse the sequence including the (CA)$_n$ repeats the following primers were used: 5′-AGGTTATTCACTGTCTTTTC-3′ (forward), 5′-AGAGGGAGACGCTCTCTTCTCATAG-3′ (reverse) and for the sequence including the (T)$_n$ repeats: 5′-CTACCAGTGTGCTCTCTG-3′ (forward) and 5′-AGGTTATTCACTGTCTTTTC-3′ (reverse) (Tib MolBiol), as all samples were sequenced in both directions. The second purification step was performed with the Qiaquick Nucleotide Removal Kit (Qiagen) and ExTerminator (A&A). All sequencing reactions were performed in an ABI Prism 310 capillary sequencer (Applied Biosystems).

Reporter genes construction

A 2180-bp cPLA$_2$ promoter region insert was prepared using a polymerase chain reaction (PCR) technique. DNA was obtained from peripheral blood mononuclear cells of the studied subjects. The following primers were used: the 5′ sense primer: 5′-GAGCTCTGGACAAACCAAGTGGAGAGGG-3′ and the 3′ anti-sense primer: 5′-GCTAGCAGTTCCCAGAGTTACCTGAGAATC-3′. The sense primer had a *Sac*I restriction site added to the 5′ end and the anti-sense primer had a *Nhe*I restriction site added to the 5′ end. The product was used in comparative studies of patients with variable numbers of (CA)$_n$ and (T)$_n$ repeats. Furthermore, it was used as a basic template to generate truncation constructs by PCR. Δ(CA)$_n$ and Δ(T)$_n$ mutations of the 2180 bp cPLA$_2$ basic promoter template were made using a PCR technique. The deletion of (T)$_n$ region was performed utilizing the following primers: the 5′ sense primers: 5′-CTCTCTGCTCCTCTCAACTTGGCTCACTGCAAGCTC-3′, 5′-CAACTTGGCTACTGCAAGCTC-3′ and the 3′ anti-sense primers: 5′-TTGCAGTGAGCCAAGTTGAGAGGAGCAGAGAGCACACTGG-3′, 5′-AGAGGAGCAGAGAGCACACTGG-3′. The deletion of (CA)$_n$ region was performed using the 5′ sense primers: 5-CAAGTAGCAATTTCAGACGCGC

ATATTTTCTGACTTCAAACTCC-3′, 5′-GCATATTTTCTGACTTCAAACTCCTGG-3′, 5′-CAAGTAGCAATTTCAGACGCGAAATCCACAACAGCACTCATGG-3′, GAAATCCACAACAGCACTCATGG-3′ and the 3′ anti-sense primers: 5′-TGAAGTCAGAAAATATGCGCGTCTGAAATTGCTACTTGTCC-3′, 5′-GCGTCTGAAATTGCTACTTGTCC-3′, 5′-TGAGTGCTGTTGTGGATTTCGCGTCTGAAATTGCTACTTGTCC-3′. Subsequently, the simultaneous deletion of (CA)$_n$ and (T)$_n$ regions (Δ(CA)$_n$-(T)$_n$ constructs) was prepared utilizing both sets of these primers.

The amplified PCR products were verified by gel electrophoresis and cloned into pCRII TOPO vector (Invitrogen, Carlsbad, CA, USA). The inserts were removed from the vector by digestion with *Sac*I and *Nhe*I and isolated by gel electrophoresis on a 2% agarose gel. Then, recovered inserts were subsequently subcloned into linearized pGL3 basic vector (Promega, Madison, WI, USA) using a T4 DNA ligase reaction (Promega). The identity and orientation of the inserts were confirmed by full bidirectional sequencing.

Transient transfection and reporter activity assessment

A549 cells were transfected with 1·5 μg of reporter gene and 0·5 μg of Renilla/pRL-cytomegalovirus (CMV) vector as a control for transfection efficiency. Cells were transfected in six-well dishes (PGS Scientific, Bethesda, MD, USA) using Lipofectamine Plus reagent (Invitrogen) for 4 h in serum-free Ham's F-12K medium (containing 2 mм L-glutamine). After transfection, medium was replaced with standard Ham's F-12K medium containing 10% fetal bovine serum and 2 mM glutamine and cells were maintained for 16 h.

Cells were washed three times in ice-cold phosphate-buffered saline and lysed using passive lysis buffer (Promega). Cell lysate was frozen at −80°C. Luciferase activity was measured using a double luciferase assay system (Promega) with a Turner TD20 luminometer (Promega).

Statistical analysis

Genotype and allele frequencies were compared between patients and controls using a χ^2 Yates' test for independence and Fisher's test when appropriate. Hardy–Weinberg equilibrium was determined using the test provided by the Institute of Human Genetics, Technical University Munich, Germany (http://ihg.gsf.de/cgi-bin/hw/hwal.pl/). A two-tailed P-value < 0.05 was considered to indicate statistical significance. In functional deletion studies, data are expressed as mean $\pm$ standard error of the mean (s.e.m.) of the relative luciferase activity from at least six different experiments. Student's t-test was used to compare the difference between promoter activities. The difference was considered statistically significant when $P < 0.05$. To correct for incidental significance, the P-value was multiplied by the

number of independent comparisons performed (P_{cor}). Thus, the P-values in the case–control studies were multiplied by six, because we analysed two microsatellite regions and four SNPs.

Results

Functional analysis of $(CA)_n$ and $(T)_n$ regions

Direct sequencing of the $cPLA_2$ promoter revealed the presence of one mononucleotide $(T)_n$ and one dinucleotide $(CA)_n$ repeats region. The dinucleotide purine–pyrimidine sequence consists mainly of $(CA)_n$ repeats and is located around the −256 to −221 bp position from the transcription start site. The mononucleotide sequence centred on the −1305 bp position consists of $(T)_n$ repeats. These regions appeared to be highly polymorphic in the studied population. To elucidate whether the variable number of $(CA)_n$ and $(T)_n$ repeats may effect $cPLA_2$ gene expression, we performed luciferase reporter gene studies. First, we prepared a basic reporter gene construct [basic template (BT)] from the whole 2180 bp region of the $cPLA_2$ promoter. It included the longest $(T)_n$ microsatellite repeats region and the longest $(CA)_n$ microsatellite repeats region. A series of promoter deletions was then performed employing a PCR technique: $\Delta(CA)_n$ – the construct with the $(CA)_n$ repeats region deleted, $\Delta(T)_n$ – the construct without the $(T)_n$ region and $\Delta(CA)_n$–$\Delta(T)_n$ – the construct with both of these regions deleted. These constructs were transfected to the A549 cell line and a double luciferase assay was performed to evaluate promoter activity. Figure 1 presents the results obtained. Constructs without the $(CA)_n$ repeats region revealed a $41\cdot8 \pm 7\%$ increase in reporter gene activity ($P < 0\cdot05$). Constructs with the deletion of the $(T)_n$ repeats region revealed a $22\cdot3 \pm 5\%$ increase in reporter gene activity ($P < 0\cdot05$). Promoter constructs with the simultaneous deletion of the $(CA)_n$ and $(T)_n$ repeats regions revealed a twofold increase in reporter gene activity compared to the basic template ($100\% \pm 20\%$) ($P < 0\cdot05$).

Further, considering that none of the studied patients and controls had either the whole $(CA)_n$ microsatellite region or $(T)_n$ region deleted, we performed luciferase reporter gene studies with the promoter constructs derived from polymorphic individuals. The results are shown in Figs 2 and 3. A lower number of CA or T repeats found in the $cPLA_2$ promoter derived from those individuals was associated with a higher $cPLA_2$ reporter gene activity, whereas a higher number of these repeats was associated with a decreased reporter gene activity. In case of the $(CA)_n$ microsatellite region, a significant decrease in relative luciferase activity appeared with $n = 19$ and more CA repeats in comparison to the shorter $(CA)_n$ regions. In the case of the $(T)_n$ microsatellite region, the presence of $n = 41$ and more T nucleotides caused a significant decrease in relative luciferase activity in comparison to the shorter $(T)_n$ repeats regions.
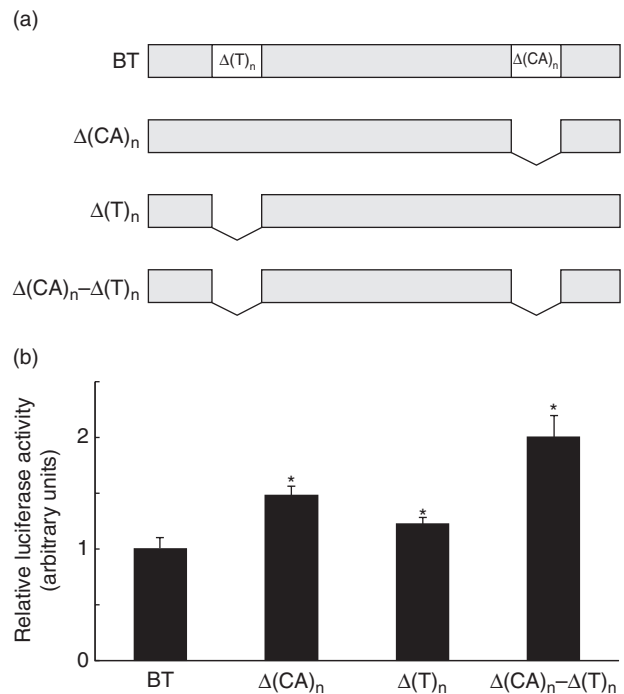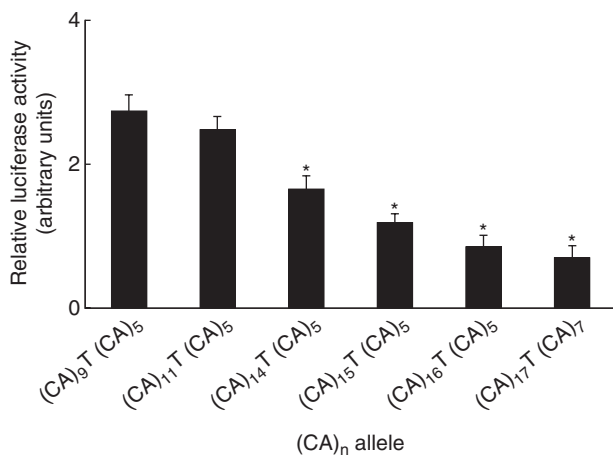

Fig. 1. Cytosolic phospholipase A_2 (cPLA2) reporter gene activity. Schematic diagram of prepared constructs with the whole region $(CA)_n$, $(T)_n$ or $(CA)_n$ and $(T)_n$ region deleted (a) and effect of these deletions on cPLA2 reporter gene activity compared to the basic template (BT) (b). (a) The 2180 bp cPLA2 promoter region insert was prepared using a polymerase chain reaction technique from DNA obtained from peripheral blood mononuclear cells of the studied subjects. It was used as a BT to generate truncation constructs by polymerase chain reaction. $\Delta(CA)_n$ – construct with the $(CA)_n$ repeats region deleted, $\Delta(T)_n$ – construct without the $(T)_n$ region and $\Delta(CA)_n$–$\Delta(T)_n$ – construct with both of these regions deleted. (b) Constructs were transfected to the A549 cell line and a double luciferase assay was performed to evaluate promoter activity. $\Delta(CA)_n$ revealed a $41\cdot8 \pm 7\%$ increase in reporter gene activity ($P < 0\cdot05$). $\Delta(T)_n$ revealed a $22\cdot3 \pm 5\%$ increase in reporter gene activity ($P < 0\cdot05$). Promoter constructs $\Delta(CA)_n$–$(T)_n$ revealed a twofold increase in reporter gene activity compared to BT ($100 \pm 20\%$) ($P < 0\cdot05$). Data are expressed as mean $\pm$ standard error of the mean of the relative luciferase activity from at least six different experiments. *$P < 0\cdot05$ as compared to BT.

Analysis of the $(CA)_n$ microsatellite region

Analysis of the number of CA repeats

The $(CA)_n$ repeats region located around −256 to −221 bp in the $cPLA_2$ promoter appears to be highly polymorphic, although we found only the homozygotic variants of this fragment in the studied populations. In the patient group, the structure of this sequence varied from $(CA)_{12}T$ to $(CA)_{17}T(CA)_7$, varying in the number of $(CA)_n$ repeats. In the control group, this sequence differed from $(CA)_{13}T(CA)_5$ to $(CA)_{13}T(CA)_{10}$. In relation to our functional gene expression data, within this microsatellite region we studied two

Fig. 2. Cytosolic phospholipase A_2 (cPLA$_2$) reporter gene activity depends on the length of the $(CA)_n$ repeats region. cPLA$_2$ reporter gene activity in promoter constructs derived from individuals polymorphic in the number of $(CA)_n$ repeats. The cPLA$_2$ promoter region insert was prepared using a polymerase chain reaction technique from DNA obtained from peripheral blood mononuclear cells of the studied subjects. A lower number of CA repeats resulted in a greater *cPLA$_2$* reporter gene activity, whereas a higher number of these repeats decreased reporter gene activity. *$P < 0.05$ compared to $(CA)_9T$ $(CA)_5$. Data are expressed as mean $\pm$ standard error of the mean of the relative luciferase activity ($n = 5$–6).

groups of alleles: the shorter ($n = 12$–18) and the longer ($n = 19$–24) ones. The comparison between the patients and controls revealed that indeed the group of shorter alleles of the $(CA)_n$ microsatellite region ($n = 12$–18) occurred significantly more often in patients with severe asthma (96·65%) than in the control group (67·67%) ($P_{cor} = 0.00006$, OR = 14·0). To correct for incidental significance, here the *P*-value was multiplied by six because we analysed independently two microsatellite regions and four SNPs. Thus, the structure of the $(CA)_n$ microsatellite region in patients with severe asthma seemed to be significantly shorter than in the control group.

Novel SNPs around the $(CA)_n$ microsatellite region of the cPLA$_2$ promoter

The direct bidirectional sequencing analysis of the whole −351 to −74 bp fragment of the *cPLA$_2$* promoter revealed that not only the $(CA)_n$ region was polymorphic in the number of CA repeats but there were also some novel single nucleotide polymorphisms (SNPs) in the close neighbourhood of this region. We found novel SNPs in positions: −292 C > G, −185 A > C, −180 T > C and −165 A > C. Frequencies of these alleles and genotypes are shown in Table 2. To determine whether there is an association between these novel SNPs and bronchial severe asthma we analysed whether each of the genotypes at each locus was consistent with Hardy–Weinberg equilibrium and then we performed

association studies. We found that −180T allele [$P_{cor} = 0.03996$, OR = 3·917, CI = (1·385 11·079)], as well as the −185 A allele [$P_{cor} = 0.03966$, OR = 3·675, CI = (1·374 9·826)] were present significantly more often in patients with severe asthma. To correct for incidental significance, here the *P*-values were multiplied by six. We found no significant difference in distribution of the other newly described polymorphisms between the patient and the control group.

Analysis of the $(T)_n$ microsatellite region

Analysis of the number of T repeats

Amplification and bidirectional sequencing of the amplified 349 bp fragment revealed that the $(T)_n$ region is highly polymorphic, but also in a homozygotic manner, in the studied populations. We found that the number of $(T)_n$ repeats varied both in the patient and the control group from 17 to 46. Here, also in relation to our functional data, two groups of alleles were isolated. The comparison between patients and controls revealed that the group of shorter alleles of $(T)_n$ repeats region ($n = 17$–38) occurred significantly more often in the patient group than in the control group (77·1% in patients *versus* 44·9% in controls; $P_{cor} = 0.0039$, OR = 4·14). The *P*-value here was also multiplied by six.

Discussion

The 85-kDa cPLA$_2\alpha$ is a key enzyme in mediating agonist-induced arachidonic acid release and regulating transcription of some inflammatory genes such as interleukin-8
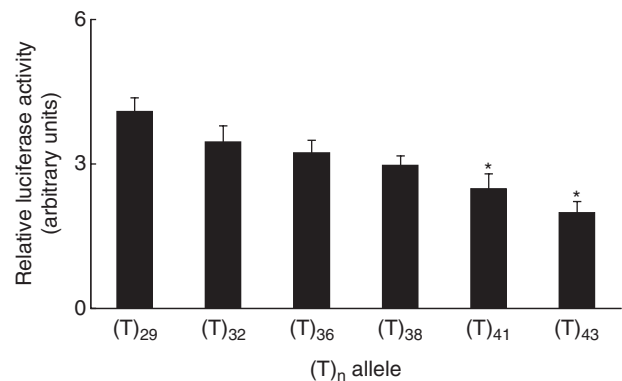
Fig. 3. Cytosolic phospholipase A_2 (cPLA$_2$) reporter gene activity depends on the length of the $(T)_n$ repeats region. cPLA$_2$ reporter gene activity in promoter constructs derived from individuals polymorphic in the number of $(T)_n$ repeats. The cPLA$_2$ promoter region insert was prepared using a polymerase chain reaction technique from DNA obtained from peripheral blood mononuclear cells of the studied subjects. Constructs with shorter forms of the $(T)_n$ repeats revealed a significant increase in reporter gene activity compared to longer forms of the $(T)_n$ repeats ($n = 41$ and more). *$P < 0.05$ compared to $(T)_{29}$. Data are expressed as mean $\pm$ standard error of the mean of the relative luciferase activity ($n = 4$–5).

Table 2. Genotype frequencies of cytosolic phospholipase A_2 ($cPLA_2$) promoter single nucleotide polymorphisms (SNPs) in groups of studied patients and controls.

SNP and sample	Allele and frequency		Genotype and frequency	P_{cor} for	
				Allele test	Genotype test
−292 C > G	C	G	CC/CG/GG	n.s.	n.s.
Patients	0·71	0·29	0·26/0·74/0·00		
Controls	0·72	0·28	0·13/0·87/0·00		
−185 A > C	A	C	AA/AC/CC	0·03966	0·0201
Patients	0·93	0·07	0·86/0·14/0·00		
Controls	0·78	0·22	0·57/0·43/0·00		
−180 T > C	T	C	TT/TC/CC	0·03996	0·02214
Patients	0·94	0·06	0·88/0·12/0·00		
Controls	0·80	0·20	0·60/0·40/0·00		
−165 A > C	A	C	AA/AC/CC	n.s.	n.s.
Patients	0·63	0·37	0·42/0·58/0·00		
Controls	0·57	0·43	0·43/0·57/0·00		

(*IL-8*) and cyclooxygenase-2 (*COX-2*) in human lung cells [16]. Together with the current effectiveness of anti-leukotriene therapy in severe asthma and of the $cPLA_2\alpha$ inhibitor (Efipladib®) in a Phase II clinical trial (Wyeth Annual Review 2005, http://ccbn.mobular.net/ccbn/7/1372/1467/index.html), these data suggest that the $cPLA_2$ and $cPLA_2$-dependent arachidonate metabolism might play an important role in inflammation.

The studies performed by Wu *et al.* [12] and Dolan-O'Keefe *et al.* [14] revealed that the CA repeat region in *cPLA2* may have a suppressor effect on *cPLA2* gene transcription. Deletion of this region from the promoter resulted in a 20–30% [12] or a 40–50% [14] increase in *cPLA2* promoter activity. The results of our functional studies with the promoters of severe asthmatic patients, with a variable number of CA repeats, provide new evidence for a regulatory function of the CA repeats. We showed that even the difference of five dinucleotide CA repeats may change luciferase activity significantly. More $(CA)_n$ repeats in the *cPLA2* promoter resulted in less relative luciferase activity. The deletion of the whole CA repeats region in our study caused a $41·8 \pm 7\%$ increase in reporter gene activity, which confirms previous results. There is also some evidence that not only *cPLA2* gene transcription might be regulated by a different number of $(CA)_n$ repeats. The first intron of the interferon-γ (*IFN-γ*) gene contains a CA microsatellite repeat that is highly polymorphic, with up to six alleles [17]. Allele 2 with 12 CA repeats was shown to be associated with a high level of *IFN-γ* production *in vitro* [17]. In the oestrogen receptor beta (*ESRB*) gene, the number of $(CA)_n$ repeats was associated with androgen and sex steroid-hormone binding globulin (SHBG) [18]. When we compared the results in patients with severe asthma to the control group, we found a significant difference in the number of $(CA)_n$ repeats in the *cPLA2* gene promoter between these groups. A lower number of $(CA)_n$ repeats occurred significantly more often in patients with severe asthma than in the control group.

Moreover, the whole region enclosing the $(CA)_n$ repeats appeared to be highly polymorphic in our studied populations as we also found novel single nucleotide polymorphisms (SNPs). Two of them −185 A > C and −180 T > C appeared to be associated with severe asthma. The −180T as well as the −185 A allele were present significantly more often in patients with severe asthma. The analysis of this region with a transcription factor database suggests that a potential octamer binding site – ATTTACAT might exist in the position of −181 bp to −174 bp. Transcription factors which bind specifically to these motifs are called octamer binding proteins (Oct). The family of these transcription factors consists of proteins such as Oct-1, Oct-2 and Oct-4, which are expressed ubiquitously and involved in the transcription regulation of numerous genes [19,20]. Oct proteins can therefore act as a repressor or an activator of transcription. Although there is no evidence for the influence of Oct proteins on *cPLA2* transcription, authors describing the *cPLA2* 5′-flanking region have reported two octamer binding sites within the *cPLA2* promoter [12,21,22]. Therefore, our finding that the existence of polymorphisms within this sequence may be associated with the severe asthma phenotype might support the hypothesis of the importance of Oct transcription factors in the regulation of cPLA2 expression.

Deletion studies performed by Dolan-O'Keefe *et al.* [14] revealed that the −2271 bp promoter construct is characterized by slightly lower luciferase activity in comparison to the −1294 bp construct, which lacks the $(T)_n$ region. However, their results did not reach statistical significance. Here, we showed that constructs with $(T)_n$ repeats deleted revealed a $22·3 \pm 5\%$ increase in reporter gene activity. Moreover we demonstrated, similar to $(CA)_n$ repeats, that *cPLA2* reporter gene activity depends on the number of $(T)_n$ repeats. Promoter constructs with shorter forms of the $(T)_n$ repeats revealed a significant increase in reporter gene activity in comparison to longer forms of the $(T)_n$ repeats. This obser-

vation indicates that the mononucleotide $(T)_n$ repeats in the *cPLA$_2$* promoter region might exert some inhibitory effect on the transcription of the *cPLA$_2$* gene. Further elucidation of this observation revealed that not only was the group of $(CA)_n$ shorter alleles associated with the severe asthma phenotype, but also the group of shorter alleles of $(T)_n$ repeats region $(n = 17–38)$ occurs significantly more often in patients.

We are aware that the groups enclosed to our study are relatively small and could lack power to measure accurately an association. Moreover, the asthma severity criteria used in GINA 2005 [15] were sometimes difficult to establish. When the patient had already been on treatment, the classification of asthma severity was performed by a daily medication regimen and response to treatment. It was also consistent with GINA 2005 [15] recommendations. However, there was a potential for misclassification and bias in our studied populations. This might be the reason why, in the latest guidelines, other issues such as the level of asthma control are introduced. Furthermore, an intriguing issue that should be investigated further is the lack of heterozygosity of $(T)_n$ and $(CA)_n$ repeats regions in our results, which was confirmed by bidirectional sequencing. Nevertheless, the results of our case–control study, together with the higher *cPLA$_2$* transcription in these patients, might suggest an important role of cPLA$_2$ in asthma pathogenesis or severity. However, studies involving larger populations and perhaps different geographic and ethnic groups may be needed to address this phenomenon.

In conclusion, there are two important observations from this study. First, the $(T)_n$ repeats and $(CA)_n$ repeat regions may exert an effect on the level of *cPLA$_2$* gene transcription. Secondly, there might be an association of these regions, either by SNPs around the $(CA)_n$ repeats region or by different-length variants of the $(CA)_n$ and the $(T)_n$ repeats regions with severe asthma. Nevertheless, further studies are needed to elucidate the role of the described polymorphisms in the *cPLA$_2$* promoter in the development of different asthmatic phenotypes.

Acknowledgements

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