Biomarkers, 2010; 15(2): 158–166

informa
healthcare

ORIGINAL ARTICLE

Functional polymorphism in *CTLA4* gene influences the response to therapy with inhaled corticosteroids in Slovenian children with atopic asthma

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Abstract

We genotyped *CTLA4* CT60 (rs3087243) functional single nucleotide polymorphism (SNP) in children with asthma and in healthy controls and correlated the genotype data with asthma clinical data, including treatment response with inhaled corticosteroids measured by forced expiratory volume in the first second (FEV_1). FEV_1 increased by 21.7% after 4 weeks of therapy in atopic asthmatics with the A/A genotype compared with an 8.6% increase in heterozygotes and a 5.8% increase in G/G homozygotes (p <0.01). Genotype and allele frequencies in asthmatics did not differ significantly from those in the control group. SNP CT60 in the *CTLA4* gene is significantly associated with the response to treatment with inhaled corticosteroids in children with atopic asthma and could be a useful biomarker for personalized therapy in asthmatic children. SNP CT60 in the *CTLA4* gene plays only a minor role in genetic susceptibility to childhood asthma in the Caucasian population.

Keywords: CTLA4 *CT60; childhood asthma; inhaled corticosteroids; asthma pharmacogenomics*

Introduction

Asthma is the most common serious chronic disease of childhood with a prevalence of between 6% and 10% in developed countries. Asthmatic inflammation underlies hyper-responsiveness of the airways and reversible bronchial obstruction to various stimuli. In an atopic patient the T helper (Th) 2 immune response to common allergens persists instead of the Th1 response characteristic for non-atopics (Busse & Lemanske 2001, Martinez 2002).

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is expressed on activated T cells as a member of the immunoglobulin superfamily. CTLA4 is homologous to CD28, a T-cell co-stimulatory molecule. Both molecules bind to B7-1 and B7-2 antigens on antigen-presenting cell (APCs). CTLA4 blocks the CD28-mediated co-stimulatory signal for T-cell activation and thereby transmits an inhibitory signal to T cells (Magistrelli et al. 1999). Human *CD28* and *CTLA4* genes are located at the same locus, 2q33 (Buonavista et al. 1992). CTLA4 protein exists in two forms. The membrane-bound form is biologically more active. The soluble form of the CTLA4 protein is the result of alternative splicing and deletion of the exons which code for the transmembrane region (Linsley et al. 1995). CTLA4 over-rides the T-cell receptor (TCR)-induced signal required for stable conjugate formation between T cells and APCs, thereby decreasing the proliferation of T cells and cytokine production. As a gatekeeper of conjugation CTLA4 limits dwell times and provides suboptimal or altered signalling which may result in non-responsiveness to lower affinity autoantigens (Schneider et al. 2006). Application of exogenous

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(Received 24 August 2009; revised 02 October 2009; accepted 04 October 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK Ltd
DOI: 10.3109/13547500903384318

CTLA4 blocks allograft rejection, causes improvement of psoriasis and thus promotes immune tolerance (Grohmann et al. 2002, Abrams et al. 1999).

APC–T-cell interaction, leading to either activation or suppression of T cells, represents an important event in controlling the delicate balance between allergic sensitization and tolerance. Airway inflammation after inhaled allergen exposure requires the recruitment, activation and differentiation of antigen-specific T cells. The CD28/CTLA4-B7 signalling pathway appears to be one of the most important regulators of T-cell responses in airways to inhaled antigens, including common allergens. B7-1 interaction with CTLA4 is crucial for the induction of low-dose tolerance to peanut. By limiting T-cell–APC contact times, CTLA4 would reduce the efficiency of the allergen presentation (van Wijk et al. 2007). Therefore CD28 and CTLA4 molecules are important regulatory components in the development of allergic airway inflammation (Burr et al. 2001, Green 2000). Plasma concentration of soluble CTLA4 is elevated in atopic and non-atopic asthma and correlates with the severity of the disease (Shi et al. 2005). However the increased expression of CTLA4 is probably the consequence of aberrant recruitment and activation of T cells in asthma and not its cause (Ip et al. 2006, Shi et al. 2005, Ueda et al. 2003). The *CTLA4* gene has been implicated as a general susceptibility gene for autoimmune diseases. The A49G polymorphism in exon 1 is the most extensively studied, as this is the only polymorphism in the *CTLA4* gene that alters an amino acid (Kristiansen et al. 2000). Single nucleotide polymorphism (SNP) *CTLA4* A49G has also been associated with asthma or bronchial hyper-responsiveness in the Korean population (Lee et al. 2002, Sohn et al. 2007), but not in Caucasians (Jasek et al. 2006). A fine-mapping association study in the *CTLA4* region revealed a SNP termed CT60 (rs3087243) to be the SNP in the *CTLA4* gene region that is most significantly associated with autoimmune diseases including Graves' disease, autoimmune hypothyroidism and type 1 diabetes (Ueda et al. 2003, Kavvoura et al. 2007). The 3' untranslated region (UTR) of human *CTLA4* mRNA including the CT60 polymorphism is responsible for mRNA stability and translational efficiency (Malquori et al. 2008). The haplotype including the CT60 G allele is linked with susceptibility to autoimmune diseases and lower expression of CTLA4 compared with the haplotype including the CT60 A allele, which protects against the autoimmune diseases (Ueda et al. 2003).

Genotype probably contributes 80% of the overall variability in the response to antiasthmatic treatment between individuals (Drazen et al. 2000). However to date there are few pharmacogenomic data confirming the particular genotype influence on the antiasthmatic effect of corticosteroids (Wechsler & Israel 2005). In the present study we searched for an association of *CTLA4* CT60 polymorphism with asthma, atopy and with the antiasthmatic effect of inhaled corticosteroid therapy.

Materials and methods

Patients and study design

Between 1 January and 31 December 2008, 102 children with asthma, aged 5–18 years were enrolled in the study. All had mild or moderate persistent asthma and were treated in the pulmonary and allergic outpatient consultation, Department of Pediatric Medicine, General Hospital Murska Sobota, Slovenia. Asthma was diagnosed according to American Thoracic Society (ATS) criteria (ATS 1987).

All the children treated in the outpatient clinic during the period of study were included according to the inclusion and exclusion criteria. Patients with other chronic inflammatory diseases except asthma and atopic diseases were excluded from the study. Patients were free from any acute disease or asthma exacerbation at the time when blood samples were taken. Parents signed informed consent for children younger than 15 years, while older children gave informed consent themselves. As a control group we used genotype data from 84 non-atopic, non-asthmatic Slovenians. The study was carried out in accordance with the Helsinki declaration of the World Medical Association (1975) and approved by the Slovenian National Medical Ethics Committee (KME 31/12/06).

Measurements and laboratory tests

We measured some important clinical and laboratory parameters in asthmatics: forced expiratory volume in the first second (FEV_1, as a percentage of that predicted for sex, height and age) before therapy (FEV_1% b.t.), after therapy (FEV_1% a.t.), difference of both (dFEV_1%) (FEV1% a.t. minus FEV_1% b.t.), the provocative concentration (PC) of methacholine causing a fall in FEV_1 of 20% (PC_{20}) and its base 10 logarithm ($logPC_{20}$), total immunoglobulin class E (total IgE) concentration, eosinophil count in peripheral blood and the fraction of nitric oxide in exhaled air (FENO) in parts per billion (ppb).

Allergic status was determined with the skin prick tests (Allergopharma, Reinbek, Germany) to the most common aeroallergens – house dust mite, pollens, feathers, animal epithelia and moulds. The test was positive if the diameter of the weal was at least 3 mm greater than the negative control weal. We also determined specific IgE to those allergens (CAP-RAST; Pharmacia &

Upjohn, Freiburg, Germany). We considered the patient to be atopic if the value of specific IgE to any allergen was >0.35 kU l^{-1} or if they had at least one positive skin prick test.

Pulmonary function was measured with a Vitalograph 2150™ spirometer (Compact, Buckingham, UK), and the baseline was defined as the best of three recordings. For the purposes of our study we recorded values of forced vital capacity (FVC) and FEV_1 and calculated the FEV_1/FVC ratio. All the patients had been tested by spirometry immediately before treatment with inhaled corticosteroids and repeated the test 4 weeks later. According to National Asthma Education and Prevention Program (NAEPP) guidelines for asthma treatment we prescribed 200 µg of fluticasone dry powder (Flixotide Diskus™, GlaxoSmithKline, Uxbridge, UK) daily for children younger than 12 years and 400 µg daily for older children (EPR-3 2007).

Bronchial hyper-reactivity was assessed with a methacholine bronchoprovocation challenge test with dosimeter controlled jet nebulizer method, described in detail elsewhere (Beach et al. 1993). We considered the precautions and contraindications to the provocation test according to ATS guidelines (Sterk et al. 1993, Crapo et al. 2000). Aerosols of methacholine were generated using a jet nebulizer. A dosimeter Provojet™(Ganshorn Medizin Electronic, Niederlauer, Germany), with a valve system enabling the administration of the aerosol only during inspiration, was used. We considered the methacholine challenge test to be positive if the provocative concentration of methacholine that caused a fall in FEV_1 of 20% from baseline (PC_{20}) was 8 mg ml^{-1} or less. For the purposes of statistical analysis, subjects who had a fall of FEV_1 of less than 20% with a methacholine concentration of 8 mg ml^{-1} were considered to have a PC_{20} of 8 mg ml^{-1}.

For online measurement of the FENO we used a Niox™ analyser (Aerocrine, Inc., New Provodence, NJ, USA). The patient takes a deep breath through the mouth to total lung capacity from ambient (nitric oxide-free) air and then exhales immediately. The subject exhales against an expiratory resistance with a constant expiratory flow rate for at least 6 s, as laid out in ATS/European Respiratory Society (ERS) guidelines (ATS/ERS 2005). The analyser uses the chemiluminescence method for gas analysis. We measured FENO before the institution of the antiasthmatic therapy.

Twelve millilitres of blood was drawn from each patient into tubes with EDTA for genetic analysis, an eosinophil count and total IgE and specific IgE analysis.

DNA extraction and genotyping

Whole blood from patients and controls was used for isolation of the total genomic DNA. First we isolated lymphocytes using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. With TRI reagent (Sigma, Steinheim, Germany) we isolated DNA and dissolved it in the water at a final concentration of 50 ng $µl^{-1}$.

Genotyping of *CTLA4* SNP CT60 was performed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). Primers and restriction enzymes were selected according to a previous study (Torres et al. 2004). The PCR reaction was carried out in a 10-µl reaction volume containing 50 ng of genomic DNA, 250 nM of each of oligonucleotide primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 10 mM Tris–HCl and 0.25 U Taq polymerase (Fermentas, Vilinus, Lithuania). PCR conditions were as follows: preincubation at 95°C for 10 min followed by 35 cycles of 1 min denaturation at 95°C, 30 s annealing at 52°C, 30 s extension at 72°C and final extension at 72°C for 5 min. The PCR product was 216 bp long. PCR products were digested using 1 unit of *Nco*I restriction enzyme (Fermentas) at 37°C overnight. The PCR products were electrophoresed on 2% agarose gel and visualized using ethidium bromide under UV fluorescence.

Samples demonstrating a 216-bp band were assigned genotype G/G, while samples demonstrating a 196-bp band were typed as A/A and samples demonstrating two bands of 216-bp and 196 bp were typed as A/G.

Statistical analysis

Data analysis was carried out using SPSS™ version 15.0 (SPSS Inc., Chicago, IL, USA). Genotype and allele frequencies were calculated for the patient and control group. The χ^2 test and two-sided Fisher's exact test were used to calculate the significance of the difference in allele and genotype frequencies between asthmatics and controls. We calculated the odds ratio (OR) for asthma with 95% confidence intervals (CI). With the ANOVA test and the *t*-test for two independent samples we analysed the influence of genotype on some clinical parameters which are quantitative traits: FENO, PC_{20} of methacholine, blood eosinophil count, total serum IgE and change of FEV_1 after inhaled corticosteroid treatment ($dFEV_1$). Because PC_{20} is not normally distributed in the population we used its base 10 logarithm (log PC_{20}) in statistical analysis. In all tests $p < 0.05$ was considered to indicate statistical significance.

Results

Demographic data

In the group of asthmatics 52.9% (n = 54) were male and 47.1% (n = 48) were female. The mean age was

10.77 years (SD 3.39); 71.6% of patients (n = 73) were atopics and 28.4% (n = 29) had non-atopic asthma.

Genotype and allelic frequencies of SNP CTLA4 CT60

The allelic and genotype frequencies in both the asthmatics and in the control group are presented in the Table 1. In the groups of asthmatics we found 13.7% (n = 14) of A/A homozygotes, 60.8% (n = 62) of heterozygotes and 25.5% (n = 26) of G/G homozygotes. Genotype frequencies are just in Hardy–Weinberg equilibrium (p = 0.063). In the control group there were 25.0% (n = 21) of A/A homozygotes, 40.5% (n = 34) of heterozygotes and 34.5% (n = 29) of G/G homozygotes. Genotype frequencies in the control group are also in Hardy–Weinberg equilibrium (p = 0.24). We found a difference between genotype frequencies in the asthmatic and control groups when considering all three genotypes (p <0.01). However for the purposes of the risk (OR) calculation we first associated all A/A homozygotes and heterozygotes in one group (A/A + A/G) and left the G/G homozygotes in the other group (dominant model for A allele and recessive model for G allele). Then we associated heterozygotes and G/G homozygotes (A/G + G/G) in one group and left A/A homozygotes in the other group (dominant model for G allele and recessive model for A allele). In both case we found no differences in the genotype frequencies between asthmatics and the control group (p = 0.18, OR for genotype with A allele 1.14 with 95% CI 0.94–1.38 and p = 0.06, OR for A/A genotype 0.55 with 95% CI 0.30–1.01, respectively). The frequency of the A allele in asthmatics was 44.1%, which is not significantly different from 45.2% in the control group (p = 0.83, OR for A allele 0.98 with 95% CI 0.78–1.22).

We also compared genotype and allelic frequencies with controls, separately for each asthma phenotype (atopic and non-atopic). In the group of atopic asthmatics the frequency of the A/A genotype was 15.1% (n = 11), of the A/G genotype 58.9% (n = 43) and of the G/G genotype 26.0% (n = 19). For the purposes of statistical analysis we associated genotype groups (first A/A + A/G and then A/G + G/G) as described previously for the whole group of asthmatics. In whichever case we found no differences in the genotype frequencies between atopic asthmatics and the control group (p = 0.25, OR for genotype with the A allele 1.13 with 95% CI 0.92–1.39 and p = 0.12, OR for A/A genotype 0.60 with 95% CI 0.31–1.17, respectively). The frequency of the A allele in atopic asthmatics was 44.5%, which is not significantly different from the control group (p = 0.90, OR for the A allele 0.98 with 95% CI 0.77–1.26).

Non-atopic asthmatics had genotype frequencies of A/A, A/G and A/A of 10.3% (n = 3), 65.5% (n = 19) and 24.1% (n = 7), respectively. When we compared genotype frequencies with controls in either of the previously described ways (A/A + A/G or A/G + G/G) we also found no significant differences (p = 0.30, OR for genotype with the A allele 1.16 with 95% CI 0.90–1.50 and p = 0.41, OR for the A/A genotype 0.41 with 95% CI 0.13–1.29, respectively). The frequency of the A allele in non-atopic asthmatics was 43.1%, which was also not significantly different from the control group (p = 0.78, OR for A allele 0.95 with 95% CI 0.68–1.34).

Influence of genotype on clinical and laboratory parameters

Clinical and laboratory parameters listed above in Methods were measured in all 102 asthmatics, except the FENO, which was measured in only 63 of the patients. Results of the clinical and laboratory parameters in asthmatics are presented in Table 2.

The results of clinical and laboratory parameters in different genotype groups are shown in Table 3. Asthmatics with genotype A/A had a 18.9% mean increase of FEV_1 after 4 weeks of therapy with inhaled corticosteroids, compared with 9.1% in heterozygotes and 6.8% in G/G homozygotes (p = 0.01, ANOVA test). We confirmed this difference when we compared the effect of treatment in the allelic groups using the t-test. Carriers of the A allele had a 12.1% mean increase of FEV_1 after therapy with inhaled corticosteroids which is significantly higher than the 8.0% found in asthmatics with the G allele (p = 0.036). Total IgE, blood eosinophil, $logPC_{20}$ and FENO values in different genotype or allelic groups were not significantly different.

Table 1. Genotype and allelic frequencies of single nucleotide polymorphism *CTLA4* CT60.

Phenotype	A/A	A/G	G/G	A/A + A/G p-value (OR, 95% CI)[a]	A/G + G/G p-value (OR, 95% C)[b]	A allele (p-value, OR, 95% CI)[c]
Asthmatics together	13.7%	60.8%	25.5%	0.18 (1.14, 0.94–1.38)	0.06 (0.55, 0.30–1.01)	44.1% (0.83, 0.98, 0.78–1.22)
Non-atopic asthmatics	10.3%	65.5%	24.1 %	0.30 (1.16, 0.90–1.50)	0.41 (0.41, 0.13–1.29)	43.1% (0.78, 0.95, 0.68–1.34)
Atopic asthmatics	15.1 %	58.9 %	26.0 %	0.25 (1.13, 0.92–1.39)	0.12 (0.60, 0.31–1.17)	44.5% (0.90, 0.98, 0.77–1.26)
Controls	25.0 %	40.5 %	34.5 %			45.2%

[a]p-Value and odds ratio (OR) with 95% confidence interval (CI) from cross-tabulation of genotype frequencies (A/A homozygotes and heterozygotes first united in one group) in asthmatics and controls. [b]p-Value and OR with 95% CI from cross-tabulation of genotype frequencies (G/G homozygotes and heterozygotes first united in one group) in asthmatics and controls. [c]p-Value and OR with 95% CI from cross-tabulation of allelic frequencies in asthmatics and controls.

Table 2. Clinical and laboratory parameters in all asthmatics.

Parameter	*n*[a]	Minimum	Maximum	Mean	Standard deviation
FEV_1 b.t.[b]	102	45	113	81.0	13.3
FEV_1 a.t.[c]	102	42	126	90.9	14.1
$dFEV_1$[d]	102	-27	61	9.9	12.1
PC_{20} (mg ml^{-1})	102	0.04	8.0	2.2	2.60
$logPC_{20}$	102	-1.39	0.90	0.22	0.71
FENO (ppb)	63	5	119	42.1	32.1
Total IgE (IU ml^{-1})	102	2	3735	530.8	651.7
Eosinophils[e]	102	40	1520	520.4	342.8

[a]Number of asthmatics at which the particular clinical or laboratory parameters was measured; [b]value of forced expiratory volume at 1 s (FEV_1) before the treatment (percentage of that predicted for age, height and sex); [c]value of FEV_1 after the 4 weeks of treatment (percentage of that predicted for age, height and sex); [4]change of FEV_1 in 4 weeks of antiasthmatic therapy; [e]number of eosinophils in mm^3 of blood. $PC_{20,}$ provocative concentration (PC) of methacholine causing a fall in FEV_1 of 20%; FENO, fraction of nitric oxide in exhaled air.

We also tested the influence of *CTLA4* CT60 polymorphism on clinical and laboratory parameters separately in atopic and non-atopic asthmatics. In atopic asthmatics with the A/A genotype FEV_1 increased by 21.7% after therapy, which is significantly higher than 8.6% in heterozygotes and 5.8% in G/G homozygotes (p <0.01, ANOVA test). We confirmed this difference using the *t*-test in the allelic group. In atopic asthmatics with the A allele FEV_1 increased by 13.0% after antiasthmatic therapy, which is significantly higher than 7.3% in those with the G allele ($p = 0.019$). There were no differences in bronchial hyper-reactivity ($logPC_{20}$) FENO, total IgE and blood eosinophil values between genotype groups in atopic asthmatics. All the results were confirmed using the *t*-test in the allelic groups and are presented in Table 4.

In non-atopic asthmatics we did not find any influence of genotype on measured clinical and laboratory parameters. Non-atopic asthmatics with the A/A, A/G and G/G genotypes had a 8.8%, 10.5% and 9.6% post-treatment increase of FEV_1, respectively ($p = 0.87$, ANOVA test). Results in non-atopic asthmatics are presented in Table 5.

Discussion

In our investigation we found a significantly better response to antiasthmatic therapy with inhaled corticosteroids in atopic asthmatics who are carriers of the A allele of the *CTLA4* 60CT polymorphism compared with those who are carriers of the G allele. Despite the very diverse effects of corticosteroids in individuals which are mostly genotype dependent (Drazen et al. 2000), to date only a few asthma pharmacogenomic studies concerning corticosteroids have been carried out. SNP rs242941 in the gene for corticotrophin-releasing hormone receptor 1 modifies the effect of inhaled corticosteroids (Tantisira et al. 2004). The effect of inhaled corticosteroids is also glucocorticoid receptor (GR) dependent (Hamid et al. 1999). Our research is the first pharmacogenomic study of *CTLA4* genotype influence on the effect of antiasthmatic therapy. We have shown that *CTLA4* CT60 polymorphism determines the response to inhaled corticosteroids, which are currently

Table 3. Influence of *CTLA4* CT60 genotype on clinical and laboratory parameters in all asthmatics.

Parameter	*CTLA4* CT60 genotype	*n*[a]	Mean	*p*-Value[b]
FEV_1 b.t.[c]	A/A	14	80.0	0.65
	A/G	62	80.7	
	G/G	26	82.4	
FEV_1 a.t.[d]	A/A	14	98.9	0.07
	A/G	62	89.8	
	G/G	26	89.2	
$dFEV_1$[e]	A/A	14	18.9	**0.01**
	A/G	62	9.1	
	G/G	26	6.8	
PC_{20} (mg ml^{-1})	A/A	14	2.1	0.87
	A/G	62	2.2	
	G/G	26	2.2	
$logPC_{20}$	A/A	14	0.20	0.96
	A/G	62	0.23	
	G/G	26	0.23	
FENO (ppb)	A/A	9	44.6	0.43
	A/G	34	46.2	
	G/G	20	34.3	
Total IgE (IU ml^{-1})	A/A	14	562.1	0.65
	A/G	62	570.2	
	G/G	26	423.6	
Eosinophils[f]	A/A	14	609.8	0.52
	A/G	62	520.2	
	G/G	26	475.0	

[a]Number of asthmatics in each genotype category; [b]*p*-value from comparison of means with ANOVA test; [c]value of forced expiratory volume in 1 s (FEV_1) before the treatment (percentage of that predicted for age, height and sex); [d]value of FEV_1 after the treatment (percentage of that predicted for age, height and sex); [e]change of FEV_1 in 4 weeks of antiasthmatic therapy; [f]number of eosinophils in mm^3 of blood. $PC_{20,}$ provocative concentration (PC) of methacholine causing a fall in FEV_1 of 20%; FENO, fraction of nitric oxide in exhaled air.

Table 4. Influence of *CTLA4* CT60 genotype on clinical and laboratory parameters in atopic asthma.

Parameter	*CTLA4* CT60 genotype	*n*[a]	Mean	*p*-Value[b]
FEV_1 b.t.[c]	A/A	11	79.5	0.41
	A/G	43	80.2	
	G/G	19	84.7	
FEV_1 a.t.[d]	A/A	11	101.2	0.06
	A/G	43	88.8	
	G/G	19	90.5	
$dFEV_1$[e]	A/A	11	21.7	**<0.01**
	A/G	43	8.6	
	G/G	19	5.8	
PC_{20} (mg ml^{-1})	A/A	11	2.1	0.83
	A/G	43	2.0	
	G/G	19	1.8	
$logPC_{20}$	A/A	11	0.19	0.62
	A/G	43	0.16	
	G/G	19	0.14	
FENO (ppb)	A/A	7	54.3	0.47
	A/G	25	55.6	
	G/G	14	42.6	
Total IgE (IU ml^{-1})	A/A	11	644.5	0.63
	A/G	43	719.3	
	G/G	19	523.8	
Eosinophils[f]	A/A	11	659.3	0.58
	A/G	43	548.8	
	G/G	19	524.0	

[a]Number of asthmatics in each genotype category; [b]*p*-value from comparison of means with ANOVA test; [c]value of forced expiratory volume in 1 s (FEV_1) before the treatment (percentage of that predicted for age, height and sex); [d]value of FEV_1 after the treatment (percentage of that predicted for age, height and sex); [e]change of FEV_1 in 4 weeks of antiasthmatic therapy; [f]number of eosinophils in mm^3 of blood. $PC_{20,}$ provocative concentration (PC) of methacholine causing a fall in FEV_1 of 20%; FENO, fraction of nitric oxide in exhaled air.

Table 5. Influence of *CTLA4* CT60 genotype on clinical and laboratory parameters in non-atopic asthma.

Parameter	*CTLA4* CT60 genotype	*n*[a]	Mean	*p*-Value[b]
FEV_1 b.t.[c]	A/A	3	81.8	0.41
	A/G	19	81.8	
	G/G	7	76.2	
FEV_1 a.t.[d]	A/A	3	90.7	0.72
	A/G	19	92.3	
	G/G	7	85.7	
$dFEV_1$[e]	A/A	3	8.8	0.87
	A/G	19	10.5	
	G/G	7	9.6	
PC_{20} (mg ml^{-1})	A/A	3	2.1	0.25
	A/G	19	2.6	
	G/G	7	3.3	
$logPC_{20}$	A/A	3	0.24	0.07
	A/G	19	0.39	
	G/G	7	0.47	
FENO (ppb)	A/A	2	10.5	0.76
	A/G	9	19.8	
	G/G	6	15.0	
Total IgE (IU ml^{-1})	A/A	3	260.3	0.57
	A/G	19	219.4	
	G/G	7	106.2	
Eosinophils[f]	A/A	3	337.5	0.68
	A/G	19	455.1	
	G/G	7	342.0	

[a]Number of asthmatics in each genotype category; [b]*p*-value from comparison of means with ANOVA test; [c]value of forced expiratory volume in 1 s (FEV_1) before the treatment (percentage of that predicted for age, height and sex); [d]value of FEV_1 after the treatment (percentage of that predicted for age, height and sex); [e]change of FEV_1 in 4 weeks of antiasthmatic therapy; [f]number of eosinophils in mm^3 of blood. $PC_{20,}$ provocative concentration (PC) of methacholine causing a fall in FEV_1 of 20%; FENO, fraction of nitric oxide in exhaled air.

the mainstay of the antiasthmatic therapy (Hawkins & Peters 2008). Topical and systemic steroids can modulate the traffic of APCs through the airway epithelial tissues. The influence of the CT60 polymorphism on the response to antiasthmatic therapy could be explained by the direct effect of corticosteroids on *CTLA4* gene expression. Initially Holt & Thomas (1997) found the main effect of corticosteroids on APC to be upstream from the presentation of the processed antigen and in this study CTLA4 ligand expression on APCs was unaffected by dexamethasone. However further studies have found the percentage of CTLA-4 positive regulatory T cells in sputum to be increased after fluticasone propionate treatment, coincident with improvements in airway inflammation and hyper-responsiveness (Qin et al. 2005, Kawayama et al. 2008). Corticosteroids inhibit the expression of co-stimulatory molecules, which are necessary for the effective antigen presentation (Pan et al. 2001). The results of our study probably reflect the direct action of corticosteroids on *CTLA4* expression, an effect which is *CTLA4* genotype dependent. Therefore the polymorphism CT60 in the 3′ non-coding region must be close or functionally linked to binding sites of some corticosteroid-dependent transcription factors. In our study, the influence of *CTLA4* CT60 on the effect of antiasthmatic therapy is limited to the atopic asthma phenotype. This finding confirms the presumed inhibitory role of corticosteroids in allergen presentation, which is one of the first steps in the pathogenesis of atopy (Holt & Upham 2004). The absence of any influence of *CTLA4* CT60 on the effect of antiasthmatic therapy in non-atopic asthma indicates differences in the pathogenesis of different asthma phenotypes and probably reflects the lesser importance of antigen presentation in the pathogenesis of this asthma phenotype. Whereas aeroallergens are the most important triggers of exacerbations in atopic asthma, viral infections also often provoke asthma attacks, especially in non-atopic asthma (Johnston 2007, Jayaratnam et al. 2005). Despite different triggers and natural courses of the conditions only a few association

studies have tried to describe the genetic contribution to atopic versus non-atopic asthma. In one such study we found an association of the chemokine receptor CCR5 delta32 mutation with non-atopic but not with atopic childhood asthma (Berce et al. 2008).

Functional studies suggested that *CTLA4* might play an important role in the pathogenesis of autoimmune diseases and other immune diseases including asthma. It has already been known that the B7–CD28 (CTLA4) co-stimulation pathway is a critical regulator of T-cell responses and an important step in allergic airway inflammation after the sensitization and inhalation of allergens (Green 2000, Burr et al. 2001). The haplotype including the CT60 G allele is linked with susceptibility to autoimmune diseases and with lower expression of CTLA4 compared with the haplotype including the CT60 A allele, which protects against the autoimmune diseases (Ueda et al. 2003). In a Dutch population the CT60 G allele was associated with the celiac disease. The haplotype including the CT60 G allele has been shown to be associated with lower mRNA levels of the soluble CTLA4 isoform, providing a possible mechanism for the T cell-mediated destruction of the small intestine (van Belzen et al. 2004).

CTLA4 polymorphisms A49G and C-318T were first associated with atopic asthma in the haplotype C-G in Korean children (Sohn et al. 2007). In the same population the *CTLA4* C-318T promoter polymorphism was associated with asthma severity and the *CTLA4* A49G polymorphism with bronchial hyper-responsiveness (Lee et al. 2002). However association studies in Japanese and Caucasian populations have not confirmed an association of the *CTLA4* polymorphisms A49G, C-318T and AT repeat in the 3′ UTR of exon 4 with asthma (Nakao et al. 2000). We have not confirmed an association of the SNP *CTLA4* CT60 with asthma or atopy in Slovenian asthmatic children. We found a general association with asthma when comparing the general distribution of all three genotypes in healthy controls and all asthma patients. However, when we grouped the genotypes according to recessive and dominant models in order to calculate risk (odds ratio) we did not confirm the significance of the association. Also the frequency of the minor (A) allele was not significantly different between asthmatics (or asthma phenotypes) and the controls. Our results are in accordance with the only previous association study in Caucasian populations concerning the role of *CTLA4* in asthma (Jasek et al. 2006) suggesting the *CTLA4* gene plays only a minor role in genetic susceptibility to childhood asthma in Caucasian populations. The results of our association study also suggest that *CTLA4* does not play as important a role in asthma pathogenesis as in autoimmune diseases. Djukanovic (2000) found that interaction of CTLA4 with its ligands on APCs above all downregulates the Th1 subtype proliferation and thereby could tilt the Th1/Th2 balance. According to these findings, activation of CTLA4 could protect against Th1-mediated autoimmune diseases but not against Th2-mediated atopy. The absence of an association of SNP *CTLA4* CT60 with asthma could also be explained in view of previous studies which have shown that plasma concentrations of soluble CTLA4 are elevated in asthma as a consequence of the dysregulation of T-cell activation and not as its cause (Ip et al. 2006, Shi et al. 2005). Blockade of CTLA4 with monoclonal antibody in a murine model of allergic asthma further increased specific IgE production and augmented blood eosinophilia. Such treatment also resulted in more severe airway inflammation and increased airway hyperresponsiveness to methacholine, bronchial eosinophilia and an increase of interleukin (IL)-4 and IL-5 levels in bronchoalveolar lavage fluid following repeated allergen inhalations (Hellings et al. 2002). Intranasal delivery of the cytoplasmic domain of CTLA4 fused with transcriptional factor Hph-1 resulted in reduced allergic inflammation in a mouse model (Choi et al. 2006). According to these findings and to our results, the role of CTLA4 in asthma is to counteract the allergic inflammation and not to participate in asthma pathogenesis.

Our finding is important as age-related genetic susceptibility and pharmacogenetic studies in phenotypically well-characterized cohorts are likely to make a major contribution to understanding the basic mechanisms in asthma and/or asthma treatment; however due to the limited number of children with asthma in our study more independent association and meta-analysis studies are warranted to replicate our initial finding. This is especially important in the stratified analysis of different asthma phenotypes. Evidence is growing that asthma may be syndromic rather than a single disease (Holloway & Koppelman 2008). This finding suggests stratified genetic analysis, which on one hand splits the sample, but is necessary on the other hand to elicit the genetic background of different asthma phenotypes. According to the international HapMap project data SNP *CTLA4* CT60 (rs3087243) is in considerable linkage disequilibrium (LD) with at least 11 SNPs (r2 >0.9) in the *CTLA4* gene extending almost 50 kb upstream of rs3087243. Despite much evidence that *CTLA4* CT60 is in fact a functional polymorphism and most significantly associated with other inflammatory diseases even when tested in haplotypes, it is possible that other SNPs in high LD with the *CTLA4* CT60 SNP may also be functional and contribute to the observed association in our study. So far in most association studies in complex diseases the causal SNP, particularly if located in a non-coding region, has been hard to pinpoint. Therefore haplotype analysis using several SNPs could be useful in an association study. In our study however haplotype analysis using several SNPs would not have

enough statistical power due to the limited number of patients available in our study. Therefore a selection of the best known functional candidate SNP was reasonable in our association study. We also expect some genome-wide asthma pharmacogenetic studies which are necessary to direct future research to define more precisely the genetic loci. Further research is also necessary on the mRNA, protein and/or metabolite level to elicit the exact mechanism of interaction between corticosteroids and CTLA4. Despite the limitations listed above, the results of our research, particularly the influence of SNP *CTLA4* CT60 on the effect of antiasthmatic treatment, could serve for future optimization and individualization of antiasthmatic treatment based on genotype testing.

Acknowledgements

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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