

ORIGINAL ARTICLE

Genetic effect of single-nucleotide polymorphisms in the *PPARGC1B* gene on airway hyperreactivity in asthmatic patientsS.-H. Lee¹, A.-S. Jang¹, S. Woo Park¹, J. -S. Park¹, Y. K. Kim², S.-T. Uh², Y. H. Kim³, I. Y. Chung⁴, B.-L. Park⁵, H. D. Shin^{5,6} and C.-S. Park¹¹Genome Research Center for Allergy and Respiratory disease, Soonchunhyang University Hospital, Wonmi-gu, Bucheon, Gyeonggi-do, Republic of Korea, ²Division of Allergy and Respiratory Medicine, Soonchunhyang University Seoul Hospital, Yongsan-gu, Seoul, Korea, ³Division of Allergy and Respiratory Medicine, Soonchunhyang University Cheonan HospBongmyeong-dong, Cheonan, Chungcheongnam-do, Korea, ⁴Division of Molecular and Life Sciences, College of Science and Technology, Hanyang University, Ansan, Gyeonggi-do, Republic of Korea, ⁵Department of Genetic Epidemiology, SNP-Genetics Inc., Gasan-Dong, Geumcheon-Ku, Seoul, Korea and ⁶Department of Life Science, Sogang University, Sinsu-dong, Mapo-gu, Seoul, Korea

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Summary

Background Peroxisome proliferator-activated receptor gamma coactivator 1 beta (*PPARGC1B*) is a co-activator for intracellular receptors such as the estrogen receptor, PPAR, and glucocorticoid receptor, which are involved in asthma development.**Objectives** Genetic association of single-nucleotide polymorphisms (SNPs) in the *PPARGC1B* gene with the risk of asthma and airway hyperreactivity (AHR) was investigated, as well as the functional effects of these SNPs on *PPARGC1B* gene and protein expression.**Methods** Direct sequencing of DNA from 24 Korean was performed to identify *PPARGC1B* SNPs. Genotyping was done in 264 controls and 949 asthmatics using single-base extension methods. *PPARGC1B* mRNA levels were measured using real-time PCR methodology.Luciferase and electrophoretic mobility shift assays (EMSA) were performed to functionally analyse *PPARGC1B* SNPs on promoter.**Results** Eighteen SNPs and one insertion/deletion polymorphism were identified, and seven SNPs were genotyped. No significant difference existed in the distribution of SNPs and haplotypes between the asthmatics and controls. However, the allele frequency of $-427C > T$ and $+102525G > A$; $R265Q$ showed a significant association with log-transformed PC_{20} methacholine values in the asthmatics ($P = 0.005$ – 0.0004). Real-time PCR demonstrated higher *PPARGC1B* mRNA levels in asthmatics having $-427CC$ allele than in those having $-427TT$ or CT alleles ($P = 0.048$). The ratio of the mRNA expression for each *PPARGC1B* exon4-mRNA compared with the wild type was similar in peripheral blood mononuclear cells carrying the $+102525G > A$ allele. Luciferase reporter assays revealed that $-427C$ allele caused higher promoter activity than $-427T$ allele. EMSA demonstrated that $-427C$ allele exhibited stronger binding activity to a nuclear protein in 293T cells than did the $-427T$ allele.**Conclusions and Clinical Relevance** Polymorphisms of $-427C > T$ on the promoter and those of $+102525G > A$ on exon 5 of the *PPARGC1B* gene may affect the development of AHR through the modulation of *PPARGC1B* gene products. The *PPARGC1B* genotypes may serve as genetic markers for AHR.**Keywords** asthma, methacholine, PCR, *PPARGC1B*, SNPs

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Correspondence:

Choon-Sik Park, Genome Research Center for Allergy and Respiratory Diseases, Division of Allergy and Respiratory Medicine, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, 1174, Jung-Dong, Wonmi-Ku, Bucheon, Gyeonggi-Do 420-767, Korea. E-mail: mdcsipark@unitel.co.kr

Introduction

Asthma is a common and heterogeneous respiratory disease characterized by chronic airway inflammation and remodeling [1]. Airway remodeling is a complex

process that involves the entire airway, including the epithelium, basement membrane, smooth muscle, blood vessels, and adventitia [2, 3]. Each change can alter the airway physiology to promote the severity of airway narrowing and hyperresponsiveness [4, 5]. Airway

remodeling is caused by aberrant synthesis of mediators from inflammatory cells and structural cells and is linked to altered epithelial–mesenchymal signaling and proliferation of biosynthetically active myofibroblasts [6]. These processes are caused by the increased expression of several inflammatory and fibrogenic mediators, including cytokines, chemokines, adhesion molecules, enzymes, and chemical mediators [7]. The induction of the genes responsible for these mediators is regulated by transcription factors.

The inflammatory and remodeling processes in asthma are modulated by transcription factors such as the glucocorticoid receptor (GR), signal transducer and activator of transcription (STAT)-6, nuclear factor-kappa-B (NF-kappa-B), activator protein-1 (AP-1), nuclear factor of activated T-cells (NFAT), cyclic AMP response element-binding protein, CCAAT/enhancer-binding protein (C/EBP), peroxisome proliferator-activated receptor (PPAR), and the bZIP transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [8, 9]. The activation of these transcription factors requires co-activators to exert their function. The activity of hepatic nuclear factor-4, PPAR, GR, and estrogen-related receptor are enhanced by the interaction with peroxisome proliferator-activated receptor gamma coactivator 1 beta [PPARGC1B, MIM: 608886; Symbols: PERC; PGC1B; PGC-1(beta)] [10, 11]. Therefore, nucleotide substitutions in the *PPARGC1B* gene may contribute to the functional changes of these transcription factors, which may lead to airway inflammation and remodeling in asthma.

The human *PPARGC1B* gene is located on chromosome 5q33.1 and comprises 12 exons spanning approximately 117 kb [12, 13]. A genome-wide linkage study identified chromosome 5q31 as a major candidate region that was linked to asthma and airway hyperreactivity (AHR) in several ethnic groups [14–16]. To reveal the genetic role of the *PPARGC1B* gene in asthma development, we identified single-nucleotide polymorphisms (SNPs) in the *PPARGC1B* gene by direct sequencing, and then performed genetic association analyses of the risk of asthma and AHR in a Korean asthmatic cohort. In addition, we performed functional analyses to validate the associated SNPs.

Materials and methods

Subjects

The study subjects were recruited from three tertiary hospitals in Korea (Soonchunhyang University Bucheon, Seoul, and Cheonan Hospital). All patients were diagnosed with asthma by a physician according to the Global Initiative for Asthma (GINA) guidelines [1]. All patients had a history of dyspnea and wheezing during the previous 12 months, plus one of the following: (1) >15% increase in forced expiratory volume in 1 s (FEV1) or

Table 1. Clinical profile and laboratory findings of the study subjects

Profiles	NC	BA
Number of subjects	264	949
Age [year (range)]*	42(5–80)	45(5–80)
Onset age [year (range)]		38.29(1–77)
BMI (kg/m ²)	23.78±3.04	24.16±3.50
Sex (F/M)	151/113	582/367
Atopy (%)†	36.33%	60.41%
Smoker (%)	29.72%	33.83%
FVC (% predicted)†	93.28±12.27	82.90±18.36
FEV1 (% predicted)†	102.30±14.63	78.85±22.57
Blood eosinophils (%)†	2.42±2.00	5.52±5.38
Log [PC20, methacholine (mg/mL)]†	1.38±0.06	0.17±0.68
Log [total IgE (IU/mL)]†	1.70±0.61	2.15±0.64

Values are mean±SE. *P* values are obtained using *t*-test or χ^2 test between bronchial asthmatics (BA) and normal controls (NC).

**P*<0.05.

†*P*<0.001 for difference between the two groups.

>12% increase plus 200 mL following inhalation of a short-acting bronchodilator, (2) <10 mg/mL PC₂₀ methacholine, or (3) >20% increase in FEV1 following 2 weeks of treatment with inhaled steroids and long-acting bronchodilators. Normal controls were recruited from among the spouses of the patients and from members of the general population who responded negatively to a screening questionnaire for respiratory symptoms [17] and had an FEV1>75% of the predicted value, a PC₂₀ metacholine >10 mg/mL, and normal findings on a simple chest radiograph. The total immunoglobulin E (IgE) was measured using the CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Atopy was defined by the presence of an immediate skin reaction >3 mm in diameter or >1 mg/mL histamine reactivity to one or more of 24 common aeroallergens, including dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*), fungi, cat fur, dog fur, cockroaches, grass, and tree and weed pollens [18]. The study protocols were approved by the Institutional Review Board of Soonchunhyang University Hospital, and written informed consent was obtained from all the subjects. The clinical parameters are summarized in Table 1.

Sequencing and genotyping analysis

We sequenced the entire *PPARGC1B* gene, including the promoter region (~1.5 kb), to identify SNPs in DNA samples from 24 Korean subjects using the ABI PRISM 3700 DNA analyser (Applied Biosystems, Foster City, CA, USA). The primer sets for the amplification and sequencing analyses were designed based on GenBank sequences (supporting information Table S1). The sequence variants were verified by chromatograms. Among the identified SNPs, seven in the *PPARGC1B* gene were genotyped using TaqMan assays (Applied Biosystems) [19]. Primer

Express software (Applied Biosystems) was used to design polymerase chain reaction (PCR) primers and minor groove binder (MGB) probes for TaqMan genotyping. One probe was labeled with FAM (6-carboxyfluorescein) and the other with VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein). PCRs were run using the TaqMan Universal Master Mix without uracil-DNA-glycosylase UNG (Applied Biosystems) at primer concentrations of 900 nM and probe concentrations of 200 nM. Reactions were performed in a 384-well format in a total volume of 5 µL using 20 ng of genomic DNA. The plate was then placed in a PE 9700 thermal cycler (Applied Biosystems), heated at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The plate was then transferred to a Prism 7900HT Sequence Detection System (Applied Biosystems), in which the fluorescence intensity of each well was measured. The fluorescence data files from each plate were analysed using SDS Ver. 2.1 automated software (Applied Biosystems).

Epstein-Barr virus transformation of B cells

B cell lines were established from peripheral blood mononuclear cells (PBMCs) of the study subjects following transformation with the Epstein-Barr virus (EBV) from supernatants of B95-8 cell lines [American Type Culture Collection (ATCC); CRL-10624]. The cells were grown in RPMI 1640 medium (JBI, Daegu, Korea) containing 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 2 mM glutamine (Invitrogen).

RNA preparation, reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA of B cell and PBMC of the study subjects was extracted using TRI Reagent (Molecular Research Center, Toyama, Japan) [20]. Any potential DNA contamination was eliminated by treatment with 1 µL of RNase-free DNase I (10 000 U/mL; Stratagene, La Jolla, CA, USA) at 37 °C for 15 min. The reaction was stopped with 0.5 µL of 2 mM EDTA. Three micrograms of RNA were denatured at 65 °C for 5 min with 1 µL of oligo(deoxythymidine)15 primer (containing 0.5 µg) and 10 mM dNTP mix and then quickly chilled on ice. The samples were then incubated at 42 °C for 2 min with 5 mM MgCl₂, 0.1 mM DTT and 1 µL of SuperScriptII RT reverse transcriptase (200 U/µL; Invitrogen) and heat-inactivated at 70 °C for 15 min. After reverse transcription, aliquots of cDNA from B cells were placed into tubes containing specific primer pairs for *PPARGC1B* and beta-actin (*ACTB*) genes.

Primer information is available on-line in supplementary information Table S3. PCR amplification was performed in a thermocycler for 27 cycles (94 °C for 30 s,

58 °C for 30 s, 72 °C for 30 s) with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 7 min. The amplified PCR products were subjected to electrophoresis on 1% agarose gels and were then visualized by ethidium bromide staining.

Real-time PCR was performed with the StepOne Real-Time PCR System (Applied Biosystems). Twenty-microliter PCR reaction mixtures were prepared consisting of 10 µL master mix (iQ SYBR Green Supermix; Bio-Rad Laboratories Inc., Hercules, CA, USA), 1 µg/µL cDNA from PBMC template, and 1 µL of each *PPARGC1B* primer pair. The reactions were denatured at 95 °C for 10 min and then amplified for 40 cycles at 95 °C for 15 s, 57 °C for 32 s, and 72 °C for 30 s. A melting curve for all PCR products was obtained immediately after amplification by increasing the temperature in 0.3 °C increments from 60 °C for 85 cycles of 15 s each. The threshold cycle (*C_t*) of *PPARGC1B* or an endogenous reference gene was defined as the fractional cycle number at which the fluorescence of PCR products passed the fixed threshold. Using this $2^{-\Delta\Delta C_t}$ method, the data are presented as the fold change in gene expression, normalized to an endogenous reference gene (*ACTB*) and relative to a control ($-427C > T$ or $+102525G > A$ genotypes of *PPARGC1B*). For the control sample, $\Delta\Delta C_t$ equals zero and 2^0 equals 1, so that the fold change in gene expression relative to the control equals 1 [21]. For the other samples, the evaluation of $2^{-\Delta\Delta C_t}$ indicates the fold change in gene expression relative to the control. In addition, fold differences in *PPARGC1B* mRNA expression in each asthmatic subject were calculated by comparing $2^{-\Delta\Delta C_t}$.

Construction of PPARGC1B promoter-luciferase reporter plasmids

Genomic DNA was extracted from B cell lines using a commercial DNA isolation kit (Puregene D5000, Qiagen, Alameda, CA, USA). The first PCR product (1107-bp site -883 to $+231$ on the promoter) was amplified from the genomic DNA fragments using forward primer and reverse primer sequences. Subsequently, a 582-bp fragment in the promoter region (-613 to -42) of the *PPARGC1B* gene was amplified using a forward primer containing a *Bgl*II restriction enzyme site and a reverse primer containing a *Hind*III restriction enzyme site. The amplified PCR product was cloned into a pGEM-T Easy vector and digested with *Bgl*II and *Hind*III (New England Biolabs, Ipswich, MA, USA). The resulting fragment was cloned into the *Bgl*II and *Hind*III sites of the promoterless luciferase reporter pGL3-Basic vector (Promega, Madison, WI, USA). To generate the $-427T$ construct, a point mutation was introduced. The pGL3-T Easy vector containing *PPARGC1B* $427T$ was amplified using the forward primer containing a *Bgl*II restriction enzyme site and a reverse primer. A second PCR fragment was amplified

using a forward primer and the reverse primer containing a *HindIII* restriction enzyme site. Overlap PCR was performed with the first and second PCR constructs using a forward primer containing a *BglII* restriction enzyme site and a reverse primer containing a *HindIII* restriction enzyme site. This amplified PCR product was cloned into a pGEM-T-Easy vector and digested with *BglII* and *HindIII*. The resulting fragment was cloned into the *BglII* and *HindIII* sites of the pGL3-Basic vector, and the construct was deemed pGL3-*PPARGC1B* 427T. The plasmids were purified using the QIAGEN Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany). At each step, the sequences were confirmed by direct sequencing. The primers used are listed on-line in supporting information Table S3.

Transient transfection and luciferase reporter assays

293T cells (ATCC; CRL-11268) were grown in Dulbecco's modified Eagle's medium (JBI) with L-glutamine (2 mM), HEPES (25 mM), 10% heat-inactivated FBS (Invitrogen), 100 U/mL penicillin, and 100 ng/mL streptomycin (Invitrogen) at 37 °C in humidified air containing 5% CO₂. Twenty-four hours before transfection, 5 × 10⁵ cells in 2 mL were seeded per well without antibiotics to obtain 90–95% confluence at the time of transfection. The 293T cells were transiently transfected with the pGL3-promoter of the *PPARGC1B* gene using Lipofectamine 2000 (Invitrogen recommended DNA (μg): lipofectamine (μL) = 1 : 2) in Opti-MEM (Invitrogen). One microgram of pSV-β-galactosidase control vector (Promega) was cotransfected to normalize the transfection efficiencies. After 48 h of transfection, the cultured cells were washed twice with phosphate-buffered saline (PBS) and then resuspended by mechanical scraping with 400 μL of reporter lysis buffer (Promega). Cells were centrifuged at 12 000 rpm for 2 min, and then the supernatant was stored at –70 °C. Beta-galactosidase (β-gal) activities were assessed by ortho-nitrophenyl-D-galactopyranoside (ONPG) hydrolysis using the β-Galactosidase Enzyme Assay System Kit (Promega), and the total protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Luciferase activity was measured using the Luciferase Assay System (Promega) and a luminometer (20/20n Luminometer, Turner BioSystems, Sunnyvale, CA, USA) and was expressed as relative luciferase activity calculated as the ratio to that of the empty pGL3-basic vector. Variations in transfection efficiency were corrected by normalization using β-gal activity.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared by the modified method of Schreiber et al. [22]. 293T cells were harvested after washing with Hank's balanced salt solution (HBSS). Cells

were washed with buffer A (10 mM HEPES, 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL aprotinin; Calbiochem, Darmstadt, Germany). The cell pellet was resuspended in buffer B (buffer A containing 0.2% Nonidet P40) and incubated for 2 min. Nuclei were pelleted by centrifugation and then resuspended in buffer C (buffer A containing 0.25 M sucrose). Nuclei were again pelleted and then resuspended in buffer D (50 mM HEPES, 400 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) and incubated with shaking for 30 min. All procedures were performed on ice. The mixture was centrifuged, and the supernatant was stored at –70 °C. DNA-protein binding assays were performed using the Gel Shift Assay System (Promega) according to the manufacturer's instructions. Aliquots of 15 μg of nuclear extracts were incubated in a 10-μL total reaction volume containing binding buffer (Promega) for 10 min with or without unlabeled oligonucleotide probe. The ³²P-labeled oligonucleotide probe was added to the reaction mixture and then incubated for 20 min. The reaction products were analysed by electrophoresis on 4% polyacrylamide gels with 0.5 × TBE buffer. The gels were dried and analysed by autoradiography. The sequences of the oligonucleotide probes are shown in supporting information Table S3.

Statistical analyses

Linkage disequilibrium (LD) was measured as Lewontin's D' ($|D'|$) and r^2 [23]. The haplotype of each individual was inferred using the EM algorithm (PHASE v2.0) developed by Stephens et al. [24]. The association of the various *PPARGC1B* genotypes and haplotypes with the risk of asthma was analysed using logistic regression models while controlling for age (continuous value), sex (male = 0, female = 1), smoking status (non-smoker = 0, ex-smoker = 1, smoker = 2), and atopy (positive = 0, negative = 1) as covariates. All statistical analyses were performed using SAS v9.1 (SAS Institute, Cary, NC, USA) and SPSS v11 software (SPSS Inc., Chicago, IL, USA). Blood log-transformed PC₂₀ methacholine levels were compared using linear regression models. To correct for multiple testing, the effective number of independent markers in *PPARGC1B* was calculated using SNPSpD software (<http://genepi.qimr.edu.au/general/daledN/SNPSpD>), which is based on the spectral decomposition (SpD) of matrices of pair-wise LD between SNPs [25]. The number of independent marker loci in *PPARGC1B* was 6.0464, and this was applied to correct for multiple testing ($P\text{-value} \times 6.0464$).

Results

Genotype distribution between asthmatics and normal controls

Following direct sequencing of DNA from 24 Korean subjects, 18 SNPs and one insertion/deletion polymorphism in

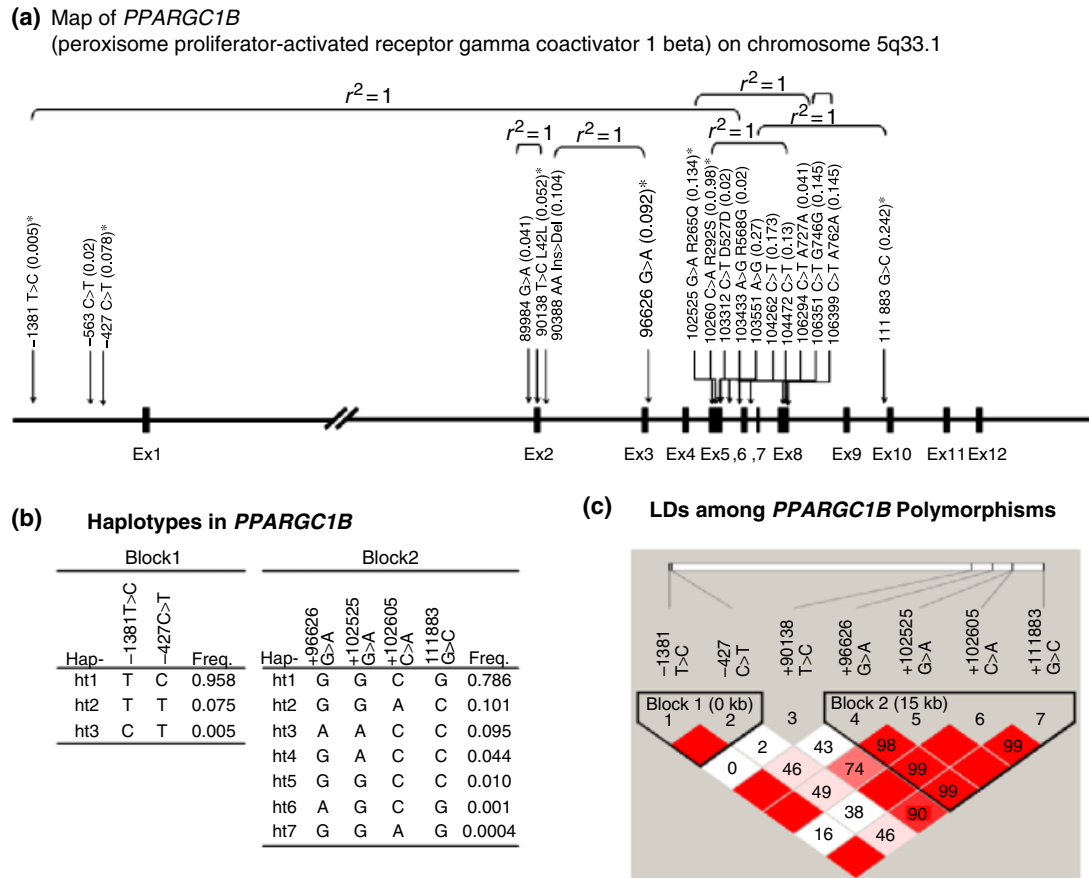


Fig. 1. The location of SNPs on *PPARGC1B*, haplotypes and pair-wise linkage disequilibrium. (a) Gene map of *PPARGC1B* gene on chromosome 5q32 and the location of SNPs. The first base of the translation site is denoted as nucleotide +1. Black and white blocks represent the coding region and untranslated region of *PPARGC1B*. Asterisks indicate SNPs selected for large-scale genotype analysis. (b) Haplotypes of *PPARGC1B* gene. (c) Diagram of linkage disequilibrium coefficient among *PPARGC1B* SNPs.

the *PPARGC1B* gene were identified (Fig. 1): three SNPs in the promoter ($-1381T>C$, $-563C>T$, and $-427C>T$), one SNP in exon 2 ($+90138T>C$, $Leu>Leu$), four SNPs in exon 5 ($+102525G>A$, $Arg>Gln$; $+102605C>A$, $Arg>Ser$; $-103312C>T$, $Asp>Asp$; and $-103433A>G$, $Arg>Gly$), three SNPs in exon 8 ($+106294C>T$, $Ala>Ala$; $+106351C>T$, $Gly>Gly$; and $+106399C>T$, $Ala>Ala$), and seven SNPs in intron 3 ($+89984G>A$, $+90388AA$ ($Ins>Del$), $+96626G>A$, $+103551A>G$, $+104262C>T$, $+104472C>T$, and $+111883G>C$). 7 SNPs ($-1381T>C$, $-427C>T$, $+90138T>C$, $+96626G>A$, $+102525G>A$, $+102605C>A$, and $+111883G>C$) were selected for larger-scale genotyping based on the general criteria, including SNPs with $>5\%$ of minor allele frequency and one of polymorphism in absolute LD ($|D'| = 1$ and $r^2 = 1$, Fig. 1). 6 SNPs among 7 SNPs were used for haplotype construction. Haplotypes were parsed into two blocks, and $+90138T>C$ was excluded for haplotype construction due to its low linkage with adjacent SNPs (Fig. 1). The block1-ht1 and block1-ht2 were not analysed due to their almost equivalency to $-427C>T$. The block2-ht1, and block2-ht3 were also not analysed due to their almost equivalency to

$+111883G>C$, $+102605C>A$ and $+96626G>A$, respectively. The frequencies of the seven *PPARGC1B* polymorphisms were calculated and are shown in supporting information Table S4. Three major haplotypes existed comprising over 86.7% in frequency (Fig. 1b), and they were further analysed for association analysis. *PPARGC1B* polymorphisms were analysed with the risk of asthma using logistic regression models with covariates of age, sex, atopy, and smoking status, and no significant difference was observed in the distribution of SNPs and the two haplotypes between the asthmatics and normal controls (supporting information Table S5). The *PPARGC1B* polymorphisms were also analysed for the association with AHR by general linear models in the asthmatics (Table 2). $+102525G>A$ was most significantly associated with log-transformed PC_{20} methacholine values in a gene dose-dependent manner (0.13 ± 0.67 mg/mL of the GG allele, 0.27 ± 0.66 mg/mL of the GA allele, and 0.66 ± 0.70 mg/mL of the AA allele; $P = 0.002$ – 0.0004 , $P^{corr} = 0.012$ – 0.006). $-427C>T$ was also significantly associated with log-transformed PC_{20} methacholine values in a gene dose-dependent manner in the asthmatics ($P = 0.005$, $P^{corr} = 0.030$ in the codominant

Table 2. The association of genotypes and haplotypes of *PPARGC1B* gene with log [PC20] methacholine in asthmatics*

Position	rs	Loci	C/C	C/R	R/R	P_a	P_d^{corr}	P_b	P_b^{corr}	P_c	P_c^{corr}
Promoter	Novel	-1381T>C	818 (0.17±0.67)	9 (0.48±0.63)		0.184	1	0.184	1		
Promoter	Novel	-427C>T	703 (0.16±0.67)	122 (0.30±0.69)	3 (0.41±0.57)	0.005	0.030	0.054	0.327	0.022	0.133
Exon2 (L42L)	rs32588	+90138T>C	758 (0.15±0.67)	66 (0.37±0.73)	6 (0.36±0.66)	0.01	0.060	0.010	0.060	0.401	1
Intron3	rs45560442	+96626G>A	686 (0.15±0.68)	139 (0.29±0.65)	4 (0.59±0.70)	0.023	0.139	0.009	0.054	0.224	1
Exon5 (R265Q)	rs455209371	+102525G>A	638 (0.13±0.67)	176 (0.27±0.66)	17 (0.66±0.70)	0.0004	0.002	0.001	0.006	0.002	0.012
Exon5 (R292S)	rs11959820	+102605C>A	669 (0.18±0.68)	145 (0.14±0.65)	10 (0.18±0.75)	0.46	1	0.420	1	0.995	1
Intron9	rs26127	+11183G>C	483 (0.14±0.68)	286 (0.20±0.68)	50 (0.37±0.67)	0.03	0.181	0.109	0.659	0.027	0.163
2SNP	Loci	Minor allele	C/C	C/R	R/R	P_a	P_d^{corr}	P_b	P_b^{corr}	P_c	P_c^{corr}
-427/102525	2SNP_h11(CG)	Dht1	20 (0.45±0.83)	222 (0.27±0.67)	589 (0.13±0.67)	0.0007	0.004	0.06	0.340	0.001	0.008
-427/102525	2SNP_h12(CA)	ht2	704 (0.14±0.67)	120 (0.35±0.67)	7 (0.29±0.87)	0.002	0.013	0.002	0.009	0.59	1
-427/102525	2SNP_h13(TA)	ht3	755 (0.16±0.68)	76 (0.32±0.67)		0.04	0.241	0.04	0.241		
-427/102525	2SNP_h14(TG)	ht4	781 (0.17±0.67)	48 (0.21±0.75)	2 (-0.56±0.72)	0.86	1	0.90	1	0.13	0.772

*C/C, C/R, and R/R represent homozygote for common allele (C/C), heterozygote (C/R), and homozygote for minor allele (R/R). Linear regression models were used for calculating odds ratios [95% confidence interval (CI)] and corresponding *P* values, controlled for age (continuous value), sex (male = 0, female = 1), smoking status (non-smoker = 0, ex-smoker = 1, smoker = 2) and atopy status (non-atopy = 0, atopy = 1) as co-variables. Mean±SE of log [PC20, methacholine] values (mg/mL), and *P* values for regression analyses of three alternative models (codominant, dominant and recessive models) were shown. Bold faces mean values showing statistical significance ($P < 0.05$).

For correction for multiple testing, the effective number of independent markers in *PPARGC1B* was calculated using the software SNPSPD (<http://genepi.qimr.edu.au/general/daleN/SNPSPD>) [25], which is based on the spectral decomposition (SpD) of matrices of pair-wise LD between SNPs. The number of independent marker loci in *PPARGC1B* was calculated as 6.0464, and this was applied to correct for multiple testing (P -value×6.0464).

model). We performed multiple regression analysis with the two SNP. *2SNP_ht1(CG)* was most significantly associated with log-transformed PC_{20} methacholine values in a gene dose-dependent manner (0.45 ± 0.83 mg/mL of the $+/+$ allele, 0.27 ± 0.67 mg/mL of the $\pm$ allele, and 0.13 ± 0.67 mg/mL of the $-/-$ allele; $P = 0.001$ – 0.0007 , $P^{corr} = 0.004$ – 0.008). *2SNP_ht2(CA)* was also significantly associated with log-transformed PC_{20} methacholine values in a gene dose-dependent manner (0.14 ± 0.67 mg/mL of the $+/+$ allele, 0.35 ± 0.67 mg/mL of the $+/+$ allele, and 0.29 ± 0.87 mg/mL of the $-/-$ allele; $P = 0.002$, $P^{corr} = 0.013$ – 0.009).

Comparison of the PPARGC1B mRNA levels between the $-427C > T$ and $+102525G > A$ alleles

To investigate the genetic effect of $+102525G > A$ and $-427C > T$ SNPs on *PPARGC1B* mRNA expression, we measured *PPARGC1B* mRNA levels in B cell lines generated from the peripheral blood of asthmatics. As $+90138T > C$, $+96626G > A$, and $+102525G > A$ are located on exon 2 (L42L), intron 3, and exon 5 (R265Q), respectively, we amplified mRNA containing exons 1 to 4 and exons 3 to 5 of the *PPARGC1B* gene. We identified two RT-PCR products of 602 and 485 bp (Fig. 2a). To identify the RT-PCR products, the sequence was deter-

mined using the BLAST search program after direct sequencing. The amplified fragment of 485 bp shared 100% sequence identity with the human *PPARGC1B* gene (data not shown), but 117 bp was deleted in exon 4 of the isoform, as previously reported by Kressler et al. [10]. To evaluate the genetic effect of the SNPs on the transcription of wild-type and exon4-mRNA of *PPARGC1B* genes, real-time quantitative PCR was performed on the PBMCs of asthmatics to quantify the *PPARGC1B* mRNA. Expression levels were examined using primers that amplified a fragment detected by a probe within exon 1 to 2 for the total RNA of the *PPARGC1B* gene and within exon 3 to 4 for the wild type and exon4-mRNA (392–409Forward/976–993Reverse). The mRNA levels of the wild type and exon4-mRNA were higher in SNPs having the $-427C > T$ CC allele than in those having the TT or CT allele ($P = 0.048$ and 0.033 , respectively; Fig. 2b). Regarding the exonic allele of $+102525G > A$, the ratio of exon 4-mRNA *PPARGC1B* mRNA to the wild-type mRNA was similar between the PBMCs carrying the $+102525G > A$ allele (Fig. 2b). These results indicate that the expression of the *PPARGC1B* gene may be transcriptionally regulated at the promoter region and that exonic $+102525G > A$ SNPs have no influence on the synthesis of the wild-type and exon4-mRNA forms of *PPARGC1B* mRNA.

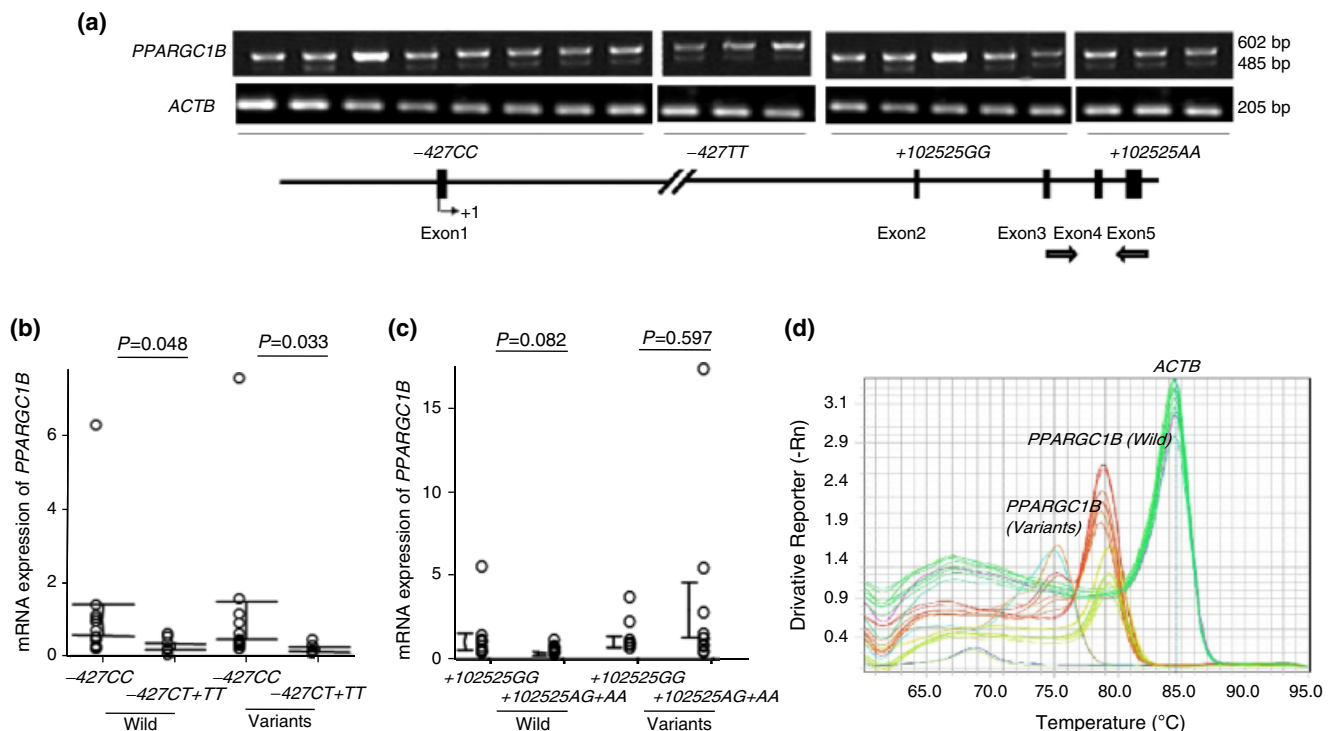


Fig. 2. mRNA expression of *PPARGC1B* gene according to $-427C > T$ and $+102525G > A$ genotypes in B-cell lines and PBMC of asthmatics. (a) B-cell lines containing common allele or minor allele were harvested and *PPARGC1B* mRNA was measured using by RT-PCR. *GAPDH* was used to ensure an equal loading of RNA samples. Data were expressed in B-cell lines as (b) and (c) *PPARGC1B* mRNA was measured using real-time PCR. Data are expressed as the ratio of *PPARGC1B* mRNA over β -actin mRNA. The experiments were performed in duplicate. (d) Melt curve using real-time PCR is shown. The experiments were performed in triplicate. The data shown represent the mean $\pm$ SE of six independent experiments.

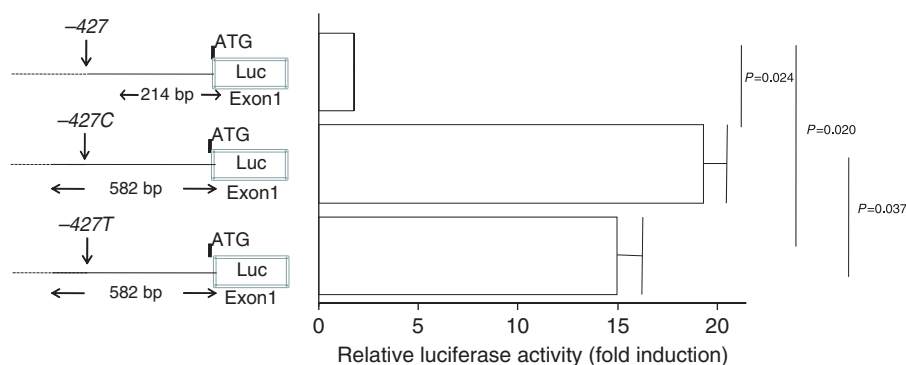


Fig. 3. Comparison of *PPARGC1B* promoter activities for the $-427C$ and $-427T$ alleles of the $-427C > T$ SNP. *PPARGC1B* promoters of each allele were inserted into a pGL3-Basic luciferase reporter system, and the constructs were transfected into 293T cells using lipofection as described in Methods. As an internal control, a pSV- β -galactosidase control vector was cotransfected with the reporter constructs. The data are presented as the ratio of the normalized luciferase activity of each construct to that of the parent pGL3-Basic vector. The data shown represent the mean $\pm$ SE of six independent experiments.

Effect of $-427C > T$ on the activity of the *PPARGC1B* promoter

As the $-427C > T$ polymorphism was located in the promoter region of the *PPARGC1B* gene, we assessed its effect on the promoter activity using luciferase reporter constructs. 293T cells were transfected with $-427C$ or $-427T$ *PPARGC1B*-pGL3 constructs, and luciferase activity was measured. The luciferase activities of the $-427C$ -pGL3 constructs were significantly higher than those of the $-427T$ -pGL3 constructs (relative luciferase activity = 19.27 ± 1.2 vs. 14.94 ± 1.28 , respectively; $P = 0.03$; Fig. 3). These results corroborated the real-time PCR results described above.

The difference in binding of the transcription factors depends on $-427C > T$

To investigate the presence of bound transcription factors in the $-427C > T$ region, we performed electrophoretic mobility shift assays of the *PPARGC1B* promoter and assessed the differential binding ability between the *CC* and *TT* alleles. Twenty-one-base allele-specific oligonucleotides were synthesized and annealed to double-stranded DNA; then, electrophoretic mobility shift assays were performed using nuclear extracts from 293T cells. The $-427C$ allele had stronger binding activity to a nuclear protein or a multiprotein complex than did the $-427T$ allele (Fig. 4). Formation of the DNA-protein complex on the radiolabeled $-427C$ allele was specifically inhibited by competition with the cold probe for $-427C$.

Discussion

The human *PPARGC1B* gene is located on chromosome 5q33.1 and comprises 12 exons spanning approximately 117 kb [13]. Genome-wide linkage studies identified chromosome 5q31 as a major candidate region that was

linked to asthma and AHR in several ethnic groups [14–16]. Alleles carrying a $649G > C$ transversion in exon 4 of the *PPARGC1B* gene, resulting in an Ala-to-Pro (A203P) substitution, were significantly associated with the development of obesity [26] and familial breast cancer risk [27]. Although Ala203Pro was important factor with development of obesity in European, it was not detected in Korean subjects. It may be due to the ethnic variability given that *PPARGC1B* is a transcriptional co-activator involved in airway inflammation and positive linkage analysis, polymorphisms in the *PPARGC1B* gene may have a genetic effect on the development of asthma and subphenotypes. However, no study of the association of *PPARGC1B* gene polymorphisms with asthma development has been reported to date. To further investigate the polymorphism association, we directly sequenced 24 DNA samples from Korean subjects. Eighteen SNPs and one insertion/deletion polymorphism were identified, and among them, 11 polymorphisms were novel SNPs. We selected seven SNPs for genotyping based on the absolute linkage and frequency. Among the SNPs of the *PPARGC1B* gene, we could not find any association with the risk of asthma development. However, the distribution of $-427C > T$ and $+102525G > A$ were significantly associated with log-transformed PC_{20} methacholine levels in the asthmatic patients after a correction for multiple comparisons. The asthmatics having common alleles of $-427C > T$ or $+102525G > A$ had the most AHR, whereas those having minor alleles had the least AHR. In addition, *2SNP_h1(CG)* was most significantly associated with log-transformed PC_{20} methacholine values in a gene dose-dependent manner. These novel data indicated that the common alleles of $-427C > T$ and $+102525G > A$ may be susceptible to AHR in asthmatics through the regulation of the *PPARGC1B* gene.

Initially, *PPARGC1B* was identified in the mouse using genomic homology search methods and was deemed

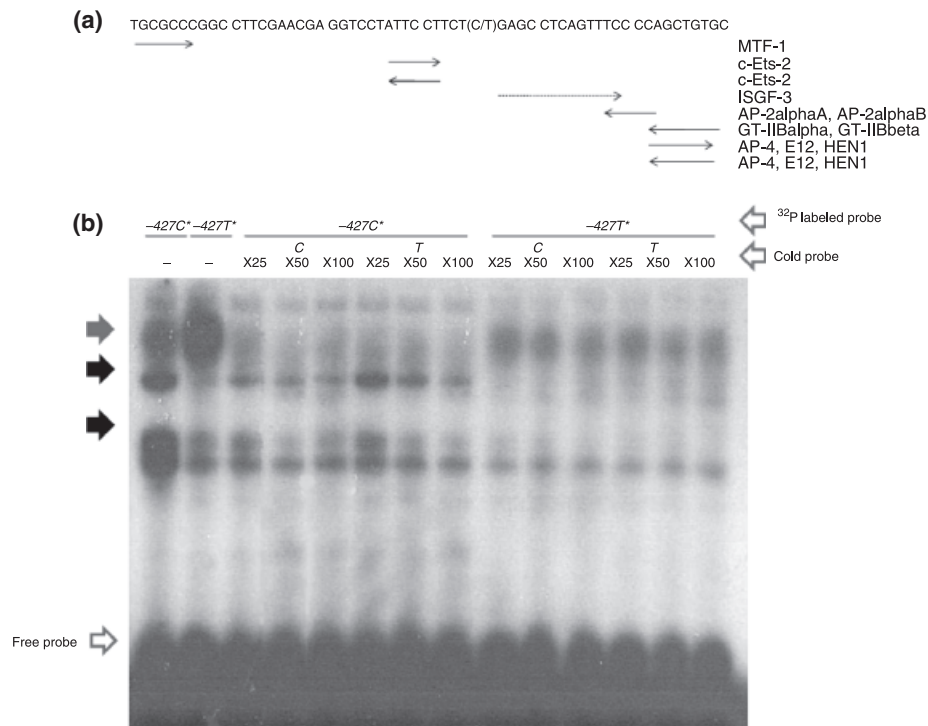


Fig. 4. Gel shift assay for SNP *PPARGC1B* $-427C>T$ using nuclear extract from 293T cells. 10^6 /mL of 293T cells were cultured for 24 h. The nuclear extracts of 293T cells were incubated with the ^{32}P -labeled probe, of which sequences include $-427C>T$ (see online supporting information). To identify specific binding complexes, nuclear extract from 293T cells was preincubated with unlabeled probe before addition of labeled probe for competition. The filled arrow indicates the specific DNA protein. This figure is representative of three different experiments.

Ppargc1b. Co-transfection assays demonstrated that the nuclear receptors associated with the potent coactivator function of *PPARGC1B* were the estrogen receptor alpha (ERa) and the glucocorticoid receptor [10]. In our study, no difference existed in the association of the SNPs with log-transformed PC_{20} methacholine levels according to the gender of the asthmatics (data not shown). This suggested that polymorphisms in the *PPARGC1B* gene may have no genetic effect on ERa binding to nuclear receptor responsive elements in DNA.

Because $+90138T>C$, $+96626G>A$, and $+102525G>A$ are located in exon 2 (L42L), intron 3, and exon 5 (R265Q), respectively, alternative splicing can occur in the *PPARGC1B* gene. Therefore, we amplified mRNA containing exons 1 to 4 and exons 3 to 5 of the *PPARGC1B* gene to identify the isoforms. We identified two RT-PCR products 602 bp and 485 bp in size (Fig. 2a). Direct sequencing revealed that the amplified fragment of 485 bp is an isoform with a 117-bp deletion of exon 4, as previously reported by Kressler et al. [10]. *PPARGC1B* interacts via two LXXLL motifs, nuclear receptor (NR)1 and NR2, with a ligand-dependent conformational change of the ERa activation function-2 (AF-2) domain [10]. NR1 is missing in the *PPARGC1B* isoform, which lacks 39 amino acids encoded by exon 4, as demonstrated in our

study. Therefore, this alternatively spliced *PPARGC1B* exon4-mRNA may lead to the loss of *PPARGC1B* gene function [9], and may cause the development of AHR.

To investigate the genetic effect of the polymorphisms on the mRNA expression of the *PPARGC1B* gene in B cell lines, we measured the *PPARGC1B* mRNA levels of the wild-type and exon4-mRNA forms by real-time PCR, and compared them with the SNPs. We found no association of $+102525G>A$ with the ratio of the isoform to the wild-type form. These results indicated that the change from Q to R caused by $+102525G>A$ may induce a structural change in *PPARGC1B* rather than in the amount of *PPARGC1B* or its isoforms. The structural modification may cause an interaction via the two NR1 and NR2 motifs, with a ligand-dependent conformational change in the intracellular transcription factors binding to nuclear receptor responsive elements on DNA. The strongest association of the *PPARGC1B* SNPs with AHR was found in exon 5 ($+102525G>A$) among the SNPs. This missense mutation led to an asynonymous codon and to an amino acid change during translation, which may affect transcription or protein structure.

As the $-427C>T$ SNP on the promoter may have a regulatory effect on *PPARGC1B* gene transcription, we

evaluated the genetic effect of the SNP using real-time PCR and luciferase assays. The PBMCs having $-427CC$ alleles expressed a greater amount of *PPARGC1B* mRNA than did those having $-427CT$ and *CC* alleles by real-time PCR. These results indicated that the protein expression of the *PPARGC1B* gene may also be transcriptionally regulated at the promoter region. To validate the genetic effect of the $-427C>T$ polymorphism, the difference in the promoter activity was analysed using luciferase assays. The luciferase activity was significantly higher in the reporter constructs containing the *CC* allele than in those containing the *TT* allele. In addition, the difference in the promoter activity was reconfirmed by the difference in binding affinity of the transcription factor(s) to the -427 loci according to the $C>T$ substitution by EMSA. The region surrounding the $-427C>T$ alleles contains no consensus binding sites for transcription factors using the Transcription Element Search Software (TESS; <http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and Searching Transcription Factor Binding Sites (TF; <http://www.cbrc.jp/research/db/TFSEARCH.html>) search systems (Fig. 4a). In our study, a DNA-protein complex was detected with the $-427C>T$ probe. The intensity of this complex was higher when hybridized with the probe for the *CC* allele than with the probe for the *TT* allele, and the intensity was inhibited by treatment with unlabeled probe. The results indicated that the *PPARGC1B* promoter region surrounding $-427C>T$ may be the binding site for a transcription factor, and the DNA binding factor may be a regulator. The identity of transcription factor remains unknown.

To date, breast cancer and obesity are associated with polymorphisms of the *PPARGC1B* gene. Analyses of *BRCA1/2* mutation-negative familial breast cancer patients revealed that the *Ala203Pro* variant is associated with increased familial breast cancer risk for heterozygous and homozygous variant allele carriers [27]. In addition, the common *Ala203* allele is a risk factor for the development of obesity [26]. Considering the biological effect of the *PPARGC1B* gene on ER, PPAR, and GR [also known as nuclear receptor subfamily 3, group C, member 1 (NR3C1)], which are essential for energy and lipid metabolism [13], the association of the *PPARGC1B* gene with the development of breast cancer and obesity is biologically plausible. In our study, we did not identify the *PPARGC1B Ala203Pro* variant by direct sequencing of DNA from 24 Korean subjects, although the frequency is around 8% in the Caucasian population [13]. This may be because of ethnic differences related to the polymorphism.

Induction of many of the genes for these mediators is regulated by transcription factors. Thus, the inflammatory and remodeling process in asthma may be modulated by the several transcription factors, including the glucocorticoid receptor (GR), STAT-6, NFkappaB, Activator Protein-1 (AP-1), Nuclear Factor of Activated T-cells (NF-AT),

cyclic AMP Response Element Binding Protein, the CCAAT/Enhancer Binding Protein (C/EBP), and Peroxisome Proliferator-activated Receptor (PPAR) and the bZIP transcription factor, Nrf2 [8, 9]. Activation of some transcription factors need co-activators to exert their function. Co-transfection assay demonstrated that the nuclear receptors where *PPARGC1B* functioned as a potent coactivator are estrogen receptor alpha and glucocorticoid receptor activity [10].

The effect of estrogen on asthma has been clinically observed. Sex differences exist with respect to the prevalence, incidence, and severity of asthma. In addition, female subjects appear also to exhibit more severe airway hyperresponsiveness (AHR) [28, 29]. Twenty-five to forty percent of reproductive female subjects experienced perimenstrual worsening of asthma, possibly induced by an increase in airway wall inflammation caused by hormonal changes [30–32], indicating that estrogen levels may play a role of bronchial hyperreactivity. Interestingly, SNP on ER1 is associated with AHR, particularly in women [33–35]. However, in our study, there was no difference in the association of these SNPs with log transformed PC20 methacholine according to the gender of the study subjects (data not shown). This suggests that polymorphism on *PPARGC1B* gene may have no genetic effect on Era binding to nuclear receptor responsive element on DNA.

In addition, *PPARGC1B* plays a critical role in regulating multiple aspects of energy metabolism, including adaptive thermogenesis, mitochondrial biogenesis, and fatty acid β -oxidation [12]. This coactivator of nuclear receptors/transcription factors has been shown to control hepatic gluconeogenesis, an important component of the pathogenesis of both type-1 and type-2 diabetes [12]. The diabetes-related changes in BHR has been evidenced in human and experimental studies. BHR is increased in patients with type 2 diabetes during the first 3 months of insulin therapy [36]. Experimental animal data also demonstrated the effect of insulin on mast cell degranulation [37] via enhancement of Th2-mediated responses and activation of NKT cells [38, 39]. Furthermore, PPARs decrease antigen-induced airway hyperresponsiveness, lung inflammation, eosinophilia, cytokine production, and GATA-3 expression as well as serum levels of antigen-specific IgE in a murine model of human asthma [40]. The expression of PPAR is augmented in the bronchial submucosa, the airway epithelium, and the smooth muscle of steroid-untreated asthmatics, as compared with control subjects [41]. Recently, we found The $+82466C>T$ polymorphism and haplotype 1 of the *PPARG* gene may be linked to increased risk for asthma and aspirin hypersensitivity in asthma [42, 43]. Based on these data, dysregulation of the *PPARGC1B* gene may decrease PPAR binding to nuclear receptor-responsive elements in DNA. This may lead to attenuation of the anti-inflammatory action of PPAR on allergic inflammation resulting from AHR.

Clinical relevance

We report that the $-427C > T$ SNP in the promoter and the $+102525G > A$ SNP in exon 5 of the *PPARGC1B* gene may affect AHR development via the modulation of *PPARGC1B* gene products. These data on the genetic polymorphisms of the *PPARGC1B* gene may be useful for developing new methods for identifying genetic markers for AHR.

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Conflict of interest: The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences for *PPARGC1B* sequence variants screening.

Table S2. Sequences of amplifying, Taqman probe for SNP genotyping.

Table S3. PCR primer sequences used in this study.

Table S4. The minor allele frequency (MAF), heterozygosity, Hardy-Weinberg equilibrium of *PPARGC1B* gene polymorphisms.

Table S5. Logistic regression analysis of *PPARGC1B* polymorphisms with the risk of asthma in Korean subjects.

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Supplementary table 1. Primer sequences for *PPARGC1B* sequence variants screening

Name	Region		Primer Sequence (5' → 3')
<i>PPARGC1B</i> -1F	Promoter	Forward	GAGAAGGAAGCTTTTGCCCT
		Reverse	AATCTCTTCGGGGCATCTTT
<i>PPARGC1B</i> -2F	Promoter	Forward	GGAGAGGAAATGGCTTGACA
		Reverse	AAAATTAGCCAGGCATGGTG
<i>PPARGC1B</i> -3F	Promoter-Ex1	Forward	TTCAGGAGGGATATGAAGAGTGA
		Reverse	GAGAGCTCTTCGTCCAGCAG
<i>PPARGC1B</i> -4F	Ex2	Forward	AGAGATGTATGTGGGCAGGG
		Reverse	GGGAGGCCTAGAGAGCAGTT
<i>PPARGC1B</i> -5F	Ex3	Forward	GGTATGGCAGGAGGGTGTT
		Reverse	CCATGTGTGTGCAATCTTCA
<i>PPARGC1B</i> -6F	Ex4	Forward	CCCAAGACCTGCTTGAGTTT
		Reverse	CTGGCCACACTTTCCTCATC
<i>PPARGC1B</i> -7F	Ex5	Forward	GGGAGAGCTGTTAGGAGGCT
		Reverse	CGTCTTGAGCCAGAAGTTCC
<i>PPARGC1B</i> -8F	Ex5	Forward	TGCAACTCATACGCTACATGC
		Reverse	CAGCTCAGGGTTCAGTCTCC
<i>PPARGC1B</i> -9F	Ex5	Forward	GGAAGCTGGAGAGCTCTGTG
		Reverse	CTGTCACATCATCCCCAGTG
<i>PPARGC1B</i> -10F	Ex6	Forward	GCAAATCAAGTCGCTCTCC
		Reverse	ATCCCGGCTGTTAGAGGAAT
<i>PPARGC1B</i> -11F	Ex7	Forward	TACCCTCCAGTGAACCTTGC
		Reverse	GAACCTCCCAGTCATCCAAA
<i>PPARGC1B</i> -12F	Ex8	Forward	GAGCTCCTGGCCTCAAGTAA
		Reverse	AGCCCAAAGTGTTTGGTGAG
<i>PPARGC1B</i> -13F	Ex8	Forward	CAAAGGAAGGTGCTGAGGTC
		Reverse	CTTCAAGGCTCCTGACCAAG
<i>PPARGC1B</i> -14F	Ex9	Forward	GCAGTAGGCATGTGTCCAGA
		Reverse	ACATAGAAGGTGGTGGCAGG
<i>PPARGC1B</i> -15F	Ex10	Forward	GTAAGCAGTGGCTTCCTTGG
		Reverse	GCTGCCCAGCTAACTTGTCT
<i>PPARGC1B</i> -16F	Ex11	Forward	ACAGTACCCACCTCACAGGC
		Reverse	GGCTGTGCACATGACTGAAT
<i>PPARGC1B</i> -17F	Ex12	Forward	GCCCACTCATAGTGGCAGAT
		Reverse	GGCACAGTACATCTGAGCCA

Supplementary Table 2. Sequences of amplifying, Taqman probe for SNP genotyping

Locus		Primer Sequence (5' → 3')
<i>-1381T>C</i>	Forward	TGCTTTCATGGTGTAGAGTCTTGT
	Reverse	AGTTGCCGTTTTACATTTGTTGCA
	Probe-1 (VIC)	ACTTAATCTTTGTATCTCTCC
	Probe-2 (FAM)	TTAATCTTTGTGTCTCTCC
<i>-427C>T</i>	Forward	GCCTTCGAACGAGGTCCTATTC
	Reverse	GCGAGACAACCTGCTTCTTCT
	Probe-1 (VIC)	AAACTGAGGCTCGAGAAG
	Probe-2 (FAM)	CTGAGGCTCAAGAAG
<i>+90138T>C</i>	Forward	GAGCAACTCTATGCTGACTTTCCA
	Reverse	TCAAAGTCGCTGGCATCCA
	Probe-1 (VIC)	CTGGGAGAGGTCAAGTT
	Probe-2 (FAM)	CTGGGAGAGGTCTGAGTT
<i>+96626G>A</i>	Forward	CCTGGGATTAGGTTTCTGGTTCAG
	Reverse	TGTGATCAATATTGCCAGTGATGCA
	Probe-1 (VIC)	CAGAGCAATCCGCTCCA
	Probe-2 (FAM)	CAGAGCAATCCACTCCA
<i>+102525G>A</i>	Forward	CCCCAGCCAGCTCCAG
	Reverse	TGCCCTGCCCAGAGCTA
	Probe-1 (VIC)	TCTCCCCGGGACTC
	Probe-2 (FAM)	TCTCCCCAGGACTC
<i>+102605C>A</i>	Forward	AAGACATGCAGGCGATGGT
	Reverse	CCAGGGCCGGGATCTGA
	Probe-1 (VIC)	TGCATGTAGCGTATGAG
	Probe-2 (FAM)	TGCATGTAGCTTATGAG
<i>+111883G>C</i>	Forward	GGCCCCAGGAAGCCA
	Reverse	TGCCCAAGAAGGAGGACAGA
	Probe-1 (VIC)	CAATGACGGAGTCTC
	Probe-2 (FAM)	CAATGACCGAGTCTC

Supplementary Table 3. PCR primer sequences used in this study

Approaches	Position	Locus	Definition	Primer sequence (5'-3')
RT-PCR				
<i>PPARGC1B</i>	Exon3	+392/+409	Forward	AAGACCCTGGATGACATC
<i>PPARGC1B</i>	Exon5	+976/+993	Reverse	CTACATGCACACCTACTG
Promoter construct				
Primary PCR	Promoter	-883/-862	Forward	TGATGTGCGCTGTAGCTTTGAAC
	Exon1	+216/+231	Reverse	GATGCGCTCCGTTAC
Nested PCR	Promoter	-613/-593	Forward, Nested	<u>AGATCT</u> GGCGCCCCGCCACCATGCCTG
	Exon1	-62/-42	Reverse, Nested	BglII GCTCCCTCCCCCGGGCCGGA <u>AGCTT</u> HindIII
Point-mutation overlap PCR				
<i>-427T</i>	Promoter	-442/-412	Forward	TATTCCTTCTTGAGCCTCAGT
	Promoter	-442/-412	Reverse	ACTGAGGCTCAAGAAGGAATA
Cut	Promoter	-393/-367	Forward	<u>AGATCT</u> CAGGTTGTCTCGCAGGACCC BglII
Real-time PCR				
<i>PPARGC1B</i> _ Full length	Exon1	+108/+128	Forward	TCTTCCTCAACTATCTCGCTGACA
	Exon2	+160/+185	Reverse	TATGCTGACTTTCCAGAACTTGACC
<i>PPARGC1B</i> _ Alternative splicing	Exon4	+598/+623	Forward	GCAGGCCTCAGATCTAAAAGTCAAC
	Exon5	+649/+672	Reverse	CAAGACAAGAAGGCTCCCATGAT
<i>ACTB</i>	Exon1	+747/+766	Forward	GGA ~~CTT~~ CGAGCAAGAGATGG
	Exon2	+980/+961	Reverse	AGCACTGTGTTGGCGTACAG

EMSA

<i>-427C</i>	Promoter	-442/-412	Forward	TATTCCTTCTCGAGCCTCAGT
	Promoter	-412/-442	Reverse	ACTGAGGCTCGAGAAGGAATA
<i>-427T</i>	Promoter	-442/-412	Forward	TATTCCTTCTTGAGCCTCAGT
	Promoter	-412/-442	Reverse	ACTGAGGCTCAAGAAGGAATA

Primers for *PPARGC1B* gene were designed using the Genbank sequence AF468496 (mRNA) or NT_029289 (genomic). The first base of translation site is denoted as nucleotide +1. And Primers for *ACTB* gene were designed using the Genbank sequence NM_001101 (mRNA).

Supplementary Table 4. The minor allele frequency (MAF), heterozygosity, Hardy-Weinberg equilibrium of *PPARGC1B* gene polymorphisms

Loci	rs	Position	Genotype				MAF	Heterozygotes	HWE
<i>-1381T>C</i>	Novel	Promoter	T	CT	C	N	0.005	0.009	0.874
			1196	11	0	1207			
<i>-427C>T</i>	Novel	Promoter	C	CT	T	N	0.078	0.144	0.351
			1023	178	5	1206			
<i>+90138T>C</i>	rs32588	Exon2 (L42L)	T	CT	C	N	0.052	0.099	0.006
			1093	110	8	1211			
<i>+96626G>A</i>	rs45560442	Intron3	G	AG	A	N	0.092	0.167	0.679
			995	204	9	1208			
<i>+102525G>A</i>	rs45520937	Exon5 (R265Q)	G	AG	A	N	0.134	0.233	0.134
			914	270	28	1212			
<i>+102605C>A</i>	rs11959820	Exon5 (R292S)	C	AC	A	N	0.098	0.177	0.606
			976	216	10	1202			
<i>+11183G>C</i>	rs26127	Intron9	G	CG	C	N	0.242	0.367	0.770
			691	436	72	1199			

Loci is calculated from the transcriptional start site. For an exact calculation of allele frequencies in Korean population, samples from our Korea asthma cohort (n = 1213) were also included.

Supplementary Table 5. Logistic regression analysis of *PPARGC1B* polymorphisms with the risk of asthma in Korean subjects

Loci	rs	Position	MAF		OR(95%CI)	P
			BA	NC		
<i>-1381T>C</i>	Novel	Promoter	0.005	0.002	2.39(0.29-19.48)	0.41
<i>-427C>T</i>	Novel	Promoter	0.080	0.067	1.16(0.77-1.75)	0.47
<i>+90138T>C</i>	rs32588	Exon2 (L42L)	0.050	0.057	0.90(0.58-1.38)	0.62
<i>+96626G>A</i>	rs45560442	Intron3	0.091	0.093	0.99(0.69-1.41)	0.96
<i>+102525G>A</i>	rs45520937	Exon5 (R265Q)	0.131	0.140	0.96(0.71-1.28)	0.76
<i>+102605C>A</i>	rs11959820	Exon5 (R292S)	0.101	0.082	1.32(0.91-1.91)	0.15
<i>+11183G>C</i>	rs26127	Intron9	0.243	0.230	1.11(0.87-1.41)	0.40
<i>ht1</i>	.	.	0.715	0.738	0.88(0.70-1.10)	0.27
<i>ht2</i>	.	.	0.101	0.081	1.32(0.91-1.92)	0.14
<i>ht3</i>	.	.	0.049	0.055	0.96(0.61-1.51)	0.84

Logistic regression analysis was performed for calculating odd ratio and corresponding *P* values of codominant model controlling age, sex, atopy and smoking status.