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# Functional Polymorphisms of the Arachidonic Acid Pathway Associate with Risks and Clinical Outcomes of Allergic Diseases

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**Keywords**
Asthma · Allergic rhinitis · Lung function · Arachidonic acid · Genetic polymorphism

**Abstract**
***Introduction:*** The arachidonic acid (AA) pathway plays a crucial role in allergic inflammatory diseases; however, the functional roles of allergy-associated single nucleotide polymorphisms (SNPs) in this pathway remain incompletely illustrated. ***Methods:*** This study belongs to a part of an ongoing Singapore/Malaysia cross-sectional genetics and epidemiological study (SMCSGES). We performed population genotyping on $n$ = 2,880 individuals from the SMCSGES cohort to assess the associations of SNPs in the AA pathway genes with asthma and allergic rhinitis (AR). Spirometry assessments were performed to identify associations between SNPs and lung function among $n$ = 74 pediatric asthmatic patients from the same cohort. Allergy-associated SNPs were functionally characterized using in vitro promoter luciferase assay, along with DNA methylome and transcriptome data of $n$ = 237 peripheral blood mononuclear cell (PBMC) samples collected from a subset of the SMCSGES cohort. ***Results:*** Genetic association analysis showed 5 tag-SNPs from 4 AA pathway genes were significantly associated with asthma (rs689466 at *COX2*, rs35744894 at hematopoietic $PGD_2$ synthase (*HPGDS*), rs11097414 at *HPGDS*, rs7167 at *CRTH2*, and rs5758 at *TBXA2R*, $p < 0.05$), whereas 3 tag-SNPs from *HPGDS* (rs35744894, rs11097414, and rs11097411) and 2 tag-SNPs from *PTGDR* (rs8019916 and rs41312470) were significantly associated with AR ($p < 0.05$). The asthma-associated rs689466 regulates *COX2* promoter activity and associates with *COX2* mRNA expression in PBMC. The allergy-associated rs1344612 was significantly associated with poorer lung function, increased risks of asthma and AR, and increased *HPGDS* promoter activity. The allergy-associated rs8019916 regulates *PTGDR* promoter activity and DNA methylation levels of cg23022053 and cg18369034 in PBMC. The asthma-associated rs7167 affects *CRTH2* expression by regulating the methylation level of cg19192256 in PBMC. ***Conclusions:*** The present study identified multiple allergy-associated SNPs that modulate the transcript expressions of key genes in the AA pathway. The development of a "personalized medicine" approach with consideration of genetic influences on the AA pathway may hopefully result in efficacious strategies to manage and treat allergic diseases.



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## Introduction

The arachidonic acid (AA) pathway generates a wide spectrum of pro-inflammatory mediators and is identified as one of the key drivers in the development of allergic inflammatory diseases, including asthma and allergic rhinitis (AR) [1–3]. AA can be metabolized by the cyclooxygenase (COX) enzyme pathway to produce biologically active prostaglandins (PGs) that are important mediators of inflammatory processes [1]. There are two major forms of COXs: COX1 is constitutively expressed in most cells and produces PGs that serve housekeeping functions [4]; COX2 can be either constitutive or inducible, depending on the tissue type. In acute and chronic inflammatory states, COX2 is induced by cytokines to facilitate the synthesis of $PGH_2$. $PGH_2$ is the precursor for prostanoids such as $PGD_2$ and $PGE_2$, as well as thromboxane A2 (TBXA2). $PGD_2$ is mainly produced in mast cells and eosinophils by the hematopoietic $PGD_2$ synthase (HPGDS), which can cause bronchoconstriction, induce vascular leakage, recruits and activates dendritic cells through either the chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2) or $PGD_2$ receptor (PTGDR) [2, 5]. TBXA2 is synthesized by the TBXA synthase and exerts its biological function via its receptor TBXA2R. Studies have also shown the activation of TBXA2R by its ligand leads to airway smooth muscle cell contraction, proliferation, and hypertrophy [6–8]. $PGE_2$ can bind to $PGE_2$ receptor 1 ($EP_1$), $EP_2$, $EP_3$, or $EP_4$ and was shown to have protective effect against asthma-related phenotype: inhalation of $PGE_2$ protected against bronchoconstriction [9], while reduced levels of $PGE_2$ were also observed in aspirin-exacerbated respiratory disease (AERD) patients [10].

Globally, allergic diseases including asthma and AR affect about 10–30% of the population [11, 12]. These allergic conditions result from a complex interplay between genetic and environmental factors. Prior studies have investigated the disease associations of single nucleotide polymorphisms (SNPs) in key genes of the AA pathway, including *COX2* [13, 14], *PTGDR* [15–17], *CRTH2* [18], and *TBXA2R* [19]. Nevertheless, most findings are considered inconsistent or lack significant disease association. The functional roles of these SNPs in allergic diseases also remain incompletely illustrated. Here, we performed population genotyping in a cohort of $n$ = 2,880 Singaporean Chinese to assess the associations of SNPs in *COX2*, *HPGDS*, *CRTH2*, *PTGDR*, and *TBXA2R* with asthma and AR. Allergy-associated SNPs were functionally characterized using DNA methylome and transcriptome data, along with in vitro assays. In the present study, we propose a functional role of allergy-associated genetic polymorphisms in the pathobiology of asthma and AR by modulating the AA pathway.

## Materials and Methods

*Study Design and Participant Recruitment*

This present study belongs to a part of an ongoing Singapore and Malaysia cross-sectional genetic and epidemiological study (SMCSGES). The recruitment of this cohort has been previously described [20–23]. Participants were recruited from the National University of Singapore (NUS) from Aug 2005 to Sep 2009, whereas pediatric asthmatic patients were recruited from KK Hospital in Singapore from January 2004 to December 2007. Participants from the University of Tunku Abdul Rahman (UTAR), Malaysia, were recruited from January 2018 to October 2018. The demographics were summarized in Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000530393).

An investigator-administered questionnaire was provided to participants to collect information on demographics and allergy-related medical history designed based on the Allergic Rhinitis Impact on Asthma (ARIA) [24] and International Study of Asthma and Allergies in Childhood (ISAAC) [25] guidelines. The Chinese ethnicity of participants was confirmed through survey questionnaires, as well as a previously performed principal component analysis [26]. Atopy was defined as having a positive skin prick test reaction toward common house dust mite allergens (*Dermatophagoides pteronyssinus* or *Blomia tropicalis*). A positive skin prick test reaction was defined as having a wheal of at least 3 mm in diameter 15 min after the skin prick. A positive control (1 mg/mL of histamine) and a negative control (0.9% of saline) were also used to validate the allergen-based skin prick measurements. Asthma was defined as ever having asthma positively diagnosed by a physician. AR was defined as having at least two major symptoms that include nasal congestion, rhinorrhea, nasal itching, and sneezing (based on 2008 guidelines set by the ARIA consortium [24]). Both asthma and AR conditions were further confirmed by having an atopy condition. Nonatopic asthma and nonatopic rhinitis individuals were excluded from the analysis. Spirometry measurements were performed on 75 pediatric asthmatic patients from the KK cohort according to American Thoracic Society (ATS) and European Respiratory Society (ERS) guidelines [27].

*Genotyping and Sanger Sequencing*

Genomic DNA (gDNA) samples were extracted from mouthwash samples for subsequent genotyping and sequencing assays. The extraction was performed using Axygen® AxyPrep™ Multisource Genomic Miniprep DNA Kit (Axygen, USA), according to the manufacturer's protocol. Quantification of the gDNA concentration was done in triplicates using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Singapore). Genotyping was performed on the Illumina BeadXpress platform using the GoldenGate Assay (Illumina, Inc., USA) and on the SNP Research Facility at the Washington University in St Louis for genotyping on the Sequenom platform using the MassARRAY iPLEX assay (Sequenom, Inc., USA). For the sequencing of *COX2*, *HPGDS*,

*PTGDR*, and *TBXA2R*, including the 2 kb regions flanking these genes, the process was done via Sanger sequencing, according to published methodology [28].

*In vitro Assays*

*Cell culture*: human embryonic kidney cells (HEK293T) were purchased from the American Type Culture Collection (ATCC, USA). HEK293T cells were grown in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum, 2 g/L sodium bicarbonate, and 2 mmol/L L-glutamine. Human keratinocyte cells (HaCat) were purchased from Cell Lines Service (CLS, Germany). HaCat cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 3.7 g/L sodium bicarbonate. All cell lines were cultured in a 37 °C humidified incubator with a 5% $CO_2$ environment. Transient transfection of plasmid constructs into the cell lines of interest was performed using Lipofectamine 2000 (Invitrogen, Singapore), with conditions optimized according to the manufacturer's protocol.

*Promoter luciferase assay*: the promoter luciferase assay was performed by cloning the putative promoter regions of *COX2* (−1,446 bp to −520 bp), *HPGDS* (−1,562 bp to +332 bp), and *PTGDR* (1,562 bp to +332 bp) separately into the plasmid pGL4.10 with a promoter-less luciferase gene (Promega, Singapore). Renilla luciferase control plasmid pGL4.74 was also co-transfected for assay normalization purposes. Both Firefly and Renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Singapore). Relative luciferase unit was calculated by dividing Firefly luciferase activity reading by that of Renilla luciferase. For the *COX2* promoter luciferase assay, testosterone (1 μg/mL), progesterone (1 μg/mL), or DMSO (as a treatment control) was also added at 4 h after transfection into HEK293T. All experiments were performed in triplicates with cells harvested at 24 or 48 h after transfection.

*Minigene assay*: based on the prediction data from SNPinfo (28), three *TBXA2R* SNPs (rs1131882, rs4523, and rs5758) are located in the exon splicing enhancer or silencer region of this gene. Thus, we performed an in vitro minigene assay to assess whether these SNPs can affect the splicing activity of *TBXA2R*. Based on our sequencing data, we identified 3 common haplotypes (frequency >5%) constituted by the rs1131882-rs4523-rs5758 SNP-set, including Hap1 (T-T-C), Hap2 (C-T-C), and Hap3 (C-C-T, online suppl. Fig. S1a). Constructs for the minigene assay were generated to include these 3 haplotypes separately in a 2,952 bp fragment of the TBXA2R gene spanning exon 2–4 of its isoform β (online suppl. Fig. S1a). The expected length of the transcribed mRNA of TBXA2R isoform α and β was 2,352 bp and 1,692 bp, respectively. We cloned the 2,952 bp *TBXA2R* gene fragment into the mammalian expression vector pcDNA3.1/Zeo(+) which has a *zeocin* resistance gene as an internal transfection control (Invitrogen, Singapore). Constructs were transfected into HEK293T and harvested 24 h after transfection. Total RNA was extracted from the harvested HEK293T cells using E.Z.N.A.® Total RNA Kit (Omega Bio-tek, USA) and converted to cDNA using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs); both were performed according to the manufacturer's protocol. PCR amplification followed by gel electrophoresis was done for both *TBXA2R* and *zeocin* cDNA transcripts. The forward and reverse primer sequences used for the PCR amplification of *TBXA2R* were "TAATACGACTCACTAT" and "TAGAAGGCA CAGTCGAGG," respectively, while the forward and reverse primer sequences used for the PCR amplification of *zeocin* were "GGCCAAGTTGACCAGTGCCGTT" and "CCACGAAGTGCA CGCAGTT," respectively. All experiments were performed in triplicates.

*Transcriptome Microarray*

Approximately 24 mL of whole blood sample was collected from each individual of the SMCSGES cohort, with peripheral blood mononuclear cells (PBMCs) extracted using Ficoll-Hypaque density-gradient centrifugation. Total RNA was extracted from PBMC using E.Z.N.A.® Total RNA Kit (Omega Bio-tek, USA), according to the manufacturer's protocol. Total RNA samples were sent for whole-transcriptome expression analysis using the Affymetrix Human Exon 1.0 ST Array. The assay was done in a facility provided by Origen Laboratories, Singapore. Affymetrix Power Tools were used for data extraction and quantile normalization, according to reported methodology [29]. Transcript expression data of array probe with transcript cluster ID 2448382 were retrieved for *COX2* mRNA expression analysis.

*DNA Methylome and Transcriptome Profiling*

*gDNA and total RNA extraction*: for DNA methylome and transcriptome profiling of PBMC samples collected from the SMCSGES cohort, 10 mL of whole blood was collected with PBMCs extracted using Ficoll-Hypaque density-gradient centrifugation. The extraction of gDNA from PBMC was performed using Axygen® AxyPrep™ Multisource Genomic Miniprep DNA Kit (Axygen, USA), while the extraction of total RNA from PBMC was performed using E.Z.N.A.® Total RNA Kit (Omega Bio-tek, USA); both were performed according to the manufacturer's protocol.

*SNP genotyping*: the Infinium OmniZhongHua-8 v1.3 BeadChip platform was used for SNP genotyping, according to the manufacturer's protocols. Haplotype phasing and imputations of the data were performed using the IMPUTE2 program with a reference panel comprising the 1000 Genomes Phase 1 database [30].

*DNA methylation profiling*: the gDNA samples first underwent the bisulfite conversion process, and the DNA methylome was assayed using the Infinium MethylationEPIC BeadChip Kit (Illumina, San Diego, CA), according to the manufacturer's protocols. Annotation of the array data was performed using the latest manifest file provided by Illumina, "Infinium MethylationEPIC v1.0 B4 Manifest File," which comprises information of 865,918 probes. Raw data were preprocessed and quantile normalized, with methylation levels (beta values, β) subsequently calculated using the Minfi package in the R program version 3.6.1.

*Whole-transcriptome sequencing*: for library construction, mRNA was first enriched using oligo (dT) beads, and NEBNext Ultra RNA Library Prep Kit was used according to the manufacturer's protocols. Sequencing was then performed using Illumina NovaSeq 6000 system. Mapping of raw sequence to human genomic sequence (NCBI GRCh38 Assembly) was done using TopHat version 2.1.1. Fragments per kilobase of transcript per million mapped reads were calculated using Cufflinks version 2.2.1. For both DNA methylation profiling and whole-transcriptome sequencing, all samples were measured and analyzed at the same time to prevent batch variation.

*Statistical Analysis*

The linkage disequilibrium (LD) pattern and r-square values between SNPs were calculated and drawn using software Haploview® vers4.2 [http://www.broadinstitute.org/haploview]. Fisher's tests were used to calculate genotype-phenotype associations in the PLINK program version 1.06. Fisher's *p*, odds ratios, and Hardy-Weinberg equilibrium (HWE) test were all evaluated using the same PLINK program. False discovery rate (FDR) adjustment was performed for all assessed SNPs within each of the candidate genes, based on the Benjamini and Hochberg method [31]. Association analyses of SNP genotypes with normally distributed continuous variables, including mRNA expression levels in PBMC, relative luciferase units measured from the promoter luciferase assay, lung function parameters, and DNA methylation levels (β), were performed using the independent sample *t* test or ANOVA test in the R program version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Correlation analyses between DNA methylation levels (β) and mRNA expression levels in PBMC (fragments per kilobase of transcript per million) were performed using the Pearson's correlation test in the same R program. Dot plot and box plot figures included in this report were also drawn in the same R program using R package ggplot2 [32].

## Results

*AA Pathway Genetic Polymorphisms Are Significantly Associated with AA and AR in the Singapore/Malaysia Population*

We first aim to identify common SNPs from AA pathway genes (minor allele frequency >0.05) that were presented in the SMCSGES cohort (online suppl. Table S1). We sequenced *COX2*, *HPGDS*, *PTGDR*, and *TBXA2R* on 40 individuals from this cohort (age = 19.9 ± 1.69, 45% male, online suppl. Table S1) and retrieved genotype information of *CRTH2* SNPs from the HapMap Chinese Han (CHB) population database [33]. A total of 38 common SNPs (minor allele frequency >5%) were identified, which were tagged by 21 tag-SNPs using an LD threshold of $r^2 > 0.8$ (online suppl. Fig. S2). Then, 13 tag-SNPs were successfully genotyped on 2,880 individuals from the SMCSGES cohort. Of these, 5 tag-SNPs from 4 genes were significantly associated with asthma (rs689466 at *COX2*, rs35744894 at *HPGDS*, rs11097414 at *HPGDS*, rs7167 at *CRTH2*, and rs5758 at *TBXA2R*, $p < 0.05$, Table 1), whereas 3 tag-SNPs from *HPGDS* (rs35744894, rs11097414, and rs11097411) and 2 tag-SNPs from *PTGDR* (rs8019916 and rs41312470) were significantly associated with AR ($p < 0.05$, Table 2). These SNPs were significantly associated with allergy after FDR adjustment, except for the association between rs7167 and asthma, which was only marginally significant (FDR-adjusted $p = 0.066$, Table 1, 2). Further, gender-stratified analysis has showed the asthma-associated tag-SNP rs689466 was significant only among the male subjects ($n = 1076$, FDR-adjusted $p = 0.0378$) but not among the female subjects ($n = 1087$, FDR-adjusted $p = 0.804$, online suppl. Table S2). This suggests sex hormone regulation may be involved in the association between rs689466 and asthma.

*Asthma-Associated Tag-SNP rs689466 Influences Promoter Activity and mRNA Expression of* COX2 *in the AA Pathway*

Next, we assessed if these allergy-associated SNPs have a functional effect on key genes of the AA pathway. An overdominance effect was observed for rs689466 on asthma risk: individuals carrying either genotype "AA" or "GG" have increased risk of the disease, as compared to the "AG" genotype (FDR-adjusted $p = 0.0321$, OR for AA vs. AG = 1.34, 95% CI = 1.07–1.68; OR for GG vs. AG = 1.30, 95% CI = 1.05–1.61). Given rs689466 is located at −1,192 bp of the *COX2* gene, we generated constructs containing −1,446 bp to −520 bp of *COX2* to conduct an in vitro luciferase assay. Allele "G" of rs689466 was associated with a significantly higher *COX2* promoter activity as compared to allele "A" in HEK293T ($p < 0.0001$,Fig. 1a). This significant trend was consistently observed even after the addition of sex hormones, including testosterone and progesterone (Fig. 1a). This suggests the gender-specific association of rs689466 with asthma risk might be influenced by other social and environmental factors (reviewed in [34]). Besides, we also used an expression array to measure *COX2* transcript expression data of 60 PBMC samples from the SMCSGES cohort (age = 21.28 ± 3.64, 55% male, online suppl. Table S1). Among the male subjects ($n = 33$), we observed a significant dosage effect of increasing *COX2* transcript expression level from genotype "AA" to "GG" of rs689466 ($p = 0.0173$, Fig. 1b). Concordantly, data from the eQTLgen consortium [35] also showed a significant increase in *COX2* mRNA expression in PBMC from allele "A" to "G" of rs689466 (FDR-adjusted $p = 1.235\text{x}10^{-23}$, *z*-score = 10.021 for allele "G," online suppl. Table S3).

*Allergy-Associated Tag-SNP rs11097414 Affects* HPGDS *Promoter Activity and Associates with Poorer Clinical Outcomes in Pediatric Asthma*

The allergy-associated rs11097414 is in a strong LD with rs1344612 ($r^2 = 1$) that is located 1,201 bp upstream of the *HPGDS* gene. We generated constructs comprising −1,531 bp to +242 bp of the *HPGDS* gene to conduct an in vitro luciferase assay. Allele "T" of rs1344612 was significantly associated with a higher *HPGDS* promoter activity as compared to allele "C" in both HEK293T ($p < 0.05$, Fig. 2a) and HaCat cells ($p < 0.05$, Fig. 2b). Concordantly, data from the eQTLGen consortium [35] also

**Table 1.** Association of tag-SNPs from AA pathway genes with the allergic asthma phenotype

| SNP | Nearby gene | Minor/major allele (A/B) | MAF | *p* value | *p* value (FDR adjusted) | OR (95% CI) | | | $P_{HWE}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | AA versus AB[a] | AA versus BB[a] | AB versus BB[a] | |
| *Asthma versus atopy non-asthma (n = 719 vs. n = 1,444)* | | | | | | | | | |
| rs4648302 | COX2 | T/G | 0.0705 | 0.984 | 0.984 | 0.81 (0.15–4.26) | 0.80 (0.15–4.12) | 0.98 (0.76–1.27) | 0.08592 |
| rs5275 | COX2 | C/T | 0.188 | 0.932 | 0.932 | 0.96 (0.56–1.65) | 0.93 (0.55–1.58) | 0.97 (0.80–1.18) | 0.1982 |
| rs689466 | COX2 | G/A | 0.479 | 0.0107* | 0.0321* | 1.34 (1.07–1.68) | 1.03 (0.81–1.32) | 0.77 (0.62–0.95) | 0.1441 |
| rs35744894 | HPGDS | A/G | 0.206 | 0.0179* | 0.0269* | 1.57 (1.01–2.42) | 1.79 (1.17–2.73) | 1.14 (0.94–1.38) | 1 |
| rs11097414 | HPGDS | T/A | 0.341 | 0.00609* | 0.0183* | 1.31 (0.99–1.74) | 1.57 (1.18–2.10) | 1.20 (0.99–1.46) | 0.6478 |
| rs11097411 | HPGDS | G/A | 0.291 | 0.0788 | 0.0788 | 1.34 (0.97–1.85) | 1.45 (1.05–2.00) | 1.08 (0.90–1.31) | 0.3386 |
| rs530963 | CRTH2 | G/T | 0.0880 | 0.545 | 0.545 | 1.67 (0.61–4.60) | 1.55 (0.57–4.17) | 0.93 (0.72–1.19) | 0.9071 |
| rs7167 | CRTH2 | T/C | 0.257 | 0.118 | 0.236 | 0.82 (0.55–1.23) | 0.71 (0.48–1.05) | 0.87 (0.71–1.05) | 0.5206 |
| rs8019916 | PTGDR | C/T | 0.115 | 0.979 | 1.000 | 0.98 (0.45–2.13) | 0.95 (0.45–2.04) | 0.98 (0.78–1.23) | 0.5207 |
| rs33998702 | PTGDR | T/C | 0.154 | 0.120 | 0.480 | 0.46 (0.21–1.00) | 0.45 (0.21–0.99) | 1.00 (0.81–1.22) | 0.2821 |
| rs8004654 | PTGDR | C/T | 0.319 | 0.271 | 0.542 | 0.83 (0.60–1.16) | 0.77 (0.55–1.07) | 0.92 (0.77–1.12) | 0.4907 |
| rs41312470 | PTGDR | A/G | 0.116 | 1.000 | 1.000 | 0.92 (0.42–1.99) | 0.92 (0.43–1.95) | 1.00 (0.80–1.26) | 0.466 |
| *Asthma versus non-atopy non-asthma (n = 719 vs. n = 716)* | | | | | | | | | |
| rs4648302 | COX2 | T/G | 0.0705 | 0.593 | 0.810 | 1.80 (0.16–20.17) | 2.02 (0.18–22.36) | 1.12 (0.83–1.53) | 0.08592 |
| rs5275 | COX2 | C/T | 0.188 | 0.810 | 0.810 | 0.84 (0.45–1.55) | 0.89 (0.49–1.62) | 1.06 (0.84–1.33) | 0.1982 |
| rs689466 | COX2 | G/A | 0.479 | 0.642 | 0.810 | 1.09 (0.84–1.41) | 0.98 (0.74–1.30) | 0.90 (0.70–1.15) | 0.1441 |
| rs35744894 | HPGDS | A/G | 0.206 | 0.0270* | 0.0405* | 1.63 (0.96–2.78) | 1.91 (1.14–3.21) | 1.17 (0.94–1.47) | 1 |
| rs11097414 | HPGDS | T/A | 0.341 | 0.0117* | 0.0351* | 1.51 (1.07–2.12) | 1.69 (1.19–2.39) | 1.12 (0.90–1.40) | 0.6478 |
| rs11097411 | HPGDS | G/A | 0.291 | 0.0786 | 0.0786 | 1.43 (0.97–2.11) | 1.55 (1.06–2.28) | 1.09 (0.88–1.35) | 0.3386 |
| rs530963 | CRTH2 | G/T | 0.0880 | 0.988 | 0.988 | 1.14 (0.37–3.49) | 1.15 (0.39–3.46) | 1.02 (0.76–1.36) | 0.9071 |
| rs7167 | CRTH2 | T/C | 0.257 | 0.0330* | 0.0660 | 0.75 (0.48–1.17) | 0.61 (0.39–0.94) | 0.82 (0.66–1.02) | 0.5206 |
| rs8019916 | PTGDR | C/T | 0.115 | 0.520 | 0.573 | 1.12 (0.45–2.78) | 0.97 (0.40–2.35) | 0.86 (0.67–1.12) | 0.5207 |
| rs33998702 | PTGDR | T/C | 0.154 | 0.182 | 0.573 | 0.45 (0.19–1.06) | 0.47 (0.20–1.10) | 1.05 (0.83–1.33) | 0.2821 |
| rs8004654 | PTGDR | C/T | 0.319 | 0.328 | 0.573 | 0.76 (0.52–1.1) | 0.77 (0.53–1.11) | 1.01 (0.81–1.26) | 0.4907 |

**Table 1** (continued)

| SNP | Nearby gene | Minor/major allele (A/B) | MAF | *p* value | *p* value (FDR adjusted) | OR (95% CI) | | | $P_{HWE}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | AA versus AB[a] | AA versus BB[a] | AB versus BB[a] | |
| rs41312470 | PTGDR | A/G | 0.116 | 0.573 | 0.573 | 1.11 (0.45–2.76) | 0.98 (0.40–2.36) | 0.88 (0.68–1.13) | 0.466 |
| rs5758 | TBXA2R | T/C | 0.1359 | 0.0221* | 0.0221* | 2.67 (1.03–6.92) | 3.15 (1.24–8.01) | 1.18 (0.91–1.54) | 0.8094 |

All data were evaluated based on the genotyping results of $n$ = 2,880 individuals from the SMCSGES cohort. For genetic association tests, *p* value was calculated using the Fisher's exact test. FDR adjustment was performed for all assessed SNPs within each of the candidate genes, based on the Benjamini and Hochberg method [31]. The minor allele is designed as "A," while the major allele is designated as "B" for the SNP. CI, confidence interval; *COX2*, cyclooxygenase-2; *CRTH2*, chemoattractant receptor-homologous molecule expressed on T helper 2 cells; FDR, false discovery rate; *HPGDS*, hematopoietic prostaglandin D synthase; MAF, minor allele frequency; OR, odds ratio; $P_{HWE}$, Hardy-Weinberg equilibrium *p* value for the tag-SNP assessed in the entire population, with significant cutoff at $p < 0.001$; *PTGDR*, prostaglandin D2 receptor; SMCSGES, Singapore/Malaysia cross-sectional genetics and epidemiological study; SNP, single nucleotide polymorphism; *TBXA2R,* thromboxane A2 receptor. *Fisher's $p < 0.05$ is considered as significant. [a]Reference genotype for the odds ratio calculation.

showed allele "T" of rs1344612 was associated with a higher *HPGDS* mRNA expression than allele "C" (FDR-adjusted $p$ = 0.00276, $z$-score = −4.9009 for allele "C," online suppl. Table S3). This indicates that allele "T" rs1344612 might result in a higher *HPGDS* transcript expression and upregulated AA pathway, which then contribute to the development of asthma and AR in the SMCSGES cohort.

Besides, among 75 pediatric asthmatic patients in the SMCSGES cohort (age = 10.83 ± 2.67, 72% male, online suppl. Table S1), allele "T" of rs11097414 was also significantly associated with reduced forced expiratory flow ($p$ = 0.00892, Fig. 2c), reduced forced expiratory volume in the first second (FEV1, $p$ = 0.00641, Fig. 2d), and reduced FEV1 to forced vital capacity ratio ($p$ = 0.0295, Fig. 2e). These findings suggested the allele "T" of rs11097414 that was associated with a higher *HPGDS* transcript expression level might also be associated with poorer clinical outcomes of pediatric asthma.

### *AR-Associated Tag-SNP rs8019916 Modulates* PTGDR *Promoter Activity and the Methylation Levels at cg23022053 and cg18369034*

The AR-associated rs8019916 is located at −1,005 bp of *PTGDR*. We generated constructs including −1,520 bp to +396 bp of *PTGDR* that contain 4 common haplotypes (Hap1, Hap2, Hap3, and Hap4, frequency >5%) formed by 5 SNPs (rs8019916, rs33998702, rs34236606, rs8004654, and rs803010) to conduct an in vitro luciferase assay (Fig. 3a). Allele "C" of rs8019916 was associated with a higher promoter activity of *PTGDR* in HEK293T (Fig. 3b) and HaCat cells (Fig. 3c). Further, by analyzing DNA methylome and transcriptome data of 237 PBMC samples from the SMCSGES cohort (age = 21.37 ± 4.24, 40.5% male), allele "C" of rs8019916 is also associated with reduced DNA methylation levels at two CpG sites (cg23022053 and cg18369034) located in *PTGDR* ($p$ = $1.01 \times 10^{-139}$ and $7.97 \times 10^{-13}$, respectively, Fig. 3d–f). This suggests rs8019916 might influence both promoter activity and DNA methylation levels in *PTGDR* of the AA pathway, which results in its association with AR susceptibility in the Singapore/Malaysia population.

### *Asthma-Associated Tag-SNP rs7167 Influences* CRTH2 *mRNA Expression in the AA Pathway*

The asthma-associated rs7167 is located at 264 bp downstream of the *CRTH2*. By analyzing the DNA methylome and transcriptome data from the SMCSGES cohort ($n$ = 237, online suppl. Table S1), genotype "TT" of rs7167 was significantly associated with reduced methylation levels of cg19192256 ($p = 4.95 \times 10^{-5}$), cg12528056 ($p = 5.23 \times 10^{-11}$), and cg19821841 ($p$ = 0.00105, Fig. 4a, b). Of these CpG sites, the methylation level of cg19192256 was also inversely correlated with the mRNA expression of *CRTH2* (Pearson's $R$ = −0.211, $p$ = 0.00121, Fig. 4c). Besides, data from the eQTLgen consortium [35] showed a significant increase of *CRTH2* mRNA expression in PBMC was associated with allele "T" as compared to allele "C" of rs7167 (FDR-adjusted $p$ = 0.00278, z-score = −4.8986 for allele "G," online suppl. Table S3). These findings suggested genotype "TT" of rs7167 might reduce the methylation level of cg19192256, which result in a

**Table 2.** Association of tag-SNPs from AA pathway genes with the AR phenotype

| SNP | Nearby gene | Minor/major allele (A/B) | MAF | *p* value | *p* value (FDR adjusted) | OR (95% CI) | | | $P_{HWE}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | AA versus AB[a] | AA versus BB[a] | AB versus BB[a] | |
| *AR versus atopy non-AR (n = 795 vs. n = 811)* | | | | | | | | | |
| rs4648302 | COX2 | T/G | 0.0705 | 0.851 | 0.851 | 1.11 (0.22–5.61) | 1.00 (0.20–4.99) | 0.91 (0.69–1.20) | 0.08592 |
| rs5275 | COX2 | C/T | 0.188 | 0.715 | 0.851 | 1.28 (0.71–2.31) | 1.26 (0.71–2.23) | 0.98 (0.79–1.21) | 0.1982 |
| rs689466 | COX2 | G/A | 0.479 | 0.561 | 0.851 | 0.88 (0.69–1.13) | 0.96 (0.73–1.27) | 1.09 (0.86–1.38) | 0.1441 |
| rs35744894 | HPGDS | A/G | 0.206 | 0.0113* | 0.0113* | 1.79 (1.06–3.02) | 2.07 (1.24–3.45) | 1.16 (0.94–1.43) | 1 |
| rs11097414 | HPGDS | T/A | 0.341 | 0.00934* | 0.0113* | 1.41 (1.02–1.95) | 1.65 (1.19–2.28) | 1.16 (0.94–1.44) | 0.6478 |
| rs11097411 | HPGDS | G/A | 0.291 | 0.00208* | 0.00624* | 1.79 (1.23–2.60) | 1.91 (1.32–2.77) | 1.07 (0.87–1.32) | 0.3386 |
| rs530963 | CRTH2 | G/T | 0.0880 | 0.372 | 0.445 | 1.87 (0.55–6.36) | 2.07 (0.62–6.92) | 1.11 (0.85–1.44) | 0.9071 |
| rs7167 | CRTH2 | T/C | 0.257 | 0.445 | 0.445 | 1.31 (0.86–1.97) | 1.28 (0.85–1.91) | 0.98 (0.79–1.2) | 0.5206 |
| rs8019916 | PTGDR | C/T | 0.115 | 0.00146* | 0.00582* | 2.84 (1.11–7.22) | 1.89 (0.76–4.72) | 0.67 (0.52–0.86) | 0.5207 |
| rs33998702 | PTGDR | T/C | 0.154 | 0.945 | 0.945 | 1.06 (0.52–2.16) | 1.09 (0.55–2.19) | 1.03 (0.82–1.28) | 0.2821 |
| rs8004654 | PTGDR | C/T | 0.319 | 0.315 | 0.420 | 0.99 (0.70–1.39) | 0.85 (0.60–1.20) | 0.86 (0.70–1.06) | 0.4907 |
| rs41312470 | PTGDR | A/G | 0.116 | 0.00291* | 0.00582* | 2.45 (1.00–6.00) | 1.65 (0.69–3.97) | 0.67 (0.53–0.86) | 0.466 |
| *AR versus non-atopy non-AR (n = 795 vs. n = 527)* | | | | | | | | | |
| rs4648302 | COX2 | T/G | 0.0705 | 0.337 | 0.585 | 1.61 (0.16–15.81) | 2.06 (0.21–19.83) | 1.28 (0.91–1.79) | 0.08592 |
| rs5275 | COX2 | C/T | 0.188 | 0.835 | 0.835 | 0.90 (0.48–1.68) | 0.97 (0.53–1.76) | 1.07 (0.84–1.37) | 0.1982 |
| rs689466 | COX2 | G/A | 0.479 | 0.390 | 0.585 | 0.83 (0.63–1.10) | 0.94 (0.69–1.27) | 1.12 (0.86–1.46) | 0.1441 |
| rs35744894 | HPGDS | A/G | 0.206 | 0.0932 | 0.0932 | 1.72 (0.96–3.11) | 1.86 (1.05–3.31) | 1.08 (0.85–1.37) | 1 |
| rs11097414 | HPGDS | T/A | 0.341 | 0.0380* | 0.0570 | 1.53 (1.06–2.21) | 1.61 (1.11–2.34) | 1.05 (0.83–1.33) | 0.6478 |
| rs11097411 | HPGDS | G/A | 0.291 | 0.0151* | 0.0453* | 1.78 (1.16–2.72) | 1.82 (1.19–2.77) | 1.02 (0.81–1.29) | 0.3386 |
| rs530963 | CRTH2 | G/T | 0.0880 | 0.341 | 0.682 | 1.50 (0.39–5.84) | 1.82 (0.48–6.91) | 1.21 (0.90–1.64) | 0.9071 |
| rs7167 | CRTH2 | T/C | 0.257 | 0.682 | 0.682 | 1.08 (0.70–1.68) | 0.97 (0.63–1.50) | 0.90 (0.71–1.14) | 0.5206 |
| rs8019916 | PTGDR | C/T | 0.115 | 0.382 | 0.509 | 1.37 (0.55–3.39) | 1.13 (0.47–2.71) | 0.82 (0.62–1.09) | 0.5207 |
| rs33998702 | PTGDR | T/C | 0.154 | 0.327 | 0.509 | 0.98 (0.44–2.21) | 1.19 (0.54–2.63) | 1.21 (0.94–1.56) | 0.2821 |
| rs8004654 | PTGDR | C/T | 0.319 | 0.768 | 0.768 | 1.00 (0.67–1.50) | 1.09 (0.73–1.62) | 1.09 (0.86–1.37) | 0.4907 |
| rs41312470 | PTGDR | A/G | 0.116 | 0.353 | 0.509 | 1.37 (0.56–3.40) | 1.12 (0.47–2.70) | 0.82 (0.62–1.08) | 0.466 |

All data were evaluated based on the genotyping results of $n$ = 2,880 individuals from the SMCSGES cohort. For genetic association tests, $p$ value was calculated using the Fisher's exact test. FDR adjustment was performed for all assessed SNPs within each of the candidate genes, based on the Benjamini and Hochberg method (Benjamini and Hochberg 1995 [31]). The minor allele is designed as "A," while the major allele is designated as "B" for the SNP. CI, confidence interval; *COX2*, cyclooxygenase-2; *CRTH2*, chemoattractant receptor-homologous molecule expressed on T helper 2 cells; FDR, false discovery rate; *HPGDS*, hematopoietic prostaglandin D synthase; MAF, minor allele frequency; OR, odds ratio; $P_{HWE}$, Hardy-Weinberg equilibrium $p$ value for the tag-SNP assessed in the entire population, with significant cutoff at $p < 0.001$; *PTGDR*, prostaglandin D2 receptor; SMCSGES, Singapore/Malaysia cross-sectional genetics and epidemiological study; SNP, single nucleotide polymorphism. *Fisher's $p < 0.05$ is considered as significant. [a]Reference genotype for the odds ratio calculation.

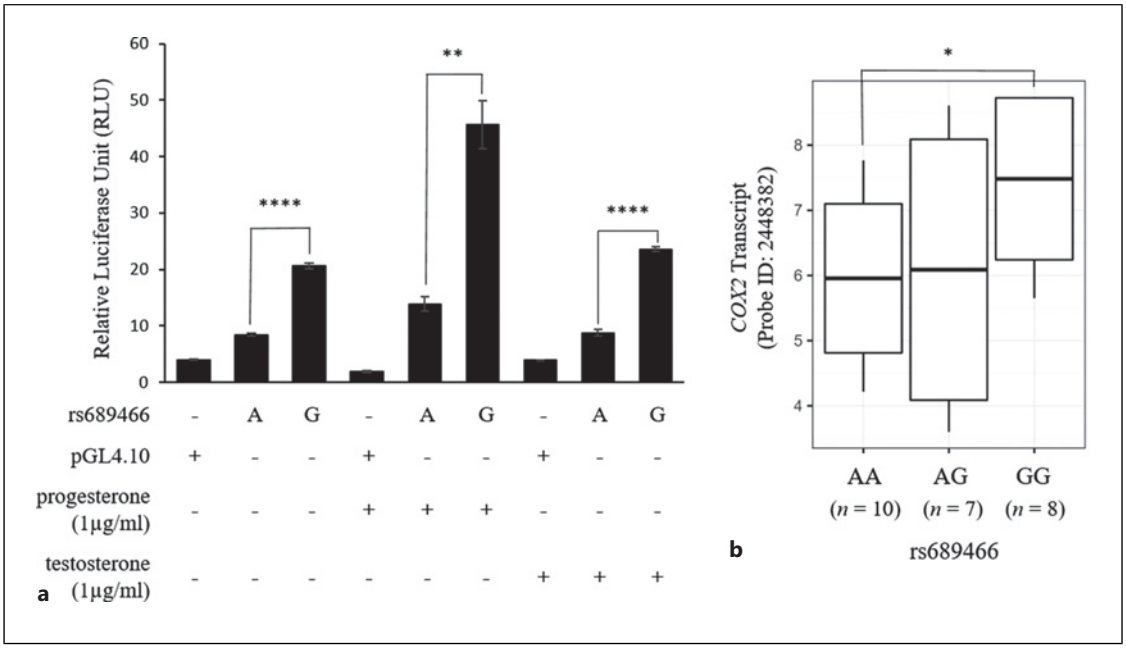



**Fig. 1.** Functional effect of rs689466 on *COX2* promoter activities and *COX2* mRNA expression levels. **a** Relative luciferase units (RLUs) of the *COX2* promoter constructs with either the allele "A" or "G" of rs689466 were compared. Promoter-less luciferase plasmid pGL4.10 serves as a background control. Luciferase activities were measured 24 or 48 h after constructs were transiently transfected into HEK293T. Progesterone (1 μg/mL), testosterone (1 μg/mL), or DMSO (as a treatment control) was also added 4 h after the transfection. Each bar represents the mean RLU ±1 standard deviation of triplicates. **b** *COX2* mRNA expression levels in PBMC were measured by a transcriptome profiling microarray (probe ID: 2448382) and then compared across rs689466 genotypes among the male subjects ($n$ = 33) in the SMCSGES cohort. Each box represents the mean, ±1 standard deviation, and the minimum and maximum values of the dataset. An independent $t$ test was conducted to determine the statistical significance ($p < 0.05$) of all associations. *COX2*, cyclooxygenase-2; PBMCs, peripheral blood mononuclear cells; SMCSGES, Singapore/Malaysia cross-sectional genetics and epidemiological study. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

higher mRNA expression of *CRTH2* and upregulation of AA pathway in PBMC.

*Asthma-Associated SNP rs5758 Does Not Influence the Splicing Activity of* TBXA2R

Based on the prediction data from SNPinfo [36], the asthma-associated rs5758 is in the exon splicing enhancer or silencer region of *TBXA2R*. Thus, we performed an in vitro minigene assay that focused on a 2,952 bp fragment spanning exon 2–4 of *TBXA2R* isoform β (online suppl. Fig. S1a) to assess whether rs5758 can affect the splicing activity of *TBXA2R*. However, as shown in online supplementary Figure S1b, we did not observe functional effect of rs5758 on the splicing activity of *TBXA2R*.

## Discussion

The present study reported association of genetic polymorphisms in key genes of the AA pathways with allergy risk and poorer clinical outcomes in pediatric asthma. By analyzing DNA methylome and transcriptome data of PBMC in the SMCSGES cohort, along with in vitro assays, we characterized the functional roles of these polymorphisms in the pathobiology of asthma and AR. Importantly, all of these variants contribute to the manifestation of allergic diseases via modulating the AA pathway (summarized in Fig. 5). These findings highlighted the complexity and importance of this pathway in the development of allergic diseases.

Our genetic association study focused on a cross-sectional cohort of Singaporean Chinese individuals. In this cohort, the allergic response is dominated by a single class of house dust mite allergen, while nonatopic asthma and nonatopic rhinitis are uncommon [21]. This promotes the detection of allergy-associated genes and reduces false-positive results [37, 38]. In the present study, allele "A" of rs689466 associates with a higher promoter activity and increased mRNA expression of *COX2* in the AA pathway. Genotypes "AA" and "GG" of rs689466 are

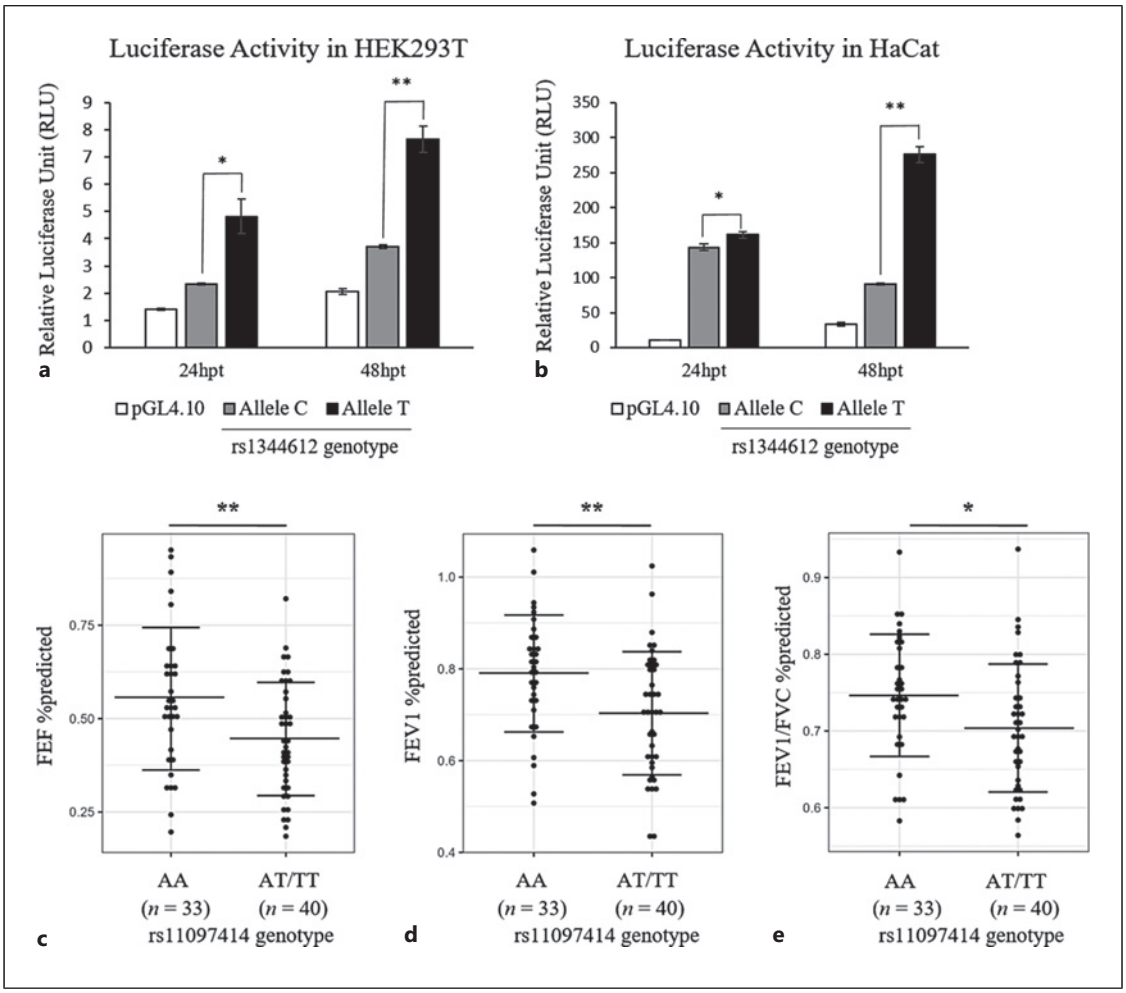
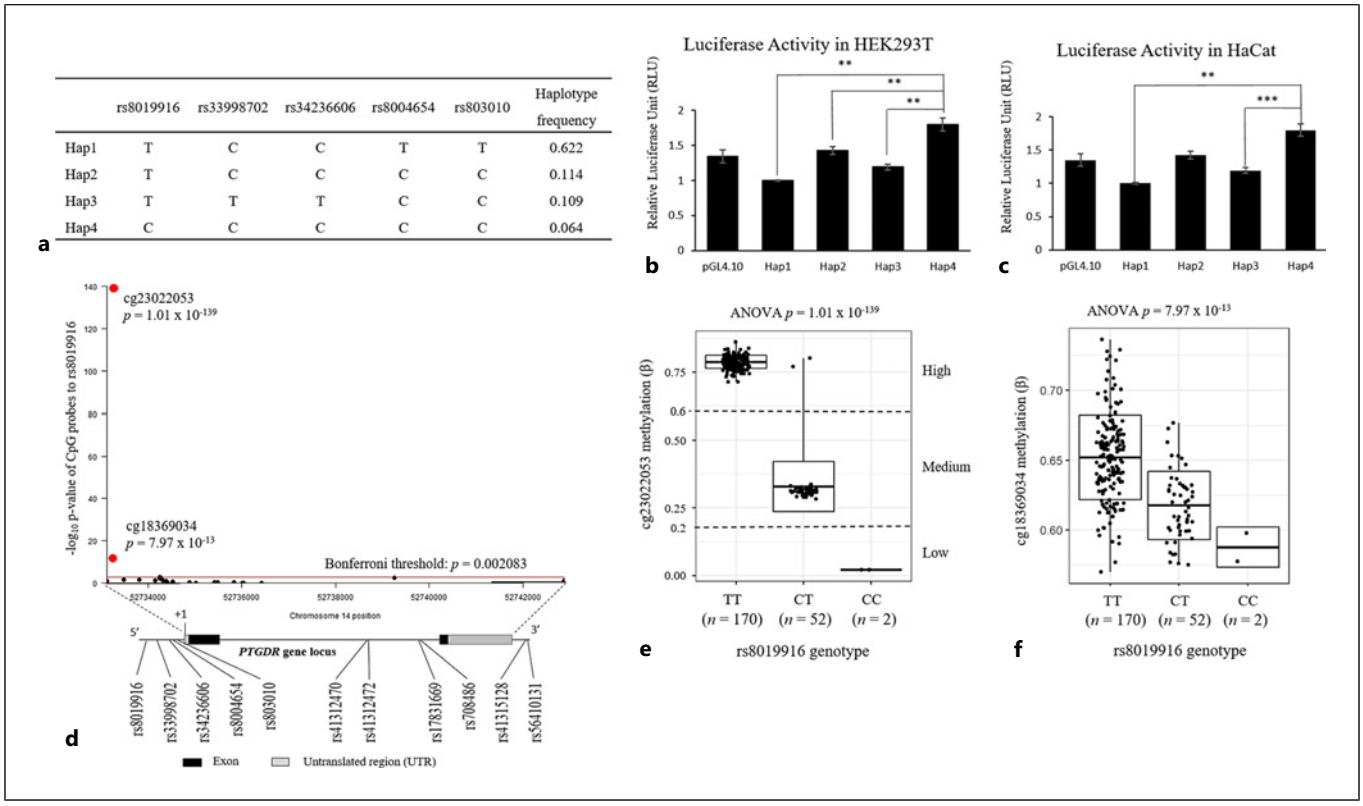



**Fig. 2.** Functional effects of allergy-associated SNPs on *HPGDS* promoter activity and their correlations with clinical outcomes in pediatric asthma. Relative luciferase units (RLUs) of HEK293T (**a**) or HaCat (**b**) cells transfected with *HPGDS* promoter constructs containing either the allele "C" or "T" of rs1344612 were compared. Promoter-less luciferase plasmid pGL4.10 serves as a background control. Luciferase activities were measured 24 or 48 h post-transfection (hpt). Each bar represents the mean RLU ±1 standard deviation of triplicates. Lung function parameters, including the percentages of predicted values of forced mid-expiratory flow (FEF) (**c**), forced expiratory volume in the first second (FEV1) (**d**), and FEV1 to forced viral capacity ratio (FEV1/FVC) (**e**), were measured on 75 pediatric asthmatic patients from the SMCSGES cohort and compared across the genotypes of rs11097414 (a proxy for rs1344612). For each group of genotypes, the mean ±1 standard deviation were shown. An independent *t* test was conducted to determine the statistical significance ($p < 0.05$) of associations in (**a**), (**b**), (**c**), (**d**), and (**e**). *HPGDS*, hematopoietic prostaglandin D synthase. **t*-test $p < 0.05$, ***t* test $p < 0.01$.

also associated with increased asthma risk among males in our study population (Table 1). Therefore, both upregulation and downregulation of *COX2* transcript expression might contribute to the development of this disease differently in the AA pathway. In agreement with this, accumulating evidence has suggested *COX2* upregulation can have either beneficial or deleterious effects on the outcome of respiratory diseases, which is largely dependent on the local concentrations of prostanoids produced by COX2 and specific interaction between these prostanoids and their individual receptors (reviewed in [2]). Studies have shown increased expression levels and immunoreactivity of the COX2 enzyme in asthmatic airways [39, 40], while other studies also indicate the pro-inflammatory $PGD_2$, which was synthesized by COX2, has a central role in allergy development and progression [41–43]. By contrast, reduced *COX2* mRNA expression levels were also observed in




| | rs8019916 | rs33998702 | rs34236606 | rs8004654 | rs803010 | Haplotype frequency |
|---|---|---|---|---|---|---|
| Hap1 | T | C | C | T | T | 0.622 |
| Hap2 | T | C | C | C | C | 0.114 |
| Hap3 | T | T | T | C | C | 0.109 |
| Hap4 | C | C | C | C | C | 0.064 |

**Fig. 3.** Functional effect of rs8019916 on *PTGDR* promoter activity and DNA methylation levels. **a** Frequencies of 4 common haplotypes (Hap1, Hap2, Hap3, and Hap4) formed by 5 SNPs in *PTGDR* (rs8019916, rs33998702, rs34236606, rs8004654, and rs803010) based on sequencing data of the SMCSGES cohort. **b**,**c** Relative luciferase units (RLUs) of HEK293T and HaCat cells transfected with *PTGDR* promoter constructs containing either Hap1, Hap2, Hap3, or Hap4 were compared. Promoter-less luciferase plasmid pGL4.10 serves as a background control. Luciferase activities were measured 24 h after transfection. An independent *t* test was conducted to determine the statistical significance ($p < 0.05$) of associations. Each bar represents the mean RLU ±1 standard deviation of triplicates. **d** Associations between rs8019916 genotype and the methylation levels of 24 CpG sites in *PTGDR* and the 2 kb flanking region of the gene (chr14: 52,732,416–52,745,442, GRCh37), using 237 PBMC samples from the SMCSGES cohort. DNA methylation levels were assessed as beta values (β). The ANOVA test was performed and a Bonferroni threshold of $p = 0.05/24 = 0.002083$ was used to determine the statistical significance of associations. **e** The methylation levels (β) of cg23022053 were compared across rs8019916 genotype using $n = 237$ PBMC samples from the SMCSGES cohort. **f** Association between DNA methylation levels of cg18369034 and *PTGDR* mRNA expression levels in $n = 237$ PBMC samples from the SMCSGES cohort. **e**,**f** Each box represents the mean, mean ±1 standard deviation, and the minimum and maximum values of the dataset. The ANOVA test was performed to determine the statistical significance ($p < 0.05$) of associations in (**e**) and (**f**). *PTGDR*, prostaglandin D2 receptor; SMCSGES, Singapore/Malaysia cross-sectional genetics and epidemiological study.

nasal polyps from aspirin-sensitive asthma patients [44], which then suppressed the synthesis of $PGE_2$ by microsomal PGE synthase and contributed to the development of AERD [2]. A prior study has also shown pro-inflammatory eicosanoids were inversely correlated with atopic sensitization in AERD patients [45].

HPGDS catalyzes the production of $PGD_2$ and is suggested to play a key role in allergic inflammatory responses such as bronchoconstriction, eosinophil survival, and leukocyte activation [41–43]. $PGD_2$ also has increased levels in patients with severe asthma [46, 47] and in response to allergen challenge [48]. Increased *HPGDS* expression was observed in the nasal mucosa of AR patients as compared to the controls, while a variety of infiltrating cells expressing *HPGDS* was also detected in this tissue [49]. In our work, associations between *HPGDS* expression and risks of allergic diseases were confirmed at the genetic level: the allele "T" of rs11097414

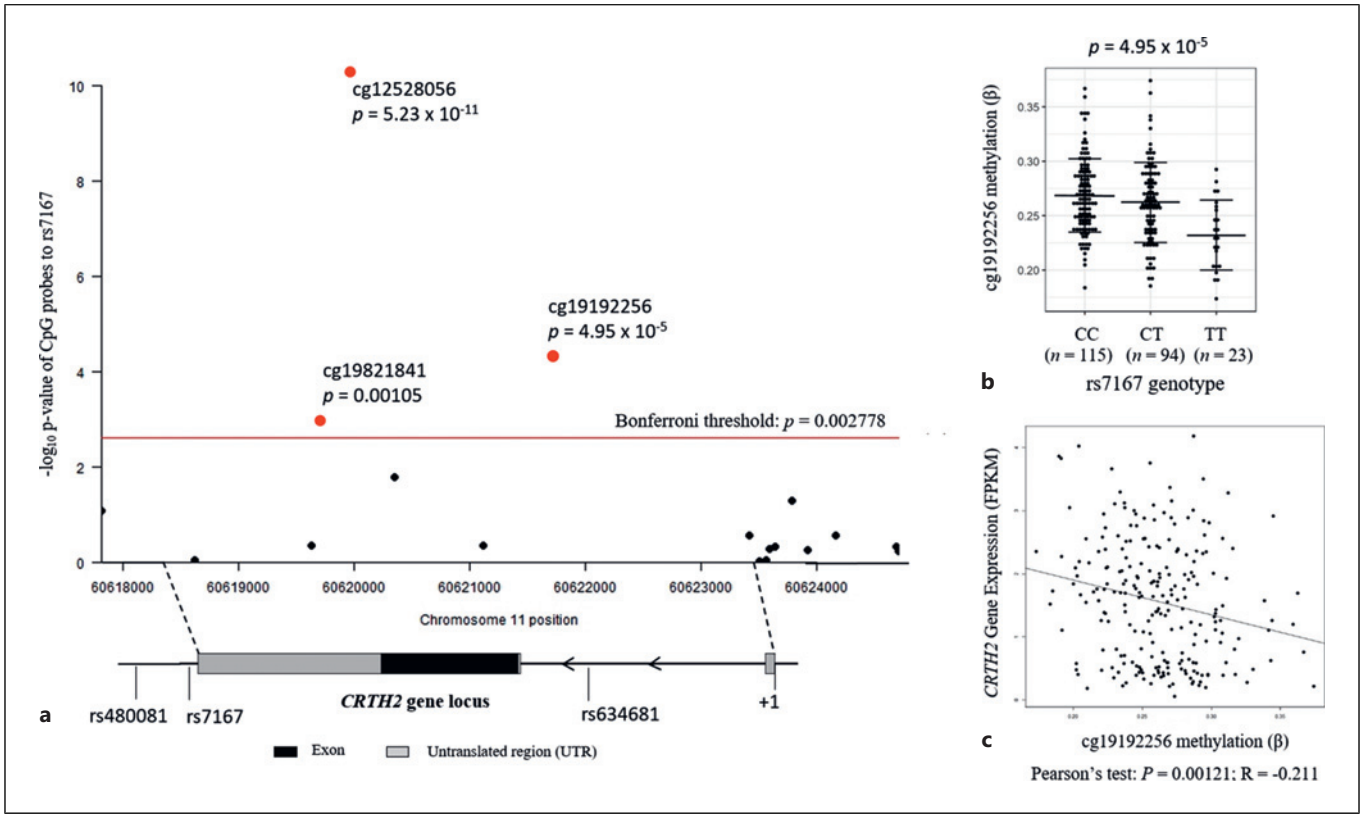



**Fig. 4.** Functional effect of rs7167 on DNA methylation and *CRTH2* mRNA expression levels. **a** Associations between rs7616 genotype and the methylation levels of 18 CpG sites in *CRTH2* and the 2 kb flanking region of the gene (chr11: 60,616,406–60,625,423, GRCh37), using 237 PBMC samples from the SMCSGES cohort. **b** The methylation levels (β) of cg19192256 were compared across rs7167 genotypes using $n$ = 237 PBMC samples from the SMCSGES cohort. For each group of genotypes, the mean and ± 1 standard deviation were shown. **a**,**b** The ANOVA test was performed and a Bonferroni threshold of $p$ = 0.05/18 = 0.002778 was used to determine the statistical significance ($p$ < 0.002778) of associations. **c** Correlation between DNA methylation levels of cg19192256 and *CRTH2* mRNA expression levels in $n$ = 237 PBMC samples from the SMCSGES cohort was shown. Pearson's correlation test was performed to determine a significant correlation ($p$ < 0.05). *CRTH2*, chemoattractant receptor-homologous molecule expressed on T helper 2 cells; PBMCs, peripheral blood mononuclear cells; SMCSGES, Singapore/Malaysia cross-sectional genetics and epidemiological study.

is associated with increased *HPGDS* promoter activity in HEK293T and HaCat cells while also associated with increased risks of asthma and AR. Besides, the allele "T" of rs11097414 was also associated with poorer clinical outcomes in our pediatric asthma cohort. In line with this, data from prior studies also showed upregulated HPGDS and $PGD_2$ production in bronchial epithelial cells during respiratory syncytial virus bronchiolitis [50], while this infection was associated with an increased risk of childhood asthma [51]. Therefore, pharmacological inhibition of HPGDS and $PGD_2$ biosynthesis might be beneficial to delay the onset of asthma, as well as improve clinical outcomes of this disease. Further, our present findings on the association of rs1107414 with lung function are considered preliminary. Future studies need to validate this by conducting a more comprehensive evaluation of asthma clinical outcomes including lung function test results, asthma control test score, and frequency of asthma symptoms in an independent cohort.

Both PTGDR and CRTH2 are the transmembrane receptors of $PGD_2$ that were frequently proposed as drug targets for the treatment of allergic disease [52, 53]. A genetic study on a multi-ethnic cohort has identified a functional haplotype that was associated with both increased *PTGDR* promoter activity and increased risk of asthma [54]. Besides, epigenetic changes, such as DNA methylation and histone modifications, have also been frequently identified to associate with allergy susceptibility and development [55, 56]. In line with these findings, the present study has shown rs8019916

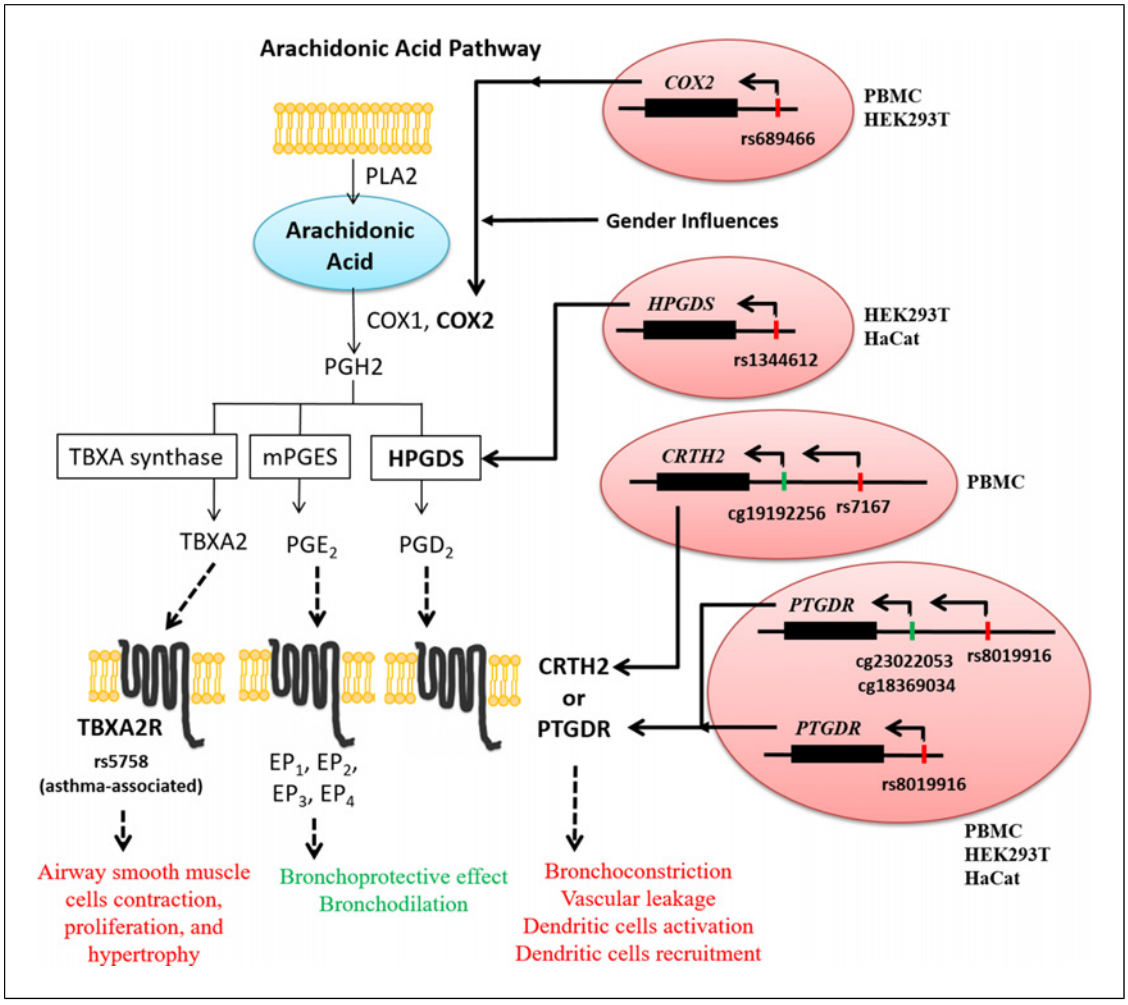



**Fig. 5.** Graphical illustration of functional SNPs that were associated with allergy-related phenotype through modulation of the expression of key genes in the AA pathway. All results described in this article were based on in vitro assay on HEK293T and HaCat cells, as well as ex vivo assay on PBMC samples. All allergy-related outcomes resulting from the activations of downstream receptors were discussed in the Introduction section of this article. COX, cyclooxygenase; CRTH2, chemoattractant receptor-homologous molecule expressed on T helper 2 cells; HPGDS, hematopoietic prostaglandin D synthase; PBMCs, peripheral blood mononuclear cells; mPGES, microsomal PGE synthase; $PGD_2$, prostaglandin $D_2$; $PGD_2$, prostaglandin $E_2$; $PGH_2$, prostaglandin $H_2$; PLA2, phospholipase A2; PTGDR, $PGD_2$ receptor; TBXA2, thromboxane A2; TBXA2R, TBXA2 receptor.

influences both promoter activity and DNA methylation levels in the *PTGDR* gene, which results in its association with allergy.

An association between rs533116 in *CRTH2* and allergic asthma was reported in an ethnically diverse population, with evidence showing this may be mediated by the elevated expression of *CRTH2* [18]. Data also showed allergen-specific CD4+ T cells expressing *CRTH2* are increased in the whole blood of allergic subjects as compared to the controls [57]. Although rs533116 was not present in our study population, we identified an asthma-associated SNP rs7167 which also modulates *CRTH2* mRNA expression via regulating the DNA methylation level in this region.

There are certain limitations to our study: First, although we demonstrated significant functional effects of asthma-associated SNPs on the promoter activities of AA pathway genes, transcription factors that contributed to these observed effects were not investigated. Further studies using electrophoretic mobility shift assay or chromatin immunoprecipitation assay might help shed light on this question. Second, in this study, the DNA methylome and transcriptome data were assessed in PBMC samples that contain different cell types. This large

variability of PBMC samples may result in confounding problems that limit the identification of true positives [55, 58]. Therefore, future studies that utilize single-cell technology for epigenome-wide association study are needed to validate our present findings. Third, although each of the allergy-associated SNPs was shown to be functionally involved in the regulation of AA pathway, we did not observe significant synergistic effect of these SNPs on the development of respiratory allergies. The sample size was too small to perform a subgroup analysis for individuals carrying multiple disease risk alleles, and we plan to address this issue in the future when we have larger numbers of participants, as a part of an ongoing cross-sectional study. Lastly, associations between these SNPs in the AA pathway genes and allergic diseases will also be validated in the future study, using an independent cohort.

## Conclusion

In summary, the present study identified multiple allergy-associated SNPs that modulate the transcript expressions of key genes in the AA pathway. Our data highlight the possible molecular mechanisms for how genetic polymorphism in the AA pathway can affect the risk and severity of respiratory allergic diseases. The development of a "personalized medicine" approach with consideration of genetic influences on the AA pathway may hopefully result in efficacious strategies to manage and treat allergy in the future.

## Acknowledgments

We thank all participants and their family members for their willingness to participate in this study and Dr. Ramani Anantharaman, Dr. Bani Kaur Suri, and Parate Pallavi Nilkanth for their help with sample collection.

## Statement of Ethics

Participant recruitment study in Singapore was approved by the Institutional Review Board (IRB) in NUS. The study protocol was reviewed and approved by NUS-IRB, approval numbers: 07–023, 09–256, 10–445, 13–075, B-10-343, and H-18-036. Participant recruitment study in Malaysia was approved by the UTAR Scientific and Ethical Review Committee (SERC). The study protocol was reviewed and approved by UTAR-SERC, approval number U/SERC/03/2016. All participants provided informed and written consent, with additional written informed consent obtained from the participants' parent, legal guardian, or next of kin for those below 21 years old. All recruitment procedures were carried out in concordance with the Helsinki Declaration.

## Conflict of Interest Statement

Fook Tim Chew has received consulting fees from Sime Darby Technology Centre, First Resources Ltd., Genting Plantation, and Olam International outside the submitted work. All funding agencies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. All other authors have no conflicts of interest to declare.

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## Author Contributions

F.T.C. planned and supervised the study. Y.Y.S., P.S., S.A.M., Y.T.R.F., W.C.C., and Y.-H.S. recruited participants for the study. Y.T.R.F. and W.C.C. collected and analyzed the lung function data. Y.Y.S. and P.S. planned and conducted the experiments and analyzed the data. Y.Y.S. wrote the manuscript. All authors reviewed and approved the manuscript.

## Data Availability Statement

All data used and included in this study are available from the corresponding author (Chew Fook Tim). Data are not publicly available due to ethical reasons. Further inquiries can be directed to the corresponding author.

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