

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Epistasis between phenylethanolamine N-methyltransferase and β 2-adrenergic receptor influences extracellular epinephrine level and associates with the susceptibility to allergic asthma

Yang Yie Sio  | Sri Anusha Matta | Yu Ting Ng | Fook Tim Chew 

Department of Biological Sciences, National University of Singapore, Singapore City, Singapore

Correspondence

Fook Tim Chew, Department of Biological Sciences, Faculty of Science, National University of Singapore, Allergy and Molecular Immunology Laboratory, Lee Hock Kwee Functional Genomics Laboratories, Block S2, Level 5, 14 Science Drive 4, Lower Kent Ridge Road, Singapore City 117543, Singapore.
Email: dbscft@nus.edu.sg

Funding information

Ministry of Education - Singapore, Grant/Award Number: R-154-000-553-112, R-154-000-565-112, R-154-000-630-112, R-154-000-A08-592, R-154-000-A27-597 and R-154-000-404-112; National Medical Research Council, Grant/Award Number: NMRC/1150/2008; Biomedical Research Council - Singapore, Grant/Award Number: BMRC/01/1/21/18/077, BMRC/04/1/21/19/315 and H17/01/a0/008

Abstract

Background: Reduced extracellular epinephrine level often associates with asthma-related symptoms; however, the correlation between asthma and genetic variants in genes participating in the epinephrine signalling pathway remains unclear.

Objective: To characterize the functions of single nucleotide polymorphisms (SNPs) in phenylethanolamine N-methyltransferase (PNMT) and β 2-adrenergic receptor (ADRB2), and to study the effects, including both direct and epistatic, of these SNPs on serum epinephrine level and asthma susceptibility.

Methods: Single nucleotide polymorphisms functions were characterized through in vitro luciferase assay. ADRB2 gene expression level in peripheral blood mononuclear cell (PBMC) was measured by transcriptome sequencing and expression microarray on two separate Asian cohorts (NUS-UTAR, n = 278 and NUS-TA, n = 58). Serum epinephrine level was assessed on a Singapore Chinese cohort (NUS-SH, n = 314) with 155 asthmatic and 159 non-asthmatic subjects. A separate Singapore Chinese cohort (NUS-G, n = 3009) was genotyped to show disease association (direct and epistatic effect) of functional SNPs in PNMT and ADRB2.

Results: Reduced serum epinephrine level was associated with increased asthma risk in Singapore Chinese. The minor allele of rs876493 was shown to increase PNMT promoter activity and reduce asthma risk. Multiple SNPs in ADRB2 forms a haplotype that was associated with the differential promoter activity of this gene. In this haplotype, rs11168070 was associated directly with ADRB2 expression in PBMCs. Both minor alleles from rs876493 and rs11168070 contribute synergistically to reduce asthma risk and increase serum epinephrine level.

Conclusion and Clinical Relevance: Epistatic interaction between genetic variants from PNMT (rs876493) and ADRB2 (rs11168070) is associated with serum epinephrine level and the susceptibility of asthma. Our findings improved the current understanding of the genetic basis of this disease, while genotypic states of these SNPs may serve as potential biomarkers to predict susceptibility to the disease.

KEYWORDS

asthma, basic mechanisms, genetics

1 | INTRODUCTION

Asthma is a syndrome of complex aetiology, characterized by reversible obstruction in the pulmonary airway. The administration of β_2 -adrenergic receptor (ADRB2) agonists, such as epinephrine, is considered the most effective bronchodilator to alleviate the symptoms during an acute asthma attack.¹⁻³ In this process, ADRB2 would be activated and subsequent relaxation of bronchial smooth muscle would take place (Figure S1).^{4,5} The effectiveness of this agonist for treating the bronchospasms during asthma exacerbation may imply asthmatic patients having intrinsically low extracellular epinephrine levels. Indeed, the failure of plasma epinephrine level to increase during acute asthmatic attacks was observed in severe asthmatic patients.⁶ Decreased plasma epinephrine level during exercise was also found in asthmatic patients compared to normal subjects.⁷ These observations suggested the association between epinephrine signalling pathway dysfunction and asthma disease.

Phenylethanolamine N-methyltransferase (PNMT) is expressed mainly in the adrenal gland and catalyses the rate-limiting step of converting norepinephrine to epinephrine (Figure S1). It has been previously shown that partial removal of the adrenal gland in asthmatic rats would result in reduced endogenous PNMT and epinephrine level; at the same time also lead to more severe pathological changes, such as increased airway wall thickness.⁸ Nevertheless, despite the importance of PNMT in epinephrine synthesis and asthma pathogenesis, no study has thus far associated genetic polymorphisms in PNMT with asthma.

As downstream effectors of the epinephrine signalling pathway, ADRB2 is expressed on almost all cell types, including bronchial epithelial cells. Several genetic variants in ADRB2 have been frequently shown to associate with asthma. Two of the most reported single nucleotide polymorphisms (SNPs) Arg16Gly (rs1042713) and Gln27Glu (rs1042714) have been significantly associated with asthma risk, nocturnal asthma and airway hyperresponsiveness in Chinese populations.⁹⁻¹² A recent meta-analysis conducted for the Chinese population has also shown associations between SNP rs1042714 with childhood asthma.¹³ However, it was also suggested that the association reported may be inconsistent as some other meta-analyses failed to show rs1042713 and rs1042714 would directly affect asthma risk.¹⁴⁻¹⁶ On the other hand, several *in vitro* studies have demonstrated multiple SNPs located upstream of the ADRB2 gene could form a functional haplotype that affects ADRB2 promoter activity, which may lead to differential gene expression.^{17,18}

Although prior reports have associated extracellular epinephrine level with asthma-related symptoms, these findings are limited to the non-Asian cohort. Besides, for genes involved in the epinephrine signalling pathway such as PNMT and ADRB2, the association between asthma and SNPs in these genes remains unclear. Here, we report epistatic interaction between SNPs rs876493 and rs11168070 from PNMT and ADRB2 genes that influence the epinephrine signalling pathway and contribute to the risk of asthma.

Functional characterizations of these SNPs were performed independently and linked with the risk of asthma in our study population. The epistatic effect between PNMT and ADRB2 SNPs contributing to disease susceptibility will be examined as well. This allows further understanding of the epinephrine signalling pathway as part of the asthma pathogenesis process and providing potential biomarkers for disease prediction.

2 | METHODS

2.1 | Study design and subjects recruitment

Subjects were recruited across two different countries (Singapore/Malaysia) in this current study. Recruitment was carried out at the National University of Singapore (NUS), multiple local hospitals within Singapore and the University of Tunku Abdul Rahman (UTAR), Malaysia. Based on the type of sample provided and measurement performed on each sample, all recruited subjects were separated into five cohorts. These are as follows: (a) Singapore Chinese individuals recruited from NUS for large scale genotyping study (NUS-G, $n = 3009$), (b) Singapore Chinese individuals recruited from NUS for the ADRB2 gene sequencing study (NUS-S, $n = 40$), (c) Singapore Chinese individuals recruited from NUS for transcriptome array measurement of the ADRB2 gene expression (NUS-TA, $n = 58$), (d) subjects recruited from NUS and local hospitals within Singapore (NUS-SH, $n = 314$) for serum epinephrine measurement and (e) subjects recruited from both NUS and the University of Tunku Abdul Rahman (UTAR), Malaysia (NUS-UTAR) for transcriptome sequencing measurement of the ADRB2 gene expression. Details on the characteristics of each cohort, including the asthma status of recruited subjects, type of sample provided and assay conducted for each sample are included in Table S1.

For recruitment of individuals who reside in Singapore (NUS-G, NUS-S, NUS-TA, NUS-SH and part of the NUS-UTAR cohorts), the recruitment study belongs to a part of an ongoing cross-sectional epidemiology and genetics study. Recruitment of participants was approved by Institutional Review Board (IRB) in National University of Singapore (NUS, NUS-IRB Ref-Code: B-10-343 for blood samples collection, B-14-150, 07-023, 09-256, 10-445 and 13-075, for the large scale epidemiology and genetics study; and the Institutional Review Board of the National Healthcare Group Domain Specific Review Board—B/04/055). Written and informed consent was obtained from every individual participated, with parental or guardian consent obtained additionally for individuals less than 21 years old. Participants with asthma disease were defined as those who had the disease ever diagnosed by physicians in local clinics or hospitals. The ethnicity of participants was confirmed through survey questionnaires and also confirmed previously through principal component analysis.¹⁹ Only Chinese individuals who reside in Singapore and unrelated to each other were included in the NUS-G, NUS-S, NUS-TA and NUS-SH cohorts. The NUS-G, NUS-S and NUS-TA cohorts were recruited from NUS

only, while the NUS-SH cohort was recruited from NUS and multiple venues in Singapore including local hospitals.

For recruitment of individuals who reside in Malaysia (NUS-UTAR cohort), peripheral blood mononuclear cell (PBMC) samples were collected from human participants at the University of Tunku Abdul Rahman (UTAR), Malaysia. The recruitment study in UTAR was approved by the UTAR Scientific and Ethical Review Committee (SERC, Ref-code: U/SERC/03/2016), while the recruitment process is the same as that carried out in Singapore.

2.2 | Measurement of serum content

Serum samples for epinephrine level analysis were collected from the NUS-SH cohort ($n = 314$). The measurement of serum epinephrine concentrations was performed using ELISA. In brief, the human epinephrine ELISA Kit was purchased from Cusabio Biotech Co., Ltd., with assay conducted according to manufacturer's instructions. All samples were diluted in 1xPBS before measurement for interpolation within the assay standard curve range. All measurements were done in triplicates.

2.3 | Cell culture and promoter-luciferase assay

Human embryonic kidney cells (HEK293T) and human bronchial epithelial cell lines HBE135-E6E7 (HBE) were both purchased from the American Type Culture Collection (ATCC). The HEK293T cell line was used for the promoter-luciferase study of both PNMT and ADRB2 genes, due to its high transfection efficiency and its frequent application in various in vitro transfection experiment. The HBE cell line was used for the promoter-luciferase study of the ADRB2 gene, as this gene was known to have a direct influence on bronchial smooth muscle relaxation. HEK293T cells were grown in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 10% foetal bovine serum, 2 g/L sodium bicarbonate and 2 mmol/L L-glutamine. HBE cells were grown in keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract, 0.005 mg/mL insulin and 500 ng/mL hydrocortisone. Both cell lines were incubated in 37°C humidified incubator with a 5% CO₂ environment.

For the assessment of PNMT promoter activity, gene region spanning -978bp to +522bp was cloned into plasmid pGL4.10 (Promega) that contains a promoterless luciferase gene. For the ADRB2 promoter assay, the gene region from -1562bp to +332bp was cloned into pGL4.10.

For promoter assay of both genes, transient transfection of plasmid construct was performed using Lipofectamine 2000 (Invitrogen) with condition optimized according to the manufacturer's protocol. Renilla luciferase control plasmid pGL4.74 was also co-transfected for assay normalization purposes. Luciferase activity was measured by the Dual-Luciferase® Reporter Assay System (Promega). Relative luciferase unit (RLU) was calculated by dividing firefly luciferase

activity reading by that of Renilla Luciferase. All transfection experiments were performed in triplicates with cells harvested 24 or 48 hours post-transfection separately.

2.4 | Genotyping and Sanger sequencing

Genomic DNA (gDNA) samples for the genotyping or sequencing assay were obtained by DNA extraction of mouthwash samples (from NUS-G, NUS-S, NUS-TA, and NUS-SH cohorts) or PBMC samples (from the NUS-UTAR cohorts). Extraction was performed using Axygen® AxyPrep™ Multisource Genomic Miniprep DNA kit, according to the manufacturer's protocol.

Before the genotyping assay, quantification of gDNA concentration was done in triplicates using NanoDrop 2000 spectrophotometer (Thermo Scientific). Genotyping was performed using PCR-based KASP genotyping assay (LGC Biosearch Technologies), according to the manufacturer's protocols. For the sequencing of ADRB2, including 2 kb upstream and 1 kb downstream of the gene region, the process was done via Sanger sequencing according to published methodology.²⁰ Sequencing assay was conducted on the NUS-S cohort ($n = 40$).

2.5 | Next-generation sequencing

For the measurement of ADRB2 transcript level by transcriptome sequencing, peripheral blood mononuclear cell (PBMC) samples were collected from human participants of the NUS-UTAR cohort ($n = 278$). Approximately 24ml of blood was collected from each subject, with PBMCs extracted using Ficoll-Hypaque density gradient centrifugation. Total RNA was extracted subsequently using EZNA® Total RNA Kit from Omega Bio-tek Inc, according to the manufacturer's protocol. 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 (FPKM) for ADRB2 transcript (NM000024.5) were calculated using Cufflinks version 2.2.1. All samples were sequenced at the same time to avoid batch variation in the sequencing results.

2.6 | Expression microarray

For the measurement of the ADRB2 gene expression level by expression microarray, PBMC samples were collected from the NUS-TA cohort ($n = 58$). Extraction procedure for PBMC and total RNA was performed with the same approaches as mentioned above. Total RNA samples were sent for downstream gene expression level analysis using Affymetrix Human Exon 1.0 ST Array. The

assay was done in a facility provided by Origen Labs, Singapore. Affymetrix Power Tools was used for data extraction and quantile normalization, according to reported methodology.²¹ Array probe with transcript cluster ID 2834743 was used for ADRB2 transcript analysis.

2.7 | Statistical analysis

The r-squared value between SNPs, linkage disequilibrium patterns and haplotype frequencies was calculated and drawn using software Haploview® vers4.2 [http://www.broadinstitute.org/haploview]. The exact test for Hardy-Weinberg equilibrium (HWE) was calculated in the PLINK program version 1.06, with a significant deviation from the HWE cut-off at $P < .001$. We performed the additive logistic regression analysis to test for SNP to disease association using R program version 3.6.1, with adjustment to characteristics significantly different

between asthmatic cases and non-asthmatic controls (Table 1). These include the test subject's age, gender, and received treatments for asthma (bronchodilators, inhaled corticosteroids and oral corticosteroids). Subject's BMI was not adjusted for the logistic regression test as 11.3% of the test cohort (NUS-G, Table 1) did not provide this information. P-value and the odds ratio for the disease association were all evaluated using the same program. $P < .05$ is considered a significant association with the disease tested. Disease or genotype association analysis with normally distributed continuous variables was conducted using the independent sample t test or ANOVA test in the R program version 3.6.1. These variables include relative luciferase units measured from the promoter-luciferase assay, test subject's serum epinephrine level and both ADRB2 gene expression level measured by transcriptome array and next-generation sequencing. The serum epinephrine level was represented as a log-transformed scale in the dot plot figure for the ease of data representation. All dot plot figures included in this report were drawn in the same R program using R

TABLE 1 Demographics of the NUS-G cohort (n = 3009) used for large scale genotyping study

	Total	Allergic asthma	Non-asthma	P-value ^a
Total, n	3009	1450	1559	-
Gender				
Male (n, %)	1248 (41.48)	794 (54.76)	454 (29.12)	ref
Female (n, %)	1756 (58.36)	655 (45.17)	1101 (70.62)	$P < .0001^*$
Age (y, mean $\pm$ 1SD) ^b	21.54 $\pm$ 4.80	20.68 $\pm$ 4.72	22.34 $\pm$ 4.73	$P < .0001^*$
BMI (kg/m ² , mean $\pm$ 1SD) ^b	20.64 $\pm$ 2.99	20.85 $\pm$ 3.28	20.46 $\pm$ 2.67	$P < .001^*$
Smoking status				
Never-smoker (n, %)	2606 (86.61)	1152 (79.45)	1454 (93.26)	ref
Current smoker (n, %)	23 (0.76)	15 (1.03)	8 (0.51)	ns
Ex-smoker (n, %)	35 (1.16)	19 (1.31)	16 (1.03)	ns
Hospital admission due to asthma (past 12 mo of study, n, %)	-	14 (0.97)	-	-
A & E admission due to asthma (past 12 mo of study, n, %)	-	49 (3.38)	-	-
Medical specialist visits due to asthma (past 12 mo of study, n, %)	-	199 (13.72)	-	-
Bronchodilator usage (n, %)	-	283 (19.52)	-	-
Inhaled corticosteroids usage (n, %)	-	200 (13.79)	-	-
Oral corticosteroids usage (n, %)	-	81 (5.59)	-	-

Abbreviations: A & E, accident and emergency department of a hospital; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; gDNA, genomic DNA; PBMC, peripheral blood mononuclear cell; Ref, reference group for the chi-squared test; SD, standard deviation.

^aFisher's exact test was used for testing categorical variables (gender and smoking status) association with disease status, while the independent samples t test was used for continuous variables (age and BMI).

^bThe mean and SD of BMI were estimated based on 88.7% of total participated subjects (n = 2670), while age was estimated based on 99.8% of total participated subjects (n = 3002).

*P-value $< .05$ is considered significant disease association.

package ggplot2.²² Synergy factor analysis was performed according to previously reported approach.²³ Coefficient of variation percentage (CV %) was calculated by dividing the standard deviation by mean of the same dataset and then multiplying the divided value by 100.

3 | RESULTS

3.1 | Serum epinephrine level correlates with the presence of asthma

Based on prior findings that have associated extracellular epinephrine level with asthma-related symptoms in European cohorts, we sought to further validate this correlation in Singapore Chinese individuals. Measurement of serum epinephrine concentration was performed on 155 allergic asthmatic and 159 non-asthmatic individuals (NUS-SH cohort, total $n = 314$). This revealed a significantly lower serum epinephrine level in allergic asthma affected individuals (14.45 ± 27.75 pg/mL), as compared to that of non-asthmatic control individuals (63.89 ± 97.98 pg/mL, $P < .0001$, Figure 1).

3.2 | SNP rs876493 influences the promoter activity of the PNMT gene

As PNMT catalyses the production of epinephrine, we next assessed whether functional SNP exists in PNMT that would influence the expression of this gene and downstream epinephrine synthesis. The only PNMT common SNP (minor allele frequency, MAF > 5%) rs876493 was identified from the HapMap CHB database.²⁴⁻²⁷

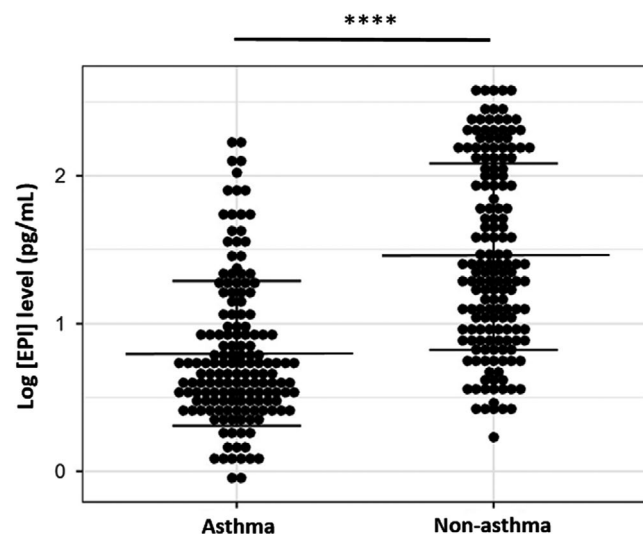


FIGURE 1 Serum epinephrine level correlates with asthma conditions among Singapore Chinese individuals (NUS-SH cohort, $n = 314$). Epinephrine level is represented in the figure as pg/mL, log10 transformed. Independent samples t test was conducted for the calculation of statistical significance between case and control group (using data before log10 transformation). **** T test P -value <.0001

It has 43% MAF for allele "A" and is located at + 39bp of the gene (Figure 2A). We generated constructs for promoter-luciferase assay containing major allele "G" and minor allele "A" of rs876493, using the PNMT gene region spanning -978bp to +522bp (Figure 2A). These constructs were transfected into HEK293T cells where PNMT promoter activity can be measured by the amount of luciferase expressed. As shown in Figure 2B, relative luciferase units (RLU) measured at 24 and 48 hours post-transfection were consistently higher in cells transfected with gene construct carrying allele "A" of rs876493 than that of allele "G" ($P < .0001$ and $P < .01$, respectively).

Having identified the functional influence of rs876493 on PNMT promoter activity, the correlation between the genotypes of this SNP and serum epinephrine level was subsequently evaluated. We genotyped 180 human subjects (145 asthmatics and 35 non-asthmatics) from the NUS-SH cohort used for serum epinephrine level study. As shown in Figure 2C, no direct association was observed between rs876493 genotype and serum epinephrine level.

3.3 | SNP rs876493 associates with asthma susceptibility

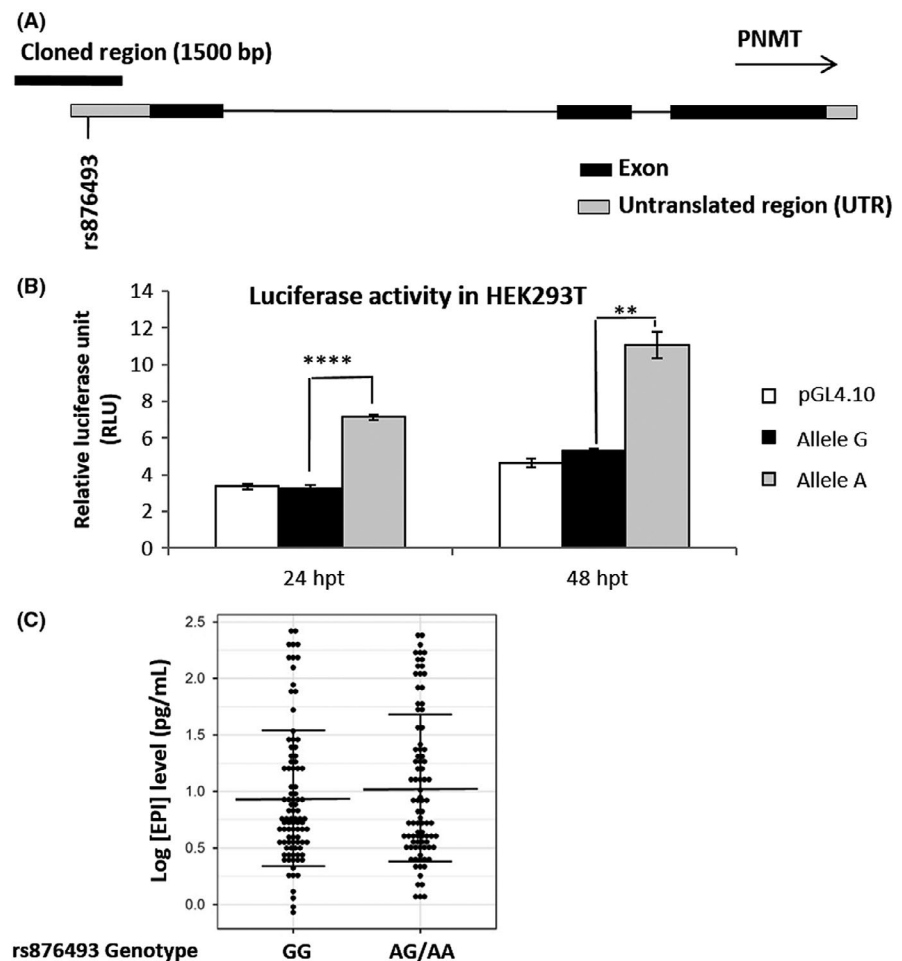
Phenylethanolamine N-methyltransferase SNP rs876493 was genotyped in the NUS-G cohort comprises 3009 Singapore Chinese individuals to show its association with allergic asthma. A total of 1450 asthmatic and 1559 non-asthmatic individuals were recruited and genotyped for rs876493. The genotyping result was tested for and passed the Hardy-Weinberg equilibrium (HWE) test. PNMT SNP rs876493 was shown significantly associated with allergic asthma disease, using logistic regression test with adjustment to test subject's age, gender and treatments received for asthma ($P = 8.39 \times 10^{-7}$, Table 2). The presence of minor allele "A" of rs876493 was shown to confer a protective effect against the disease (odds ratio, OR = 0.729 and 0.719 for heterozygous "AG" and homozygous minor "AA" genotype, respectively, both using homozygous major "GG" genotype as the reference, Table 2).

3.4 | ADRB2 haplotype affects promoter activity of the gene

Given ADRB2 acts as one of the downstream receptors in the epinephrine signalling pathway, we next investigated whether there is any SNP influencing the function of this gene. The ADRB2 gene region, including 2 kb upstream and 1 kb downstream of the gene, was sequenced on 40 Singapore Chinese individuals from the recruited NUS-S cohort. A total of 17 common SNPs (MAF > 5%) were identified in this region, with nine SNPs located upstream, two SNPs in 5'-UTR and four SNPs in exon 1 of the gene (Figure 3A).

Prior studies have already shown multiple SNPs within the upstream region of the ADRB2 gene could influence its promoter activity.^{17,18} To validate this, we assessed the promoter activity of the ADRB2 gene region spanning -1562bp to +332bp, which contains 12 common SNPs identified through sequencing assay on the NUS-S

FIGURE 2 SNP rs876493 influences the promoter activity of the PNMT gene. A, Gene structure of the PNMT gene with the relative position of SNP rs876493, and cloned PNMT gene construct (−978bp to + 522bp) for promoter-luciferase assay. B, Relative luciferase units of PNMT construct with either “C” or “T” allele of rs876493 were compared. Promoterless luciferase plasmid (pGL4.10) was also included as background control. The activity of luciferase expressed in human embryonic kidney (HEK293T) cell line was measured 24 or 48 h post-transfection (hpt). C, Serum epinephrine level of 180 Chinese ethnicity volunteers (NUS-SH cohort) sorted according to rs876493 genotype. Independent samples *t* test was conducted for the calculation of statistical significance. ***T* test *P*-value <.01, *****t* test *P*-value <.0001



cohort. A total of five common haplotypes (haplotype frequency >5%), namely Hap1, Hap2, Hap3, Hap4 and Hap5, were formed by these SNPs (Figure 3A). Plasmid constructs were then generated for all five haplotypes in the promoter region of interest and linked to a luciferase reporter gene. These constructs were transfected into HEK293T and HBE cells separately, and RLUs were assessed 24 hours post-transfection. In transfected HBE cells, RLU was significantly reduced in Hap2 as compared to that of Hap3, Hap4 and Hap5, while RLU for Hap1 construct was not significantly different from the RLUs measured from these four haplotypes. Besides, in transfected HEK293T cells, Hap4 has resulted in lower RLU than that of Hap1, Hap2 and Hap3. The RLU of Hap5 was not significantly different from RLUs of all four other haplotype constructs (Figure 4A). Taken together, our data have indicated ADRB2 haplotype differences would result in differential ADRB2 promoter activity in both of the cell lines assayed.

3.5 | SNP rs11168070 correlates with ADRB2 gene expression and asthma susceptibility

To determine which SNP in ADRB2 haplotype is influencing ADRB2 gene expression directly, we next assessed ADRB2 transcript level with genotypes of each tag-SNP within the haplotype. Using the

sequencing data obtained from the NUS-S cohort, we identified six tag-SNPs in the ADRB2 promoter region (Figure 3A). PBMC samples from our recruited Asian cohort of 278 individuals (Malaysia/Singapore, the NUS-UTAR cohort) were genotyped for these tag-SNPs and underwent transcriptome sequencing for the expression level of ADRB2 transcript (NM_000024.5). Five tag-SNPs were successfully genotyped, and a significant expression quantitative trait loci (eQTL) effect was observed for SNP rs11168070. The presence of minor allele “G” of this SNP was found correlated with increased ADRB2 transcript expression (ANOVA *P* = .0439, Figure 4B).

We next recruited a separate cohort of Singapore Chinese individuals (the NUS-TA cohort, *n* = 58) to validate this current eQTL finding and further correlate ADRB2 gene expression level with asthma disease. All recruited participants are with well-defined asthma or non-asthma characteristics, for which asthmatics patients have disease positively diagnosed by a physician. Based on these criteria, we validated the eQTL effect between rs11168070 and ADRB2 gene expression. Increased expression of the ADRB2 gene was found in subjects with the “G” allele of SNP rs11168070 (*P* = .0279, Figure 4C). Besides, we also observed a marginally significant increment in ADRB2 gene expression among non-asthmatic subjects (*P* = .06785, Figure 4C). An increase in ADRB2 gene expression is thus correlated with both the presence of minor allele “G” of SNP rs11168070 and protection against asthma in this study cohort.

TABLE 2 Association of tag-SNPs in PNMT and ADRB2 genes with allergic asthma in the Singapore Chinese population

SNP	Minor/Major Allele	MAF (Ca/Co)	No. of subjects (Ca/Co)	P-value ^a	OR _{het} (95% CI)	OR _{hom} (95% CI)	P _{HWE}
rs876493	A/G	0.260/0.320	1450/1559	8.39e-07*	0.729 (0.614-0.865)	0.719 (0.615-0.838)	0.038
rs11168070	G/C	0.0837/0.102	1450/1559	.0689	0.808 (0.653-0.997)	1.007 (0.576-1.696)	0.370

Note: All data were evaluated based on a Singapore Chinese cohort (NUS-G cohort, n = 3009).

Abbreviations: Ca, cases; CI, confidence interval; Co, controls; MAF, minor allele frequencies for asthma cases and non-asthma controls; OR, odds ratio for individuals carrying heterozygous (OR_{het}) or homozygous minor (OR_{hom}) genotype, both using homozygous major genotype individuals as reference, with adjustment to covariates same as that in the logistic regression test; SNP, single nucleotide polymorphism.

P_{HWE}: P-value of Exact Test for Hardy-Weinberg Equilibrium, significant cut-off at P < .001.

^aP-value for the additive logistic regression test of SNP to disease association, with adjustment to the test subject's age, gender, and received treatments for asthma (bronchodilators, inhaled corticosteroids and oral corticosteroids).

*P-value < .05 is considered significant disease association and also indicated in bold.

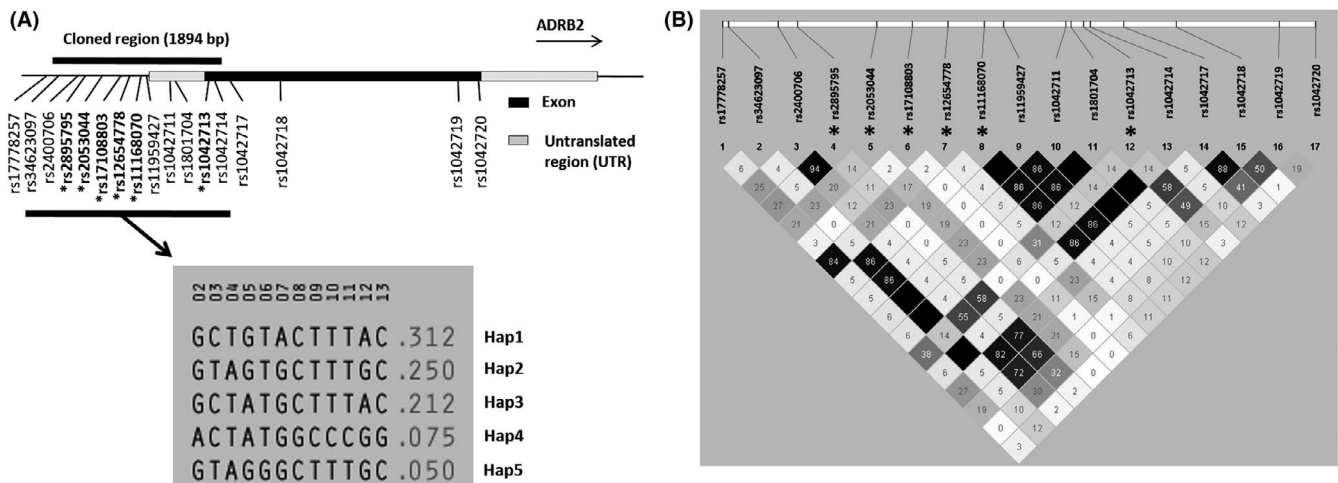


FIGURE 3 Gene structure of ADRB2 and linkage disequilibrium (LD) pattern of common SNPs in Singapore Chinese Population. A, Graphical illustration of the ADRB2 gene region, along with the relative position of common SNPs, cloned region for promoter-luciferase assay, and five common haplotypes (haplotype frequency >5%) formed by 12 SNPs in the cloned region. The frequency for each haplotype was also indicated. Tag-SNPs within the cloned region are also indicated with an asterisk (*). The frequencies of all common SNPs and haplotypes were assayed by Sanger sequencing on the NUS-S cohort (n = 40). B, LD pattern for 17 common ADRB2 SNPs identified in the NUS-S cohort

The SNP rs11168070 was then genotyped in the same NUS-G cohort (n = 3009) used in the genotyping study of rs876493. We used the logistic regression model to test for direct association of this SNP with the risk of asthma, with adjustment to test subject's age, gender and received treatments due to asthma (bronchodilator usage, inhaled and oral corticosteroids usage). As summarized in Table 2, rs11168070 was found to be in HWE, and its association with allergic asthma was marginally significant ($P = .0689$). The genotype "CG" of this SNP confers a protective effect against the disease (adjusted OR = 0.789, 95% CI = 0.653-0.997), as compared to the reference genotype "CC."

3.6 | SNPs rs876493 and rs11168070 influence asthma risk and extracellular epinephrine level via epistatic interaction

Given both PNMT and ADRB2 are involved in the epinephrine signalling pathway, we explored possible epistatic interaction between

rs876493 and rs11168070 contributing to the risk of asthma. Synergy factor (SF) analysis was performed on individuals from the Singapore Chinese cohort (n = 3009) that were genotyped for these 2 SNPs. This revealed a significant synergistic effect between rs876493 and rs11168070 that associates with the asthma disease (SF = 0.673, P -value = .0434, Figure 5A). In subjects carrying minor alleles for both SNPs of interest, 95% confidence interval of the OR was found to range from 0.393 to 0.669, suggesting a combined protective effect against allergic asthma (Figure 5A).

To further investigate the functional relevance of this epistatic effect in the epinephrine signalling pathway, we reanalysed the Singapore Chinese cohort (n = 314) used in serum epinephrine level study. A total of 180 subjects from this cohort were successfully genotyped for both rs876493 and rs11168070. Measured serum epinephrine levels in the genotyped cohort were categorized into 4 groups, according to the presence or absence of minor alleles from these SNPs. The highest serum epinephrine level was observed in the group of subjects carrying both minor alleles "A" and "G" for

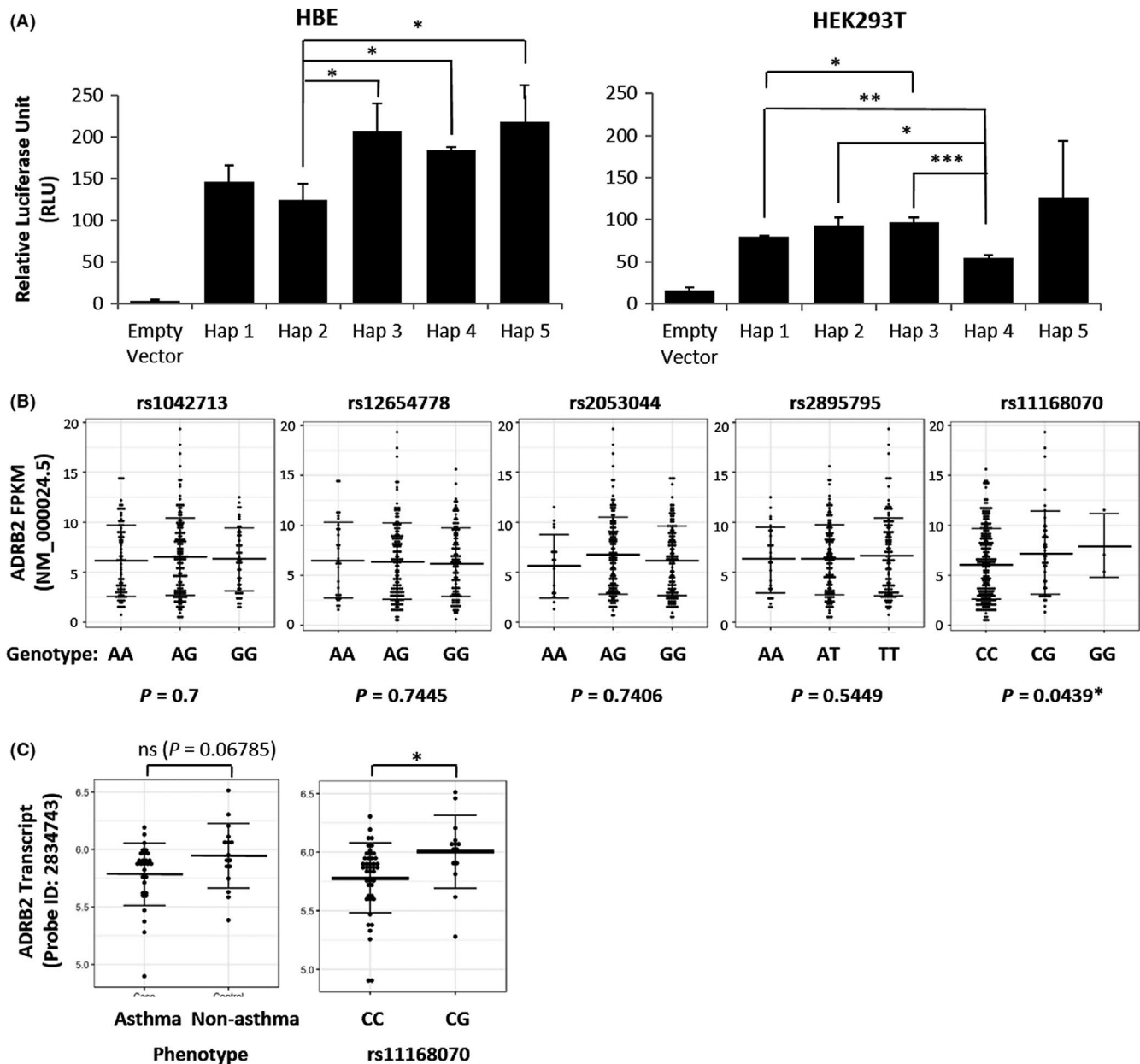


FIGURE 4 SNP rs11168070 influences promoter activity and PBMC expression of the ADRB2 gene. A, Relative luciferase unit of ADRB2 constructs compared across five different haplotypes (Hap1, Hap2, Hap3, Hap4 and Hap5) and assayed using human embryonic kidney (HEK293T) or human bronchial epithelial HBE135-E6E7 (HBE) cell lines, both on 24 h post-transfection. Independent samples *t* test was conducted for the calculation of statistical significance between each treatment group. **T* test *P*-value <0.05, ***t* test *P*-value <0.01, ****t* test *P*-value <0.001. B, Fragments Per Kilobase of transcript per Million mapped reads (FPKM) of ADRB2 transcript expression in PBMC were compared against genotypes of five tag-SNPs located within the ADRB2 promoter region, with ANOVA *P*-value indicated below each figures. An Asian cohort of 278 test subjects (Singapore/Malaysia, NUS-UTAR cohort) was studied. C, Measured relative ADRB2 transcript expressions, using Affymetrix Human Exon 1.0 ST Array, were sorted according to test subject's (left) asthma condition or (right) rs11168070 genotype. A cohort of 58 Singapore Chinese individuals was studied (NUS-TA cohort). Independent samples *t* test was conducted for calculation of statistical significance in all assays

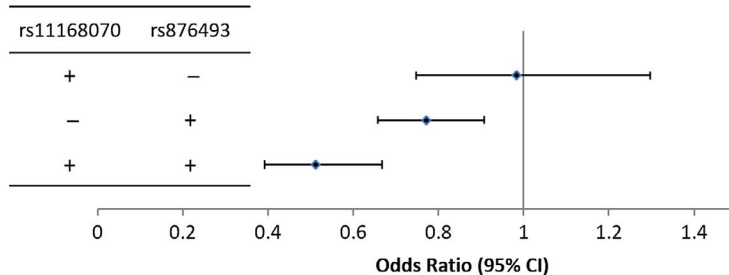
SNPs rs876493 and rs11168070 separately, as well as those with at least one minor allele in rs11168070 and no minor allele in rs876493 (Figure 5B). Besides, for subjects with at least one minor allele in rs876493 and no minor allele in rs11168070, the serum epinephrine level was found slightly lower as compared to these 2 groups, while the lowest serum epinephrine level was found in the group of subjects without minor allele for both SNPs (Figure 5B).

4 | DISCUSSION

In this study, we demonstrate epistatic interaction between SNPs from PNMT and ADRB2 can influence extracellular epinephrine level and contribute to the risk of asthma. Our study revealed that SNP rs876493 is correlated with differential PNMT promoter activity, while SNP rs11168070 is influencing the expression of the ADRB2

(A)

rs11168070 (ADRB2)	rs876493 (PNMT)	Controls	Cases	Odds ratio	95% CI of OR	SF P Value	SF (95% CI)
–	–	585	640	Reference			
+	–	117	126	0.984	(0.747-1.297)	0.0434*	0.673 (0.46-0.99)
–	+	625	528	0.772	(0.657-0.907)		
+	+	184	103	0.512	(0.392-0.668)		



(B)

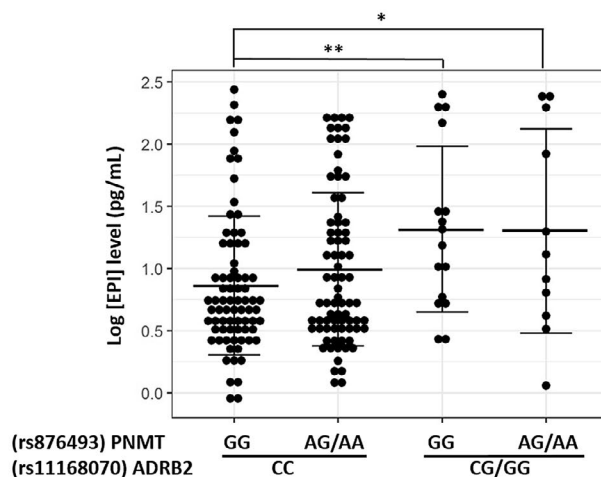


FIGURE 5 Epistatic interaction between SNPs rs876493 (PNMT) and rs11168070 (ADRB2) influences both asthma susceptibility and serum epinephrine level. A, Synergy factor (SF) analysis for allergic asthma association between rs876493 (PNMT) and rs11168070 (ADRB2), using the NUS-G cohort ($n = 3009$). CI, confidence interval; OR, odds ratio; +, presence of minor allele; –, absence of minor allele. B, Serum epinephrine level of 180 Singapore Chinese individuals (NUS-SH cohort) sorted according to rs876493 and rs11168070 genotypes. Independent samples *t* test was conducted for the calculation of statistical significance. **T* test *P*-value $< .05$, ***t* test *P*-value $< .01$

gene. The PNMT SNP rs876493 is directly associated with asthma in our Singapore Chinese study cohort, with its minor allele conferring protective effects against this disease (OR = 0.729 and 0.719 for heterozygous and homozygous minor genotype, respectively). Besides, by synergy factor analysis, we have shown a significant epistatic interaction between SNPs rs876493 and rs11168070 that results in a higher protective effect against asthma (OR = 0.512, 95% CI: 0.392-0.668) than the disease protective effect conferred from SNP rs876493 individually. Subjects carry minor alleles from rs876493 and rs11168070 have also shown increased serum epinephrine level as compared to those without any minor alleles from these SNPs.

The role of epinephrine as a β 2-adrenergic receptor agonist to improve pulmonary function has been widely exploited in the treatment of bronchospasms during asthma exacerbation.^{1,2} The effectiveness of epinephrine in asthma treatment is attributed to prior findings that associated reduced epinephrine level with asthma-related symptoms.^{6,7} In concordance with these findings, we have correlated low serum epinephrine level with increased asthma risk in a Singapore Chinese cohort (NUS-SH cohort, $n = 314$). The serum epinephrine concentrations in asthmatic cases and non-asthmatic controls in this cohort were 14.45 ± 27.75 and

63.89 ± 97.98 pg/mL, respectively. On average, this observed epinephrine level in the controls is higher than reported plasma epinephrine concentration of 20 to 55 pg/mL for adults under resting state,²⁸⁻³⁰ which suggests our study cohort may consist of individuals of various physiological states. This is also reflected by a high coefficient of variation (CV) percentage for both asthma and non-asthma group of 192% and 153%, respectively. As a limitation of this study, we were unable to determine the physiological state of each participated individuals. Despite this, when all individuals in this cohort were collectively analysed, the difference in serum epinephrine concentration between asthmatic case and non-asthmatic control group was still significant.

Phenylethanolamine N-methyltransferase is an enzyme localized in the adrenal medulla that catalyses the endogenous conversion of norepinephrine to epinephrine. Knocking out of the PNMT gene in a mouse model has been shown to completely remove the presence of endogenous epinephrine in serum, adrenal gland and urine, showing it as the essential enzyme to synthesize epinephrine.³¹ This present study focused on characterizing the only PNMT common SNP rs876493 identified in the HapMap CHB population. The transferability of SNP data

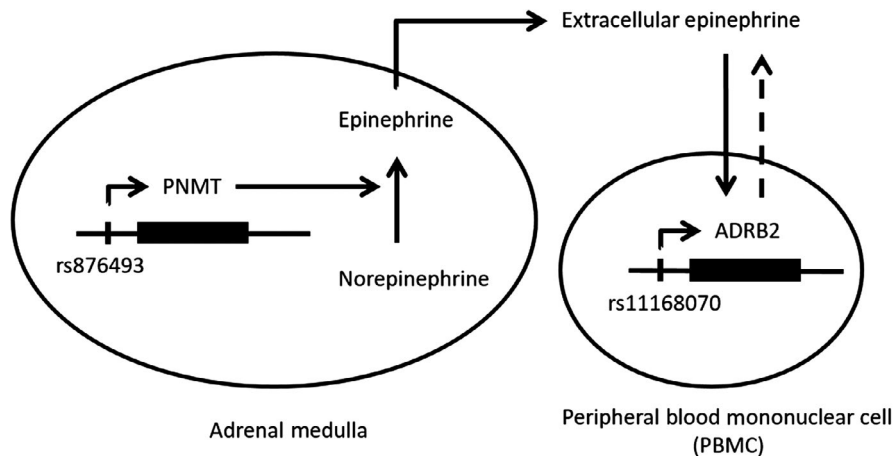


FIGURE 6 Graphical Illustration of epistatic interaction between SNPs rs876493 (PNMT) and rs11168070 (ADRB2). The promoter SNP rs876493 influences PNMT gene expression in the adrenal medulla, while its protein product catalyses the conversion of norepinephrine into epinephrine. The epinephrine is secreted into the extracellular compartment and acts as an agonist for the ADRB2 receptor. A hypothesized feedback mechanism is suggested for ADRB2 regulation of epinephrine level (indicated by a dashed arrow), while expression of the ADRB2 gene is in turn regulated by rs11168070. Taken together, both rs876493 and rs11168070 co-regulates the extracellular epinephrine level, which provided a functional explanation for epistatic interaction between these SNPs on asthma susceptibility

from CHB to our Singapore Chinese study cohort has been previously demonstrated and confirmed with a high MAF correlation between these two populations.¹⁹ Besides, our recruited cohort comprises Chinese individuals resided in the relatively uniform tropical environment of Singapore, resulting in the allergic responses observed in this population to be affected mostly by house dust mite allergen.³² This has effectively reduced the possibility of environmental factors that may confound the genetic association analysis. In this present study, the minor allele "A" of rs876493 was shown to increase PNMT gene promoter activity, while also correlated with reduced risk of asthma in our study population. These findings are in concordance with a prior study that showed a decrease in PNMT protein level has resulted in pathological changes towards asthma.⁸ Besides, given PNMT participates in the synthesis of epinephrine, we speculated this allele "A" of rs876493 may also upregulate endogenous epinephrine levels in human subjects. However, serum epinephrine level was not significantly correlated with rs876493 genotype in our study. This indicates the extracellular epinephrine level may also be regulated by other factors, thus masking the regulatory effect contributed by the PNMT protein.

β_2 -adrenergic receptor is a cell surface beta-adrenergic receptor that interacts with epinephrine.⁵ SNP rs1042714 located in the ADRB2 gene was correlated with asthma risk in multiple studies using the Chinese cohort,^{9-11,13} although some other meta-analyses have failed to replicate these results.¹⁴⁻¹⁶ In our work, we focused on characterizing 5 common ADRB2 haplotypes (frequency >5%) found in Singapore Chinese (NUS-S cohort), which consists of rs1042714 and 11 other common SNPs in the ADRB2 gene promoter region. By performing transfections assays for both HEK293T and HBE135-E6E7 cell lines, we showed these five haplotypes resulted in differential ADRB2 gene promoter activity. This finding is in concordance with previous in vitro

promoter-luciferase and gene expression assays that showed multiple SNPs in ADRB2 correlates with differential promoter activity of this gene.^{17,18} Besides, given different trends of RLUs were observed in the transfection assays between HEK293T and HBE135-E6E7 cell lines, we speculated that the ADRB2 promoter activity may be influenced by more than one SNP from the haplotype and this influence may be tissue-specific. We then proceed to examine ADRB2 transcript expression with genotypes of each SNP within the ADRB2 haplotypes, using PBMC samples of subjects from the NUS-UTAR and NUS-TA cohorts (4,4). In the haplotype used in this present study, we showed rs11168070 that tags for rs1042714 as the only tag-SNP directly associated with ADRB2 gene expression in PBMCs. The minor allele "G" of rs11168070 was correlated with increased expression of ADRB2 transcript. As a functional consequence to this, increased ADRB2 signalling in immune cells may lead to suppressed inflammatory response and decreased cytokine secretions in both innate and adaptive T cells, as revealed in prior studies.³³⁻³⁶ This supports our subsequent finding that associated allele "G" of rs11168070 with reduced asthma risk.

In this current study, we also explored the statistical perspective of epistasis, which refers to a significant deviation from the additive relationship between multilocus genotypes and phenotypic variation in a population.³⁷ The synergy factor (SF) analysis was used for the detection of statistical epistasis, as it calculates the synergistic relationship between two binary factors; in our study, these factors refer to the presence or absence of minor allele in both SNPs rs876493 and rs11168070.²³ Using the SF analysis, we revealed significant epistatic interaction between SNPs rs876493 and rs11168070, suggesting PNMT and ADRB2 may act synergistically to affect the risk of asthma. Also, stratified analysis of serum epinephrine level with genotypes of these two SNPs indicated PNMT and ADRB2 may involve in regulating the extracellular

level of epinephrine. Based on these findings, we proposed a novel epistasis mechanism between genes in the epinephrine signalling pathway that influence asthma susceptibility. As demonstrated in Figure 6, extracellular epinephrine is synthesized in adrenal medulla cells as catalysed by PNMT, while rs876493 regulates PNMT expression in adrenal medulla cells. The epinephrine also acts as a ligand for ADRB2, at the same time a feedback mechanism may exist for this receptor to regulate the extracellular epinephrine level. The expression of ADRB2 in PBMCs is in turn regulated by rs11168070. Collectively, our findings suggested that both SNPs rs876493 and rs11168070 co-regulate the level of extracellular epinephrine, for which the epinephrine may interact with ADRB2 receptors in immune cells to activate downstream anti-inflammatory pathways and reduce susceptibility to asthma. To our knowledge, this is the first report that links asthma with intercellular epistasis between genes involved in the epinephrine signalling pathway. Our present study has improved current insights on the genetic basis of asthma and identified potential biomarkers for better estimation of asthma susceptibility. Further research is required to discover how ADRB2 regulates extracellular epinephrine level and whether this epinephrine signalling pathway is associated with specific asthma endotype to better understand the asthma pathogenesis process and allow improvement of current therapeutic approaches.

ACKNOWLEDGEMENTS

We thank all participants and their family members for willing to participate in this study. FTC has received research support from the Ministry of Education Academic Research Fund, National Medical Research Council, Biomedical Research Council, Singapore, and the Agency for Science, Technology and Research; R-154-000-404-112, R-154-000-553-112, R-154-000-565-112, R-154-000-630-112, R-154-000-A08-592, R-154-000-A27-597, BMRC/01/1/21/18/077, BMRC/04/1/21/19/315, NMRC/1150/2008 and H17/01/a0/008. YYS has received a graduate research scholarship from the National University of Singapore.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

FTC planned and supervised the study. YYS, SAM and YTN recruited participants for the study. YYS planned, conducted the experiments and analysed the data. YYS 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).

ORCID

Yang Yie Sio  <https://orcid.org/0000-0002-3549-1706>

Fook Tim Chew  <https://orcid.org/0000-0003-1337-5146>

REFERENCES

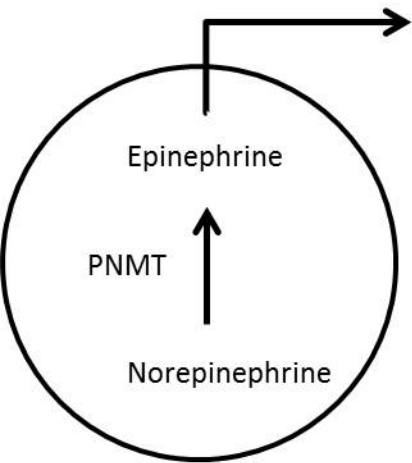
- Inoue S. Effects of epinephrine on asthmatic children. Effects of epinephrine on blood glucose, pulmonary function, and heart rate of children with asthma of varying severity. *J Allergy*. 1967;40:337-348.
- Becker LA, Hom J, Villasis-Keever M, van der Wouden JC. Beta2-agonists for acute bronchitis. *Cochrane Database Syst Rev*. 2011;7:CD001726.
- Rau JL. Inhaled adrenergic bronchodilators: historical development and clinical application. *Respir Care*. 2000;45:854-863.
- Hawkins GA, Weiss ST, Bleecker ER. Clinical consequences of ADRbeta2 polymorphisms. *Pharmacogenomics*. 2008;9:349-358.
- Johnson M. Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation. *J Allergy Clin Immunol*. 2006;117:18-24.
- Ind PW, Causon RC, Brown MJ, Barnes PJ. Circulating catecholamines in acute asthma. *Br Med J (Clin Res Ed)*. 1985;290:267-269.
- Barnes PJ, Brown MJ, Silverman M, Dollery CT. Circulating catecholamines in exercise and hyperventilation induced asthma. *Thorax*. 1981;36:435-440.
- Hu CP, Zou YQ, Feng JT, Li XZ. The effect of unilateral adrenalectomy on transformation of adrenal medullary chromaffin cells in vivo: a potential mechanism of asthma pathogenesis. *PLoS One*. 2012;7:e44586.
- Liu ZQ, Jiao Y, Liu CJ, Zhang HC, Hu BR. Association between polymorphisms and haplotypes of the beta-2 adrenergic receptor gene and asthma in a Chinese Han population. *Int J Clin Exp Med*. 2014;7:3812-3817.
- Ramphul K, Lv J, Hua L, et al. Single nucleotide polymorphisms predisposing to asthma in children of Mauritian Indian and Chinese Han ethnicity. *Braz J Med Biol Res*. 2014;47:394-397.
- Chiang CH, Lin MW, Chung MY, Yang UC. The association between the IL-4, ADRβ2 and ADAM 33 gene polymorphisms and asthma in the Taiwanese population. *J Chin Med Assoc*. 2012;75:635-643.
- Yin K, Zhang X, Qiu Y. Association between beta2-adrenergic receptor genetic polymorphisms and nocturnal asthmatic patients of Chinese Han nationality. *Respiration*. 2006;73:464-467.
- Li X, Zhang Y, Zhang J, et al. Asthma susceptible genes in Chinese population: a meta-analysis. *Respir Res*. 2010;11:129.
- Contopoulos-Ioannidis DG, Manoli EN, Ioannidis JP. Meta-analysis of the association of beta2-adrenergic receptor polymorphisms with asthma phenotypes. *J Allergy Clin Immunol*. 2005;115:963-972.
- Migita O, Noguchi E, Jian Z, et al. ADRB2 polymorphisms and asthma susceptibility: transmission disequilibrium test and meta-analysis. *Int Arch Allergy Immunol*. 2004;134:150-157.
- Thakkinian A, McEvoy M, Minelli C, et al. Systematic review and meta-analysis of the association between {beta}2-adrenoceptor polymorphisms and asthma: a HuGE review. *Am J Epidemiol*. 2005;162:201-211.
- Panebra A, Wang WC, Malone MM, et al. Common ADRB2 haplotypes derived from 26 polymorphic sites direct beta2-adrenergic receptor expression and regulation phenotypes. *PLoS One*. 2010;5:e11819.
- Johnatty SE, Abdellatif M, Shimmin L, Clark RB, Boerwinkle E. Beta 2 adrenergic receptor 5' haplotypes influence promoter activity. *Br J Pharmacol*. 2002;137:1213-1216.
- Andiappan AK, Anantharaman R, Nilkanth PP, de Wang Y, Chew FT. Evaluating the transferability of Hapmap SNPs to a Singapore Chinese population. *BMC Genet*. 2010;11:36.
- Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977;74:5463-5467.

21. Lockstone HE. Exon array data analysis using Affymetrix power tools and R statistical software. *Brief Bioinform.* 2011;12:634-644.
22. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY; London: Springer; 2009.
23. Cortina-Borja M, Smith AD, Combarros O, Lehmann DJ. The synergy factor: a statistic to measure interactions in complex diseases. *BMC Res Notes.* 2009;2:105.
24. International HapMap C. A haplotype map of the human genome. *Nature.* 2005;437:1299-1320.
25. International HapMap C, Altshuler DM, Gibbs RA, et al. Integrating common and rare genetic variation in diverse human populations. *Nature.* 2010;467:52-58.
26. International HapMap C. The International HapMap Project. *Nature.* 2003;426:789-796.
27. International HapMap C, Frazer KA, Ballinger DG, et al. A second generation human haplotype map of over 3.1 million SNPs. *Nature* 2007;449: 851-861.
28. Zhu J, Leng X, Zhao H, Chen L, Du R. Serum levels of epinephrine and norepinephrine in cirrhotic patients with portal hypertension. *Chin Med J (Engl).* 1998;111:504-506.
29. Skrabal F, Aubock J, Hortnagl H, Brucke T. Plasma epinephrine and norepinephrine concentrations in primary and secondary human hypertension. *Hypertension.* 1981;3:373-379.
30. Christensen TE, Bang LE, Holmvang L, et al. (123)I-MIBG Scintigraphy in the Subacute State of Takotsubo Cardiomyopathy. *JACC Cardiovasc Imaging.* 2016;9:982-990.
31. Bao X, Lu CM, Liu F, et al. Epinephrine is required for normal cardiovascular responses to stress in the phenylethanolamine N-methyltransferase knockout mouse. *Circulation.* 2007;116:1024-1031.
32. Andiappan AK, Puan KJ, Lee B, et al. Allergic airway diseases in a tropical urban environment are driven by dominant mono-specific sensitization against house dust mites. *Allergy.* 2014;69:501-509.
33. Tanaka S, Tsutsui M, Kishida T, Souma S, Kuroda J, Yoshida T. Salbutamol inhibits lipopolysaccharide-induced inflammatory responses in rat peritoneal macrophages. *J Toxicol Sci.* 2010;35:327-334.
34. Donnelly LE, Tudhope SJ, Fenwick PS, Barnes PJ. Effects of formoterol and salmeterol on cytokine release from monocyte-derived macrophages. *Eur Respir J.* 2010;36:178-186.
35. Panina-Bordignon P, Mazzeo D, Lucia PD, et al. Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. *J Clin Invest.* 1997;100:1513-1519.
36. Guereschi MG, Araujo LP, Maricato JT, et al. Beta2-adrenergic receptor signaling in CD4+ Foxp3+ regulatory T cells enhances their suppressive function in a PKA-dependent manner. *Eur J Immunol.* 2013;43:1001-1012.
37. Moore JH, Williams SM. Traversing the conceptual divide between biological and statistical epistasis: systems biology and a more modern synthesis. *BioEssays.* 2005;27:637-646.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Sio YY, Matta SA, Ng YT, Chew FT. Epistasis between phenylethanolamine N-methyltransferase and β 2-adrenergic receptor influences extracellular epinephrine level and associates with the susceptibility to allergic asthma. *Clin Exp Allergy.* 2020;50:352-363. <https://doi.org/10.1111/cea.13552>



Adrenal Medulla

Extracellular Epinephrine



Bronchial Smooth Muscle Relaxation

Supplementary Table S1: Study design and detailed demographics for 5 cohorts used in current study.

	NUS-G	NUS-S	NUS-TA	NUS-SH	NUS-UTAR
Total (n)	3009	40	58	314	278
Gender: Male (n, %)	1248 (41.48)	18 (45)	32 (55.17)	130 (41.40)	86 (30.93)
Female (n, %)	1756 (58.36)	22 (55)	26 (44.83)	69 (21.97)	74 (26.62)
Age (Year, Mean $\pm$ 1SD)	21.54 $\pm$ 4.80	19.9 $\pm$ 1.69	21.19 $\pm$ 3.47	13.10 $\pm$ 5.85	20.96 $\pm$ 3.47
BMI (kg·m ⁻² , Mean $\pm$ 1SD)	20.46 $\pm$ 2.67	20.51 $\pm$ 2.67	20.77 $\pm$ 3.03	19.23 $\pm$ 4.18	23.37 $\pm$ 4.18
Smoking Status					
Never-Smoker (n, %)	2606 (86.61)	-	56 (96.55)	-	151 (54.32)
Current Smoker (n, %)	23 (0.76)	-	2 (3.45)	-	1 (0.36)
Ex-Smoker (n, %)	35 (1.16)	-	0	-	5 (1.80)
Non-asthmatic (n, %)	1559 (51.81)	20 (50)	29 (50)	159 (50.64)	137 (49.28)
Asthmatic (n, %)	1450 (48.19)	20 (50)	29 (50)	155 (49.36)	22 (7.91)
Hospital admission due to asthma (past 12 months of study. n, % of asthmatics)	14 (0.97)	0	0	9 (5.81)	0
A&E admission due to asthma (past 12 months of study. n, % of asthmatics)	49 (3.38)	0	1 (1.72)	36 (23.23)	0
Medical Specialist visits due to asthma (past 12 months of study. n, % of asthmatics)	199 (13.72)	2 (10)	1 (1.72)	98 (63.23)	0
Bronchodilator Usage (n, % of asthmatics)	283 (19.52)	-	-	128 (82.58)	2 (9.09)
Inhaled Corticosteroids Usage (n, % of asthmatics)	200 (13.79)	-	-	142 (91.61)	0
Oral Corticosteroids Usage (n, % of asthmatics)	81 (5.59)	-	-	24 (15.48)	0
Type of samples (n)					
Serum	-	-	-	314	-
Mouthwash (gDNA)	3009	40	58	180	-
PBMC (gDNA)	-	-	-	-	278

PBMC (RNA)	-	-	58	-	278
Measurements (n)					
Genotyping	3009	-	58	180	278
Sequencing (ADRB2)	-	40	-	-	-
Transcriptome Array	-	-	58	-	-
Transcriptome Sequencing	-	-	-	-	278
ELISA (epinephrine)	-	-	-	314	-

*A&E: accident and emergency department of a hospital, ADRB2: β 2-adrenergic receptor, BMI: body mass index, ELISA: enzyme-linked immunosorbent assay, gDNA: genomic DNA, PBMC: peripheral blood mononuclear cell, SD: standard deviation.

Legend to supplementary figure

Supplementary Fig. S1. Graphical Illustration of the Epinephrine Signaling Pathway. In the adrenal medulla chromaffin cells, phenylethanolamine N-methyltransferase (PNMT) catalyzes the conversion of norepinephrine into epinephrine. The epinephrine is secreted into the extracellular compartment and interacts with the β 2-adrenergic receptor (ADRB2) to result in bronchial smooth muscle relaxation.