

# A functional brain-derived neurotrophic factor (*BDNF*) gene variant increases the risk of moderate-to-severe allergic rhinitis

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**Background:** Brain-derived neurotrophic factor (BDNF) is a secretory protein that has been implicated in the pathogenesis of allergic rhinitis (AR), atopic asthma, and eczema, but it is currently unknown whether *BDNF* polymorphisms influence susceptibility to moderate-to-severe AR.

**Objective:** We sought to identify disease associations and the functional effect of *BDNF* genetic variants in patients with moderate-to-severe AR.

**Methods:** Tagging single nucleotide polymorphisms (SNPs) spanning the *BDNF* gene were selected from the human HapMap Han Chinese from Beijing (CHB) data set, and associations with moderate-to-severe AR were assessed in 2 independent cohorts of Chinese patients (2216 from Shandong province and 1239 living in Singapore). The functional effects of the *BDNF* genetic variants were determined by using both *in vitro* and *ex vivo* assays.

**Results:** The tagging SNP rs10767664 was significantly associated with the risk of moderate-to-severe AR in both

Singapore Chinese ( $P = .0017$ ; odds ratio, 1.324) and Shandong Chinese populations ( $P = .039$ ; odds ratio, 1.180). The coding nonsynonymous SNP rs6265 was in perfect linkage with rs10767664 and conferred increased BDNF protein secretion by a human cell line *in vitro*. Subjects bearing the AA genotype of rs10767664 exhibited increased risk of moderate-to-severe AR and displayed increased BDNF protein and total IgE levels in plasma. Using a large-scale expression quantitative trait locus study, we demonstrated that *BDNF* SNPs are significantly associated with altered *BDNF* concentrations in peripheral blood.

**Conclusion:** A common genetic variant of the *BDNF* gene is associated with increased risk of moderate-to-severe AR, and the AA genotype is associated with increased *BDNF* mRNA levels in peripheral blood. Together, these data indicate that functional *BDNF* gene variants increase the risk of moderate-to-severe AR. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

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Allergic rhinitis (AR) is a major health issue that affects 10% to 20% of the global population, with nearly 400 million persons worldwide affected by symptoms, including itching, sneezing, rhinorrhea, and nasal obstruction.<sup>1</sup> More than 50% of patients with AR have the moderate-to-severe form of the disease, whereas the remainder exhibit only mild symptoms.<sup>2</sup> Moderate-to-severe AR can significantly impair normal daily activity and reduce health-related quality of life, and clinical management of patients with moderate-to-severe AR is extremely challenging. Hence the disease represents a substantial economic and health care burden across most of the developed world.<sup>2-5</sup> AR prevalence has been steadily increasing in recent years, leading to greater pressure on governments and health care agencies to manage the disease more effectively<sup>5</sup> and emphasizing the need to identify factors that influence AR risk and disease severity.<sup>6</sup> Allergic phenotypes, such as AR, result from a complex interplay of genetic and environmental risk factors,<sup>7-9</sup> and there are several genetic variants already known to be associated with AR risk in the Singapore Chinese population.<sup>10</sup> A potential key determinant of allergic diseases is brain-derived neurotrophic factor (BDNF), a secretory protein belonging to the neurotrophin family of molecules that plays well-established roles in a range of neural processes<sup>11,12</sup> but might also contribute to the pathophysiology of AR.<sup>13,14</sup> Eosinophils have been shown to both produce BDNF and express the corresponding receptor tropomyosin receptor kinase B, which is required to mediate the biological effects of the protein and might represent an important mediator of allergic inflammation.<sup>14-16</sup> BDNF genetic variants have also been reported to be associated with the risk of severe asthma, and BDNF protein levels are increased in the sera of patients with severe allergic phenotypes.<sup>17,18</sup> In this study we used a comprehensive approach to study the effects of BDNF genetic variants on the risk of moderate-to-severe AR in 2 independent study populations. We also used a large-scale meta-analysis to evaluate the functional effect of the risk variant identified on BDNF mRNA expression and validated our findings using *ex vivo* functional experiments. Finally, we assessed whether increased gene expression translated into increased BDNF protein levels using ELISA analysis of plasma samples that were stratified by genotype. We have previously reported that genetic variation in BDNF expression is associated with AR disease in an epidemiologic cohort in Singapore,<sup>19</sup> and other investigators have reported that BDNF protein levels in nasal mucosa predict total AR symptom scores,<sup>20</sup> whereas BDNF concentrations in plasma might represent a biomarker of clinical severity in patients with allergic asthma.<sup>17</sup> Although one previous study has identified that BDNF single nucleotide polymorphisms (SNPs) are associated with severe forms of asthma,<sup>21</sup> it is currently unknown whether these SNPs also confer an increased risk of AR. Therefore we evaluated the association of BDNF SNPs with AR risk and disease severity in 2 independent populations of Chinese patients with moderate-to-severe AR.

## METHODS

### Population-based studies in human subjects

**Shandong Chinese cohort.** A total of 481 patients (295 male and 186 female patients) aged 4 to 60 years (mean,  $22.2 \pm 15.9$  years) with

#### Abbreviations used

|         |                                        |
|---------|----------------------------------------|
| AR:     | Allergic rhinitis                      |
| BDNF:   | Brain-derived neurotrophic factor      |
| CHB:    | HapMap Han Chinese from Beijing        |
| eQTL:   | Expression quantitative trait locus    |
| HWE:    | Hardy-Weinberg equilibrium             |
| Met:    | Methionine                             |
| OR:     | Odds ratio                             |
| SNP:    | Single nucleotide polymorphism         |
| tagSNP: | Tagging single nucleotide polymorphism |
| Val:    | Valine                                 |

symptoms of AR (itchy nose, sneezing, rhinorrhea, and nasal obstruction) were recruited between 2011 and 2012 through the outpatient clinic of the Department of Otolaryngology in the Qilu Hospital, Shandong University (Jinan, Shandong Province, China; Table 1). Diagnosis of moderate-to-severe AR was based on Allergic Rhinitis and its Impact on Asthma criteria,<sup>1</sup> the patient's history of allergic symptoms, and allergy test results using the ImmunoCAP system (Phadia AB, Uppsala, Sweden). More than 92% of patients with AR were allergic to 1 or both of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, and all displayed moderate-to-severe symptoms that required medical treatment, immunotherapy, or both. Healthy control subjects ( $n = 1735$ ) were recruited from the same province.<sup>22</sup> DNA was extracted from peripheral blood samples by using a previously published protocol.<sup>19</sup> Study approval was obtained from the Institutional Review Boards of Shandong University, China (reference no. 2010G0020258).

**Singapore Chinese cohort.** The Singapore Chinese cohort has been described previously.<sup>19</sup> In the current report we used data from a subgroup of these patients with moderate-to-severe AR, as defined by Allergic Rhinitis and its Impact on Asthma guidelines ( $n = 522$ ), as well as control subjects without rhinitis ( $n = 717$ , Table 1). Study approval was obtained from the Institutional Review Board of the National University of Singapore (reference NUS10-343).

**Selection of tagging SNPs.** The HapMap Han Chinese from Beijing (CHB) genotype data set from 45 unrelated subjects was used to determine tagging SNPs (tagSNPs) for the BDNF gene.<sup>23</sup> All the SNPs within the chromosomal location 27,630 to 27,700 kb on chromosome 11 were used for the tagging approach. A schematic of the linkage disequilibrium plot of this region is provided in Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). All BDNF genetic variants in the CHB population were identified. The genotypes of these samples were used as a reference to evaluate the coverage and depth of genome-wide chips, which have previously been used for genome-wide association studies in the Chinese population. Our group has also published a report describing use of the CHB population as a reference population for our Singapore Chinese cohort.<sup>24</sup> All BDNF genetic variants identified in the CHB population were retrieved and tagged by using the algorithm of Gabriel et al,<sup>25</sup> which resulted in 7 tagSNPs covering the entire BDNF gene ( $r^2 = 0.8$  for association testing).

**Statistical analysis.** Genotypes of the tagSNPs in control subjects were tested for Hardy-Weinberg equilibrium (HWE) by using the  $\chi^2$  test. For the Shandong Chinese population, 1 SNP that failed the HWE test was excluded from further statistical analysis (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The information on SNPs and their quality control for the Singapore Chinese population has been provided in Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

The primary test of allelic association was performed by using the Cochran-Armitage test for trend, which evaluates the association of the minor allele with the disease phenotype. *P* values and odds ratios (ORs) with 95% CIs were estimated by using the PLINK program, version 1.06.<sup>26</sup> Quantitative measurements of associations with SNP genotypes were tested by using Kruskal-Wallis or Mann-Whitney tests in Prism GraphPad software (version 6.0; GraphPad Software, San Diego, Calif), except where indicated otherwise.

**TABLE I.** Demographic and clinical characteristics of the study samples

| Category                    | Patients with moderate-to-severe AR*       | Control subjects†                   |
|-----------------------------|--------------------------------------------|-------------------------------------|
| Shandong Chinese samples    |                                            |                                     |
| Subjects (no.)              | 481                                        | 1736                                |
| Median age (y)              | 22                                         | 37                                  |
| Sex (% female)              | 186 (38.66)                                | 684 (39.40)                         |
| Asthma comorbidity, no. (%) | 117 (24.32)                                | NA                                  |
| Smoking, no. (%)            | Active: 53 (11.02)<br>Passive: 109 (22.66) | NA                                  |
| Singapore Chinese samples   |                                            |                                     |
| Subjects (no.)              | 522                                        | 717‡                                |
| Median age (y)              | 22                                         | 21                                  |
| Sex (% female)              | 277 (53.07)                                | 522 (72.6)                          |
| Asthma comorbidity, no. (%) | 216 (38.66)                                | 0                                   |
| Smoking, no. (%)            | Active: 8 (1.53)<br>Passive: 8 (1.53)      | Active: 2 (0.2)<br>Passive: 4 (0.4) |

NA, Information on asthma status and smoking was not available for the population control subjects from Shandong.

\*Moderate-to-severe AR was classified based on the presence of 2 or more major symptoms (including nasal congestion, rhinorrhea, nasal itching, and sneezing), a positive skin prick test response, and a history of moderate-to-severe symptoms and affected quality-of-life outcomes (based on 2008 guidelines set by the Allergic Rhinitis and its Impact on Asthma consortium).

†Control subjects are persons of the same ethnicity and classified as healthy based on a population-based cohort.

‡Nonatopic nonrhinitis control subjects are subjects of Chinese ethnicity recruited in Singapore and classified as healthy based on negative skin prick test responses and the absence of a history of allergic symptoms.

**Genotyping.** SNP genotyping of the Shandong Chinese AR population was performed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the determination of allele-specific primer extension products with the Sequenom MassARRAY system with iPLEX technology (Sequenom, San Diego, Calif). Design of the oligonucleotides was carried out according to Sequenom guidelines and performed with MassARRAY Assay Design software. Multiplex PCR amplification of amplicons containing the 15 SNPs of interest was performed with Qiagen HotStart Taq Polymerase (Qiagen, Hilden, Germany) with 5 ng of genomic DNA. Primer extension reactions were carried out according to the manufacturer's instructions for iPLEX chemistry. Assay data were analyzed with Sequenom TYPER software. Clustering of genotype calls was evaluated to confirm that the clustering was appropriate to include in the statistical analysis. All SNPs were then quality control tested for HWE and taken forward for further statistical analysis.

### In vitro studies

**Mammalian cell culture and transfection.** HEK293T cells were purchased from the American Type Culture Collection (Rockville, Md) and maintained in RPMI 1640 medium (Sigma-Aldrich, St Louis, Mo) containing 1% L-glutamine and 10% FBS (Thermo Scientific, Waltham, Mass). Cells were cultured in a humidified incubator (5% CO<sub>2</sub> at 37°C) and enumerated by means of trypan blue exclusion with a hemocytometer. Recombinant expression vectors were transiently transfected into HEK293T cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif), and the cells were grown in 6-well culture plates (Greiner, Monroe, NC), according to the manufacturer's protocol. The pJexpress 603 mammalian vectors were used (DNA2.0, Menlo Park, Calif). Wells containing cells only served as negative controls. Recombinant plasmids containing the different constructs were transfected into triplicate cell cultures by using Lipofectamine 2000. Cells were harvested 72 hours after transfection for analysis of transcript and protein expression.

**Protein extraction.** Total cellular proteins were extracted by using NP-40 lysis buffer containing 20 mmol/L Tris, 137 mmol/L NaCl, 10%

glycerol, 1% NP-40, 3× dH<sub>2</sub>O, and 2 mmol/L phenylmethylsulfonyl fluoride adjusted to pH 8.0 with NaOH.

A total of 250 µL of lysis buffer was added to each sample before incubation on ice for 5 minutes, sonication for 5 minutes, and centrifugation of the extract at 14,000g and 4°C for 10 minutes. The samples were placed in aliquots and stored at −80°C for future analysis. Total proteins in the culture supernatants were concentrated by adding 1× HALT protease/phosphatase inhibitor cocktail together with ice-cold acetone, followed by incubation at −20°C for 30 minutes. The solution was finally centrifuged at 3500 rpm for 1 hour, and the pellet was resuspended in 1% PBS for storage at −80°C until use.

**ELISA.** BDNF protein concentrations in cell lysates, culture supernatants, and sera from healthy peripheral blood samples was determined in Nunc MaxiSorp 96-well microplates (Fisher Scientific, Loughborough, United Kingdom) by using the commercially available BDNF Emax ImmunoAssay System (Promega, Madison, Wis), according to the manufacturer's protocol. Cell-culture supernatants and cell lysates were assayed in triplicate. The raw data obtained from the ELISA measurements were processed and used in R software to fit a 5-point logistic regression fit. The fit was then used to determine the concentrations of BDNF in the analyzed samples.

### Whole blood-based expression quantitative trait locus studies

Data from a previous large-scale expression quantitative trait locus (eQTL) study of peripheral blood samples from 5,311 subjects<sup>27</sup> were used to analyze the potential association of the SNP rs6265 genotypes with transcript expression levels. This whole-genome eQTL meta-analysis was performed by using 9 data sets from 7 cohorts: EGCUT14 (n = 891), InCHIANTI (n = 611), the Rotterdam study (n = 762), Fehrmann (n = 1240 on the Illumina HT12v3 platform and n = 229 on Illumina H8v2), HVH (n = 43 on Illumina HT12v3 and n = 63 on Illumina HT12v4), SHIP-TREND (n = 963), and DILGOM (n = 509). The sample preparation and processing method and the eQTL analysis have been reported previously.<sup>27</sup> For the current study, we used summary statistic eQTL data from the original meta-analysis for rs6265 and rs10767664 and the *BDNF* gene (Illumina probe ID: ILMN\_1719820; position: chromosome 11, 27741985-27742034).

## RESULTS

### Genetic association of SNP rs10767664 with moderate-to-severe AR in Chinese populations

**Shandong Chinese cohort.** Sequenom-based genotyping of 7 tagSNPs in Shandong Chinese patients with AR revealed that 6 tagSNPs were in HWE and could be tested for association with AR risk (by using PLINK software), whereas 1 SNP was not in HWE and was therefore excluded from further analyses. The results displayed in Table II show a significant association of rs10767664 with AR disease at a *P* value of .039 and an OR of 1.17. The risk allele A was present at a higher frequency in the patients with AR (57%) than in the control subjects (53%). This result was in agreement with our previous report that the A allele is present at a higher frequency in patients with AR in an epidemiologic cohort.<sup>19</sup>

**Singapore Chinese cohort.** To validate the association of SNP rs10767664 with AR risk, we next sought to replicate our findings in the Shandong Chinese cohort by performing a similar analysis in Singapore Chinese patients with moderate-to-severe AR compared with control subjects. The association results in Table III confirmed that SNP rs10767664 was significantly associated with the moderate-to-severe AR phenotype at a *P* value of .0017 and an OR of 1.324.

**Meta-analysis in the combined Chinese populations.** We next performed a PLINK meta-analysis to combine the SNP association results from the 2 independent Chinese cohorts.

**TABLE II.** Association of *BDNF* tagSNPs with AR in the Shandong Chinese population

| CHR | SNP        | BP       | Risk allele | AF    | Case frequency | Control frequency | Other allele | CHISQ  | <i>P</i> <sub>trend</sub> | OR    | SE    | L95   | U95   |
|-----|------------|----------|-------------|-------|----------------|-------------------|--------------|--------|---------------------------|-------|-------|-------|-------|
| 11  | rs10767664 | 27682562 | A           | 0.55  | 0.5666         | 0.5256            | T            | 4.227  | <b>.039</b>               | 1.180 | 0.080 | 1.008 | 1.381 |
| 11  | rs962369   | 27690996 | G           | 0.05  | 0.05714        | 0.04522           | A            | 1.985  | .159                      | 1.280 | 0.175 | 0.907 | 1.805 |
| 11  | rs1401635  | 27650567 | C           | 0.049 | 0.05375        | 0.04378           | G            | 1.484  | .223                      | 1.241 | 0.177 | 0.876 | 1.756 |
| 11  | rs11030121 | 27692783 | T           | 0.077 | 0.08229        | 0.07114           | C            | 1.194  | .275                      | 1.171 | 0.145 | 0.882 | 1.554 |
| 11  | rs4923468  | 27682351 | A           | 0.029 | 0.03117        | 0.02563           | C            | 0.7698 | .380                      | 1.223 | 0.230 | 0.780 | 1.919 |
| 11  | rs12273539 | 27639887 | T           | 0.176 | 0.174          | 0.8209            | C            | 0.1294 | .719                      | 1.038 | 0.103 | 0.847 | 1.271 |

The *P*<sub>trend</sub> value is calculated by using the Cochran-Armitage test for trend with PLINK software (version 1.06), and a value of less than .05 for the association test for AR is shown in boldface.

AF, Allele frequency in reference to the risk allele; BP, position in base pairs for the SNP location on the chromosome; Case frequency and Control frequency, frequencies of the risk allele in affected (cases) and unaffected (control subjects) subjects; CHISQ,  $\chi^2$  statistic for the SNP association test; CHR, chromosome on which the SNP is present; L95, lower limit of the 95% CI of the OR; Other allele, allele 2 for the SNP considered for the association test; SE, SE for the Cochran-Armitage test for trend; U95, upper limit of the 95% CI of the OR.

**TABLE III.** Association of *BDNF* tagSNPs with moderate-to-severe AR in the Singapore Chinese population

| CHR | SNP        | BP       | Risk allele | AF    | Case frequency | Control frequency | Other allele | CHISQ  | <i>P</i> <sub>trend</sub> | OR     | SE      | L95    | U95   |
|-----|------------|----------|-------------|-------|----------------|-------------------|--------------|--------|---------------------------|--------|---------|--------|-------|
| 11  | rs10767664 | 27682562 | A           | 0.454 | 0.489          | 0.4195            | T            | 9.791  | <b>.0017</b>              | 1.324  | 0.08984 | 1.11   | 1.579 |
| 11  | rs962369   | 27690996 | G           | 0.035 | 0.03475        | 0.04266           | A            | 0.9943 | .3187                     | 0.8079 | 0.2142  | 0.5309 | 1.23  |
| 11  | rs12273539 | 27639887 | T           | 0.159 | 0.1541         | 0.1631            | C            | 0.3616 | .5476                     | 0.9348 | 0.1121  | 0.7505 | 1.164 |
| 11  | rs11030101 | 27637320 | T           | 0.257 | 0.2519         | 0.2612            | A            | 0.2677 | .6049                     | 0.9527 | 0.0936  | 0.793  | 1.145 |

The *P*<sub>trend</sub> value is calculated by using the Cochran-Armitage test for trend with PLINK software (version 1.06), and a value of less than .05 for the association test for AR is shown in boldface.

AF, Allele frequency in reference to the risk allele; BP, position in base pairs for the SNP location on the chromosome; Case frequency and Control frequency, frequencies of the risk allele in affected (cases) and unaffected (control subjects) subjects; CHISQ,  $\chi^2$  statistic for the SNP association test; CHR, chromosome on which the SNP is present; L95, lower limit of the 95% CI of the OR; Other allele, allele 2 for the SNP considered for the association test; SE, SE for the Cochran-Armitage test for trend; U95, upper limit of the 95% CI of the OR.

**TABLE IV.** Association of rs10767664 with the moderate-to-severe AR phenotype by using the meta-analysis approach

| CHR                          | SNP        | BP       | Risk allele | Other allele | RAF   | OR             | SE                 | L95   | U95   | <i>P</i> <sub>allele</sub> |   |
|------------------------------|------------|----------|-------------|--------------|-------|----------------|--------------------|-------|-------|----------------------------|---|
| Shandong Chinese population  |            |          |             |              |       |                |                    |       |       |                            |   |
| 11                           | rs10767664 | 27682562 | A           | T            | 0.546 | 1.180          | 0.080              | 1.008 | 1.381 | .039*                      |   |
| Singapore Chinese population |            |          |             |              |       |                |                    |       |       |                            |   |
| 11                           | rs10767664 | 27682562 | A           | T            | 0.454 | 1.324          | 0.090              | 1.11  | 1.579 | .0017*                     |   |
| CHR                          | SNP        | BP       | Risk allele | Other allele | N     | <i>P</i> value | <i>P</i> (R) value | OR    | OR(R) | Q                          | I |
| Meta-analysis                |            |          |             |              |       |                |                    |       |       |                            |   |
| 11                           | rs10767664 | 27682562 | A           | T            | 2     | .000298        | .000298            | 1.242 | 1.242 | .3397                      | 0 |

All association statistics have been calculated for the risk allele of the SNP rs10767664.

BP, Base pair; CHR, Chromosome; I, I<sup>2</sup> heterogeneity index (0-100); N, number of studies for the meta-analysis; *P* and OR, *P* value and OR calculated by using a fixed-effect model; *P*(R) and OR(R), *P* value and OR calculated by using a random-effect model; Q, *P* value for Cochran Q statistic; RAF, risk allele frequency; Risk allele, risk allele for the SNP rs10767664.

\**P*<sub>allele</sub> < .05.

Table IV displays the combined association results, which remained significant (*P* = .000298; OR, 1.242) and were thus consistent with the individual associations observed previously.

**TagSNP selection for functional studies *in vitro*.** Having established a genetic association of SNP rs10767664 with AR risk in 2 separate Chinese cohorts, we next aimed to evaluate the functional effect of this variant. When we screened all the SNPs for linkage with the tagSNP rs10767664, we detected the coding SNP rs6265 in perfect linkage (*D'* = 1 and *r*<sup>2</sup> = 1). This SNP was nonsynonymous, changing the amino acid residue from valine (Val) to methionine (Met), and thus displayed potential to alter BDNF protein expression. Therefore we used rs6265 as the “functional tag” for subsequent *in vitro* studies.

## Effects of rs6265 on BDNF protein levels in HEK293T cells

We next transfected HEK293T cells with recombinant plasmids containing either the Val or Met variants of BDNF and assessed protein levels in culture supernatants and cell lysates using ELISA. As expected, we observed overexpression of BDNF protein in both types of transfected samples compared with the mock-transfected control cells (Fig 1), and BDNF levels in the cell lysates were comparable between the wild-type Val form and the mutant Met form (Fig 1, A). However, there was a significant decrease in BDNF concentration in the culture supernatants of cells expressing Met BDNF relative to that seen in cells expressing Val BDNF (*P* < .01; Fig 1, B).

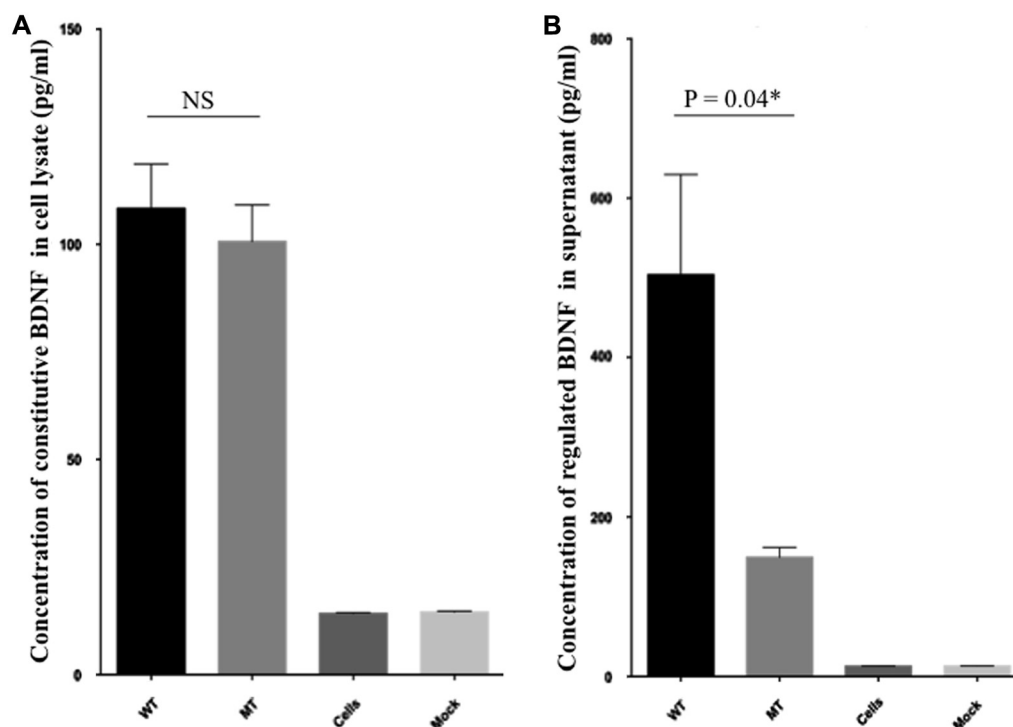


**FIG 1.** BDNF protein levels (in picograms per milliliter) in cell lysates (**A**) and supernatants (**B**) of HEK293T cells *in vitro*. *Cells*, Untransfected HEK293T cells with no vector; *Mock*, untransfected cells with the empty vector; *MT*, Met/mutant variant; *WT*, wild-type/Val variant. \* $P < .05$ , Student *t* test.

### Effects of rs6265 on BDNF concentrations in plasma

We then proceeded to assess whether the results of our *in vitro* assays could be validated in *ex vivo* samples from human volunteers. To evaluate whether the rs6265 genotype influenced plasma levels of BDNF protein, we used a Singapore Chinese cohort of 156 subjects with known BDNF plasma concentration and rs6265 genotype (43 with the CC variant, 73 with the CT variant, and 40 with the TT variant). A nonparametric Kruskal-Wallis test revealed a significant association of rs6265 with BDNF protein levels in plasma. The risk genotype CC, as determined in our genetic study, exhibited higher BDNF plasma concentrations than did the genotypes CT and TT at  $P$  values of .02 and .03, respectively (Fig 2).

### Correlation of BDNF SNPs with gene expression level in whole blood

To evaluate whether the BDNF SNPs affected mRNA expression, we used a large eQTL meta-analysis data set, which comprised 8 different sets of whole-blood expression data with corresponding whole-genome SNP information.<sup>27</sup> Table V<sup>27</sup> demonstrates a strong association of BDNF SNPs with BDNF mRNA levels in whole peripheral blood in multiple populations of unrelated subjects from Europe. Subjects with the C allele for rs6265 displayed significantly higher BDNF gene expression relative to that seen in subjects who exhibited the T allele. A representative plot showing normalized expression of BDNF stratified according to genotype rs10767664 (A) or rs6265 (B) in the SHIP-TREND cohort is displayed in Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). The nature of the SNP effect was identical in 7 of the 8 data sets, with a meta  $P$  value of .0013 for rs6265 and a  $z$  score of  $-3.20$ .

### Association of rs6265 with total serum IgE levels

We next sought to determine whether the BDNF functional variant rs6265 exhibited any association with total serum IgE levels in a Singapore Chinese population ( $n = 123$  subjects; 35 with the CC genotype, 56 with the CT genotype, and 32 with the TT genotype). Fig 3 shows a significant association of the exonic nonsynonymous variant rs6265 with total serum IgE levels, and the risk genotype CC conferred higher total IgE levels than were detected in the TT genotype group ( $P < .05$ ).

### DISCUSSION

The BDNF gene is located on chromosome 11 and exhibits several SNPs that have previously been linked with allergic phenotypes, including atopic dermatitis,<sup>28</sup> AR, and atopic asthma.<sup>19,21,29-31</sup> Previous reports suggest that increased BDNF protein levels predict total symptom scores in patients with AR<sup>20</sup> and are associated with severe asthma phenotypes in children,<sup>17,18</sup> suggesting that BDNF protein plays a significant role in determining the severity of allergic diseases. Although BDNF SNPs have now been implicated in patients with allergic diseases in multiple populations around the world, other studies have failed to replicate these associations, and the functional effect of the BDNF variants has remained unclear.<sup>21</sup> We now provide compelling new evidence that the BDNF variant rs10767664 is associated with increased risk of moderate-to-severe AR in 2 independent patient cohorts. We also confirmed that our identification of an association of BDNF tagSNPs with risk of moderate-to-severe AR was independent of asthma status (see Table E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). All BDNF tagSNPs displayed similar allele frequencies in both Chinese populations, irrespective of asthma

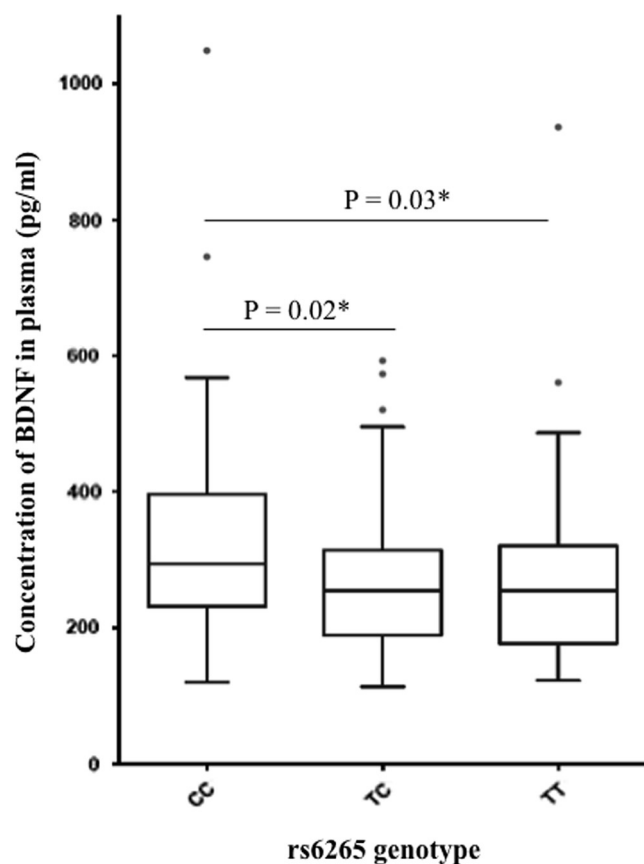


**FIG 2.** BDNF plasma levels (in picograms per milliliter) in healthy subjects, as measured by means of ELISA and stratified according to rs6265 genotype. A Kruskal-Wallis test was initially used to test whether the different genotypes were associated with altered BDNF protein levels in plasma. Pairwise Mann-Whitney tests were then carried out to determine whether there were significant differences in BDNF levels between genotypes.

comorbidity. The A allele of rs10767664 exhibits higher frequency in the Shandong Chinese (0.55) compared with the Singapore Chinese (0.45) cohorts. These data resemble those from northern Chinese subjects (CHB), who display a higher frequency of the A allele (0.521) of rs10767664 than do southern Chinese subjects (0.45; estimated by using 1000 Genomes Project data, see Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). *In silico* prediction with TF search (<http://www.cbrc.jp/research/db/TFSEARCH.html>) reveals a loss of transcription factor binding of heat shock factor protein 1 for the T allele, which could result in lower BDNF levels. However, further studies are needed to validate this *in silico* finding. Therefore the BDNF SNP rs10767664 might represent an important overall determinant of severe atopic phenotypes in the Chinese population. A similar association of BDNF with atopic asthma was also observed in our Singapore Chinese population,<sup>19</sup> suggesting that BDNF variants might also influence the risk of allergic lung inflammation.

Therefore the BDNF tagSNP rs10767664 might represent a key risk factor for atopic phenotypes in the Chinese population. When we screened all the SNPs for linkage with the tagSNP rs10767664 using HapMap data, we detected the coding SNP rs6265 to be in perfect linkage ( $D' = 1$  and  $r^2 = 1$ ). Using

**TABLE V.** Effect of BDNF SNPs on gene expression in whole blood

| SNP            | rs10767664 | rs6265   |
|----------------|------------|----------|
| EGCUT          | -0.98392   | -1.04359 |
| SHIP-TREND     | -3.13821   | -3.32569 |
| Groningen-HT12 | -1.05558   | -0.73882 |
| Groningen-H8v2 | 1.069896   | 1.311833 |
| Rotterdam      | -0.7718    | -1.1304  |
| Fehrman        | -1.35155   | -1.08089 |
| INCHIANTI      | -0.32069   | -1.33303 |
| HVH-HT12v3     | -1.20073   | -0.47799 |
| Meta z score*  | -2.97246   | -3.20519 |
| Meta P value†  | .0029      | .0013    |

Data from a previous large-scale eQTL study of peripheral blood samples from 5311 subjects<sup>27</sup> were used to analyze the potential association of SNP rs6265 genotypes with transcript expression levels. This whole-genome eQTL meta-analysis was performed over 9 data sets from 7 cohorts: EGCUT (n = 891), InCHIANTI (n = 611), Rotterdam study (n = 762), Fehrman (n = 1240 on the Illumina HT12v3 platform and n = 229 on Illumina H8v2), HVH (n = 43 on Illumina HT12v3 and n = 63 on Illumina HT12v4), SHIP-TREND (n = 963), and DILGOM (n = 509). The sample preparation and processing methods and the eQTL analysis have been reported previously.<sup>27</sup> For the current study, we used summary statistic eQTL data from the original meta-analysis for rs6265 and rs10767664 and the BDNF gene (Illumina probe ID: ILMN\_1719820; position: chromosome 11, 27741985-27742034). The z score reflects the effect size of the genotype on gene expression in the individual cohorts. The negative sign indicates that the minor allele leads to reduced expression.

\*Meta z scores and †meta P values represent the combined z scores and P values obtained in the eQTL meta-analysis when analyzing all the data sets together with data from Westra et al.<sup>27</sup> A positive z score (>0) implies an increase in expression for the allele assessed, whereas a negative z score implies the assessed allele associates to a reduction in gene expression in comparison with the other allele.

1000 Genomes Project data, we further validated that these SNPs are indeed in perfect linkage (see Table E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This SNP was nonsynonymous, changing the amino acid residue from Val to Met, and thus displayed potential to alter BDNF protein expression. Therefore we used rs6265 as the "functional tag" for subsequent *in vitro* studies. The allele frequencies of rs10767664 and rs6265 are different among different populations (see Table E4), which could result in the risk allele switching between populations. We used the HapMap data for identifying tagSNPs for association in this study. Using the 1000 Genomes Project for the BDNF locus, we identified many SNPs that were previously not described in the HapMap but were in linkage to our tagSNPs (see Table E5). However, there were other common SNPs in these 1000 Genomes Project data that were not tagged by our tagSNPs, which is a limitation of the current study. Therefore further investigations in other study populations of different ethnic origins will be required to determine the corresponding risk alleles in those groups. Future studies investigating a more comprehensive SNP architecture of the BDNF gene could yield interesting findings to finely map this disease-associated locus.

Our data revealed that the variant rs6265, which was in perfect linkage with rs10767664, was strongly associated with altered BDNF protein secretion *in vitro*. The effect of the nonsynonymous variant rs6265 on constitutive expression of BDNF was assessed in HEK293T cells transiently transfected with recombinant plasmids to induce overexpression of either the Val66 or Met66 variant of BDNF. Using this approach, we observed a significant decrease in secreted BDNF protein levels in the culture supernatants of cells overexpressing the Met66 variant, whereas BDNF levels in cell lysates were comparable for both variants.

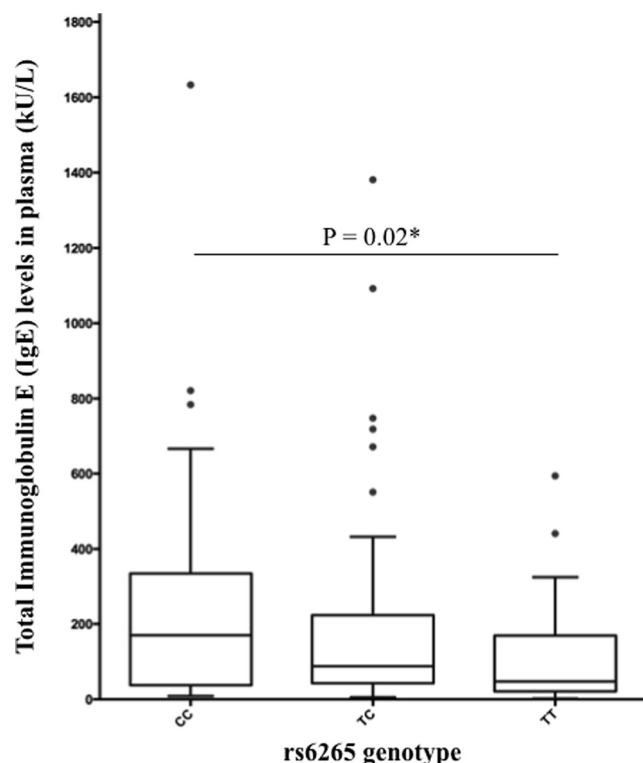


**FIG 3.** Total IgE levels in sera from healthy subjects, as measured by using ImmunoCAP and stratified according to rs6265 genotype. The Kruskal-Wallis test was initially used to test whether the different genotypes were associated with altered plasma IgE levels. The pairwise Mann-Whitney test were then carried out to determine whether there were significant differences in IgE levels between genotypes.

These data resemble those reported by Chen et al,<sup>32</sup> who demonstrated an effect of Met66 substitution on secretion of BDNF protein by neurosecretory cells and who indicate that the amino acid substitution induced by rs6265 can alter protein function. The *BDNF* SNP rs6265 is located in the prodomain and forms part of the sorting motif that directs the protein to secretory vesicles for export from the cell. The Met substitution at position 66 has previously been shown to significantly decrease BDNF interaction with sorting proteins, such as sortilin, in secretory vesicles and impairs colocalization with secretory granules, leading to reduced BDNF release by neurosecretory cells and hippocampal neurons.<sup>33</sup> These data, when taken together with our own *in vitro* observations, strongly suggest that secretion of BDNF into human plasma is influenced by the rs6265 genotype. Accordingly, when we measured BDNF protein concentrations in human plasma using ELISA, we observed significantly higher levels in subjects with the Val-coding CC genotype relative to the Met-coding TT genotype.

Although initially annotated as a neurotrophic factor, BDNF is also expressed by nonneuronal cell types, including endothelial cells and human airway epithelial cells, which produce greater quantities of the protein under inflammatory conditions.<sup>34,35</sup> Consistent with these data, patients with AR have been reported to exhibit higher levels of BDNF in the airways after nasal provocation,<sup>20</sup> and increased levels of BDNF protein are associated with increased itching in patients with eczema.<sup>36</sup> This increase in BDNF expression has functional consequences for ongoing immune responses; as a key component of the inflammatory

infiltrate in patients with allergic disease, eosinophils can produce BDNF to elicit further neurotrophin release from airway epithelial cells, thereby increasing eosinophil survival and modulating the function of multiple different cell types.<sup>20,37-39</sup> In a separate study treatment with anti-BDNF antibodies in a rodent model protected against airflow limitation, sensory hyperreactivity, and airway hyperresponsiveness in response to allergen challenge, whereas administration of recombinant BDNF was sufficient to induce these pathologic changes in the lungs of healthy animals.<sup>40</sup> Indeed, the known ability of neurotrophins to increase production of the neuropeptides that modulate epithelial cell reactivity might be a critical event in the development of airway hyperresponsiveness, which is an archetypal feature of both AR and atopic asthma. BDNF has even been shown to promote hematopoietic colony growth and participate in wound healing and tissue repair and might therefore promote remodeling of the inflamed airway.<sup>14</sup> Accordingly, leukocyte production of BDNF is preferentially associated with late-phase allergic responses and correlates with clinical symptoms, which is in line with our current findings in patients with moderate-to-severe AR and suggests a pathophysiologic role for BDNF in chronic mucosal inflammation.<sup>14</sup>

In summary, we report that a *BDNF* genetic variant is associated with moderate-to-severe AR risk in 2 large cohorts of human patients and is also likely to affect *BDNF* gene expression and protein levels, as demonstrated by using both *ex vivo* and *in vitro* approaches. Furthermore, the genotype we identified was associated with increased BDNF protein levels in blood and resulted in increased total plasma IgE levels, which represent a crucial risk factor for allergic disease manifestation. Further study of neurotrophin gene variants in the pathogenesis of AR will now be required to fully elucidate the role of these proteins in airway inflammation and perhaps uncover new therapeutic options for patients with AR and other allergic disorders.

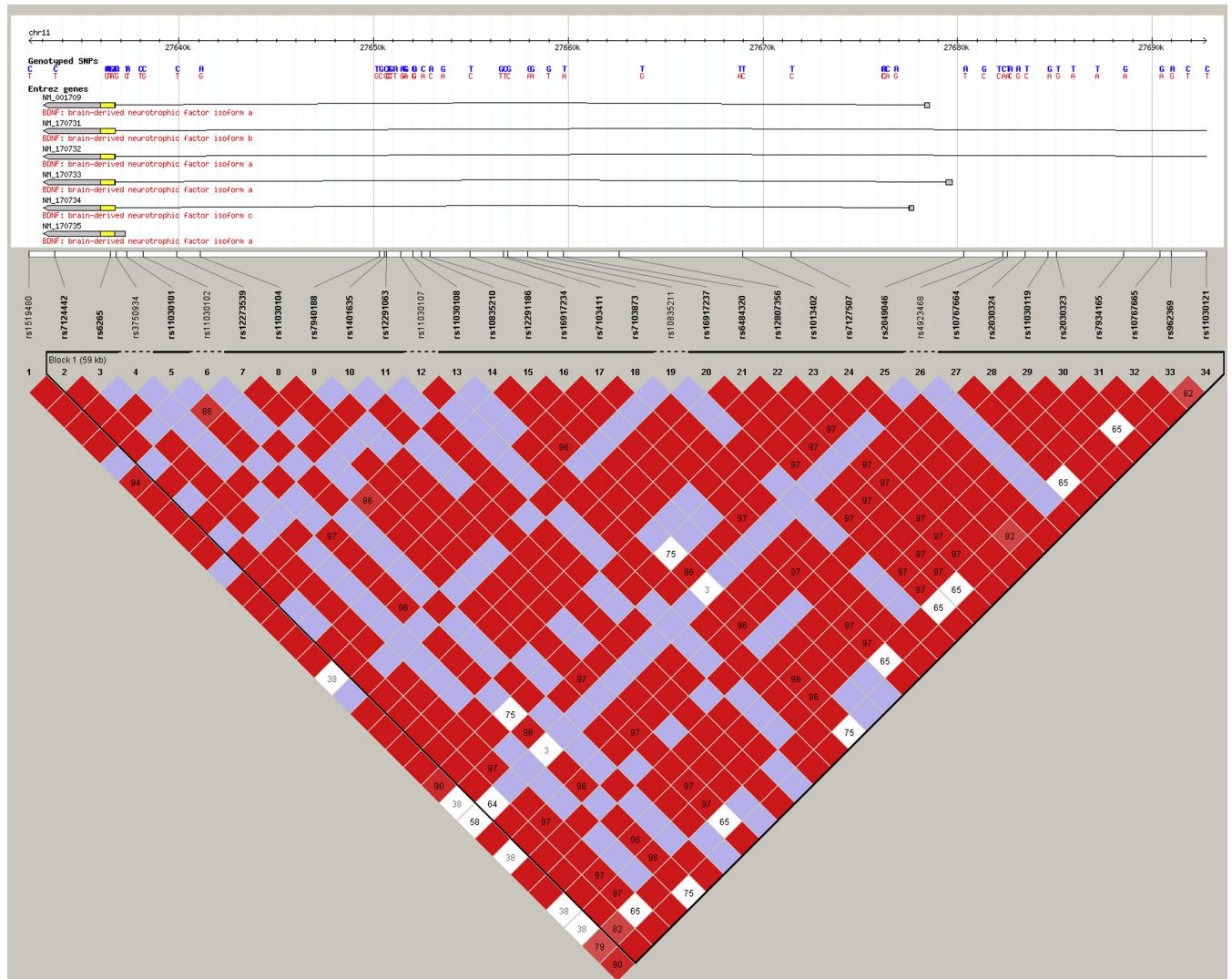
We thank all the volunteers and their family members who participated in this study. We thank Ramani Anantharaman, Parate Pallavi Nilkanth, and other members of the functional genomics laboratory at the National University of Singapore for helping in sample collection for the epidemiologic cohort. Dr Neil McCarthy of Insight Editing London provided writing assistance.

**Clinical implications: Future studies of BDNF expression in the inflamed nasal mucosa might uncover novel methods of reducing local BDNF protein levels and conferring therapeutic benefit in patients with AR.**

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FIG E1. Linkage disequilibrium plot generated by using Haploview for the *BDNF* gene locus.

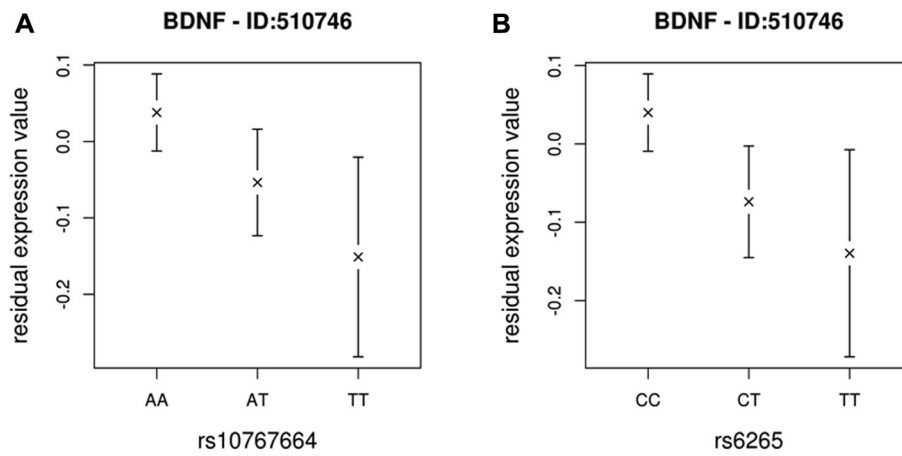


**FIG E2.** A representative plot of normalized expression of *BDNF* (probe ID: 510746) stratified according to rs10767664 (**A**) and rs6265 (**B**) genotype in the SHIP-TREND cohort.

**TABLE E1.** Information on genotype quality and HWE testing for the Shandong Chinese population

| CHR | SNP        | BP       | Risk allele | AF    | Other allele | Genotyping call rate | HWE <i>P</i> value |
|-----|------------|----------|-------------|-------|--------------|----------------------|--------------------|
| 11  | rs10767664 | 27682562 | A           | 0.55  | T            | 95.62%               | .7936              |
| 11  | rs962369   | 27690996 | G           | 0.05  | A            | 95.71%               | .1404              |
| 11  | rs1401635  | 27650567 | C           | 0.049 | G            | 96.39%               | .4488              |
| 11  | rs11030121 | 27692783 | T           | 0.077 | C            | 96.44%               | .751               |
| 11  | rs4923468  | 27682351 | A           | 0.029 | C            | 96.44%               | 1                  |
| 11  | rs12273539 | 27639887 | T           | 0.176 | C            | 96.39%               | .2367              |

rs10835210 had an HWE *P* value of 3.16E-07 and hence was removed from further statistical analysis.

*AF*, Allele frequency of risk allele; *BP*, position in base pairs for the SNP location on the chromosome; *CHR*, chromosome on which the SNP is present.

**TABLE E2.** Information on genotype quality and HWE testing for the Singapore Chinese population

| CHR | SNP        | BP       | Risk allele | AF    | Other allele | Genotyping call rate | HWE <i>P</i> value |
|-----|------------|----------|-------------|-------|--------------|----------------------|--------------------|
| 11  | rs10767664 | 27682562 | A           | 0.454 | T            | 86.77%               | .6304              |
| 11  | rs962369   | 27690996 | G           | 0.035 | A            | 99.58%               | .2452              |
| 11  | rs12273539 | 27639887 | T           | 0.159 | C            | 99.10%               | .2437              |
| 11  | rs11030101 | 27637320 | T           | 0.257 | A            | 99.27%               | .6173              |

*AF*, Frequency of the risk allele; *BP*, position in base pairs for the SNP location on the chromosome; *CHR*, Chromosome on which the SNP is present.

**TABLE E3.** Distribution of allele frequencies in the Chinese populations stratified by asthma status

| CHR                          | SNP        | BP       | Risk allele | Frequency of risk allele in AR without asthma (363 cases) | Frequency of risk allele in AR with asthma (117 cases) | Frequency of risk allele in control subjects* | Other allele |
|------------------------------|------------|----------|-------------|-----------------------------------------------------------|--------------------------------------------------------|-----------------------------------------------|--------------|
| Shandong Chinese population  |            |          |             |                                                           |                                                        |                                               |              |
| 11                           | rs10767664 | 27682562 | A           | 0.5683                                                    | 0.5611                                                 | 0.5256                                        | T            |
| 11                           | rs962369   | 27690996 | G           | 0.05631                                                   | 0.05978                                                | 0.04522                                       | A            |
| 11                           | rs1401635  | 27650567 | C           | 0.05757                                                   | 0.04167                                                | 0.04378                                       | G            |
| 11                           | rs11030121 | 27692783 | T           | 0.08553                                                   | 0.07216                                                | 0.07114                                       | C            |
| 11                           | rs4923468  | 27682351 | A           | 0.03115                                                   | 0.03125                                                | 0.02563                                       | C            |
| 11                           | rs12273539 | 27639887 | C           | 0.8148                                                    | 0.8632                                                 | 0.8209                                        | T            |
| CHR                          | SNP        | BP       | Risk allele | Frequency of risk allele in AR without asthma (277 cases) | Frequency of risk allele in AR with asthma (216 cases) | Frequency of risk allele in control subjects* | Other allele |
| Singapore Chinese population |            |          |             |                                                           |                                                        |                                               |              |
| 11                           | rs10767664 | 27682562 | A           | 0.4917                                                    | 0.4706                                                 | 0.4195                                        | T            |
| 11                           | rs962369   | 27690996 | G           | 0.03818                                                   | 0.03756                                                | 0.04266                                       | A            |
| 11                           | rs12273539 | 27639887 | C           | 0.8636                                                    | 0.8388                                                 | 0.8369                                        | T            |
| 11                           | rs11030101 | 27637320 | T           | 0.2618                                                    | 0.2371                                                 | 0.2612                                        | A            |

BP, Position in base pairs for the SNP location on the chromosome; CHR, chromosome on which the SNP is present; Other allele, allele 2 for the SNP considered for the association test; Risk allele, risk allele for the SNP tested for association.

\*All control subjects have no history of asthma.

**TABLE E4.** Allele frequency of the 2 *BDNF* genetic variants rs10767664 and rs6265 according to 1000 Genomes Project data in the different ethnicities evaluated

| Population                     | Alleles A    | Alleles T    | Genotypes A A | Genotypes A T | Genotypes T T | Allele count            | Genotype count                        |
|--------------------------------|--------------|--------------|---------------|---------------|---------------|-------------------------|---------------------------------------|
| <b>rs10767664</b>              |              |              |               |               |               |                         |                                       |
| 1000GENOMES:phase_1_ALL        | 0.744        | 0.256        | 0.582         | 0.323         | 0.094         | 1625 (A) / 559 (T)      | 636 (A A) / 353 (A T) / 103 (T T)     |
| 1000GENOMES:phase_1_AFR        | 0.929        | 0.071        | 0.874         | 0.11          | 0.016         | 457 (A) / 35 (T)        | 215 (A A) / 27 (A T) / 4 (T T)        |
| 1000GENOMES:phase_1_AMR        | 0.801        | 0.199        | 0.635         | 0.331         | 0.033         | 290 (A) / 72 (T)        | 115 (A A) / 60 (A T) / 6 (T T)        |
| 1000GENOMES:phase_1_ASN        | 0.526        | 0.474        | 0.318         | 0.416         | 0.266         | 301 (A) / 271 (T)       | 91 (A A) / 119 (A T) / 76 (T T)       |
| 1000GENOMES:phase_1_ASW        | 0.918        | 0.082        | 0.852         | 0.131         | 0.016         | 112 (A) / 10 (T)        | 52 (A A) / 8 (A T) / 1 (T T)          |
| 1000GENOMES:phase_1_CEU        | 0.741        | 0.259        | 0.529         | 0.424         | 0.047         | 126 (A) / 44 (T)        | 45 (A A) / 36 (A T) / 4 (T T)         |
| <b>1000GENOMES:phase_1_CHB</b> | <b>0.521</b> | <b>0.479</b> | <b>0.309</b>  | <b>0.423</b>  | <b>0.268</b>  | <b>101 (A) / 93 (T)</b> | <b>30 (A A) / 41 (A T) / 26 (T T)</b> |
| <b>1000GENOMES:phase_1_CHS</b> | <b>0.45</b>  | <b>0.55</b>  | <b>0.24</b>   | <b>0.42</b>   | <b>0.34</b>   | <b>90 (A) / 110 (T)</b> | <b>24 (A A) / 42 (A T) / 34 (T T)</b> |
| 1000GENOMES:phase_1_CLM        | 0.808        | 0.192        | 0.633         | 0.35          | 0.017         | 97 (A) / 23 (T)         | 38 (A A) / 21 (A T) / 1 (T T)         |
| 1000GENOMES:phase_1_EUR        | 0.761        | 0.239        | 0.567         | 0.388         | 0.045         | 577 (A) / 181 (T)       | 215 (A A) / 147 (A T) / 17 (T T)      |
| 1000GENOMES:phase_1_FIN        | 0.806        | 0.194        | 0.624         | 0.366         | 0.011         | 150 (A) / 36 (T)        | 58 (A A) / 34 (A T) / 1 (T T)         |
| 1000GENOMES:phase_1_GBR        | 0.803        | 0.197        | 0.618         | 0.371         | 0.011         | 143 (A) / 35 (T)        | 55 (A A) / 33 (A T) / 1 (T T)         |
| 1000GENOMES:phase_1_IBS        | 0.571        | 0.429        | 0.357         | 0.429         | 0.214         | 16 (A) / 12 (T)         | 5 (A A) / 6 (A T) / 3 (T T)           |
| 1000GENOMES:phase_1_JPT        | 0.618        | 0.382        | 0.416         | 0.404         | 0.18          | 110 (A) / 68 (T)        | 37 (A A) / 36 (A T) / 16 (T T)        |
| 1000GENOMES:phase_1_LWK        | 0.933        | 0.067        | 0.876         | 0.113         | 0.01          | 181 (A) / 13 (T)        | 85 (A A) / 11 (A T) / 1 (T T)         |
| 1000GENOMES:phase_1_MXL        | 0.795        | 0.205        | 0.652         | 0.288         | 0.061         | 105 (A) / 27 (T)        | 43 (A A) / 19 (A T) / 4 (T T)         |
| 1000GENOMES:phase_1_PUR        | 0.8          | 0.2          | 0.618         | 0.364         | 0.018         | 88 (A) / 22 (T)         | 34 (A A) / 20 (A T) / 1 (T T)         |
| 1000GENOMES:phase_1_TSI        | 0.724        | 0.276        | 0.531         | 0.388         | 0.082         | 142 (A) / 54 (T)        | 52 (A A) / 38 (A T) / 8 (T T)         |
| 1000GENOMES:phase_1_YRI        | 0.932        | 0.068        | 0.886         | 0.091         | 0.023         | 164 (A) / 12 (T)        | 78 (A A) / 8 (A T) / 2 (T T)          |
| <b>rs6265</b>                  |              |              |               |               |               |                         |                                       |
| 1000GENOMES:phase_1_ALL        | 0.772        | 0.228        | 0.63          | 0.283         | 0.087         | 1685 (C) / 499 (T)      | 688 (C C) / 309 (C T) / 95 (T T)      |
| 1000GENOMES:phase_1_AFR        | 0.986        | 0.014        | 0.972         | 0.028         |               | 485 (C) / 7 (T)         | 239 (C C) / 7 (C T)                   |
| 1000GENOMES:phase_1_AMR        | 0.823        | 0.177        | 0.68          | 0.287         | 0.033         | 298 (C) / 64 (T)        | 123 (C C) / 52 (C T) / 6 (T T)        |
| 1000GENOMES:phase_1_ASN        | 0.521        | 0.479        | 0.311         | 0.42          | 0.269         | 298 (C) / 274 (T)       | 89 (C C) / 120 (C T) / 77 (T T)       |
| 1000GENOMES:phase_1_ASW        | 0.959        | 0.041        | 0.918         | 0.082         |               | 117 (C) / 5 (T)         | 56 (C C) / 5 (C T)                    |
| 1000GENOMES:phase_1_CEU        | 0.788        | 0.212        | 0.612         | 0.353         | 0.035         | 134 (C) / 36 (T)        | 52 (C C) / 30 (C T) / 3 (T T)         |
| <b>1000GENOMES:phase_1_CHB</b> | <b>0.51</b>  | <b>0.49</b>  | <b>0.299</b>  | <b>0.423</b>  | <b>0.278</b>  | <b>99 (C) / 95 (T)</b>  | <b>29 (C C) / 41 (C T) / 27 (T T)</b> |
| <b>1000GENOMES:phase_1_CHS</b> | <b>0.445</b> | <b>0.555</b> | <b>0.23</b>   | <b>0.43</b>   | <b>0.34</b>   | <b>89 (C) / 111 (T)</b> | <b>23 (C C) / 43 (C T) / 34 (T T)</b> |
| 1000GENOMES:phase_1_CLM        | 0.842        | 0.158        | 0.7           | 0.283         | 0.017         | 101 (C) / 19 (T)        | 42 (C C) / 17 (C T) / 1 (T T)         |
| 1000GENOMES:phase_1_EUR        | 0.797        | 0.203        | 0.625         | 0.343         | 0.032         | 604 (C) / 154 (T)       | 237 (C C) / 130 (C T) / 12 (T T)      |
| 1000GENOMES:phase_1_FIN        | 0.823        | 0.177        | 0.656         | 0.333         | 0.011         | 153 (C) / 33 (T)        | 61 (C C) / 31 (C T) / 1 (T T)         |
| 1000GENOMES:phase_1_GBR        | 0.837        | 0.163        | 0.685         | 0.303         | 0.011         | 149 (C) / 29 (T)        | 61 (C C) / 27 (C T) / 1 (T T)         |
| 1000GENOMES:phase_1_IBS        | 0.714        | 0.286        | 0.5           | 0.429         | 0.071         | 20 (C) / 8 (T)          | 7 (C C) / 6 (C T) / 1 (T T)           |
| 1000GENOMES:phase_1_JPT        | 0.618        | 0.382        | 0.416         | 0.404         | 0.18          | 110 (C) / 68 (T)        | 37 (C C) / 36 (C T) / 16 (T T)        |
| 1000GENOMES:phase_1_LWK        | 0.995        | 0.005        | 0.99          | 0.01          |               | 193 (C) / 1 (T)         | 96 (C C) / 1 (C T)                    |
| 1000GENOMES:phase_1_MXL        | 0.795        | 0.205        | 0.652         | 0.288         | 0.061         | 105 (C) / 27 (T)        | 43 (C C) / 19 (C T) / 4 (T T)         |
| 1000GENOMES:phase_1_PUR        | 0.836        | 0.164        | 0.691         | 0.291         | 0.018         | 92 (C) / 18 (T)         | 38 (C C) / 16 (C T) / 1 (T T)         |
| 1000GENOMES:phase_1_TSI        | 0.755        | 0.245        | 0.571         | 0.367         | 0.061         | 148 (C) / 48 (T)        | 56 (C C) / 36 (C T) / 6 (T T)         |
| 1000GENOMES:phase_1_YRI        | 0.994        | 0.006        | 0.989         | 0.011         |               | 175 (C) / 1 (T)         | 87 (C C) / 1 (C T)                    |

The two populations (CHB,CHS) similar to the studied populations are shown in boldface. The allele frequencies of CHB and our Shandong Chinese and CHS and our Singapore Chinese are comparable.

**TABLE E5.** SNPs in linkage with the tagSNPs tested for association analysis, as estimated by using the 1000 Genomes Project data

| Chromosome | 1000 Genomes<br>project SNP | MAF      | POS1     | R <sup>2</sup> | TagSNP     | POS2     | N_INDV |
|------------|-----------------------------|----------|----------|----------------|------------|----------|--------|
| 11         | rs7124442                   | 0.058333 | 27692783 | 0.748428       | rs11030121 | 27633617 | 60     |
| 11         | rs1401635                   | 0.058333 | 27692783 | 0.748428       | rs11030121 | 27650567 | 60     |
| 11         | rs11030108                  | 0.058333 | 27692783 | 0.748428       | rs11030121 | 27652040 | 60     |
| 11         | rs1013402                   | 0.058333 | 27692783 | 0.748428       | rs11030121 | 27668957 | 60     |
| 11         | rs11030119                  | 0.058333 | 27692783 | 0.748428       | rs11030121 | 27684678 | 60     |
| 11         | rs962369                    | 0.058333 | 27692783 | 0.748428       | rs11030121 | 27690996 | 60     |
| 11         | rs7127507                   | 0.083333 | 27692783 | 0.662745       | rs11030121 | 27671460 | 60     |
| 11         | rs7944119                   | 0.083333 | 27692783 | 0.662745       | rs11030121 | 27678874 | 60     |
| 11         | rs7127507                   | 0.083333 | 27650567 | 0.660377       | rs1401635  | 27671460 | 60     |
| 11         | rs7944119                   | 0.083333 | 27650567 | 0.660377       | rs1401635  | 27678874 | 60     |
| 11         | rs7127507                   | 0.083333 | 27690996 | 0.660377       | rs962369   | 27671460 | 60     |
| 11         | rs7944119                   | 0.083333 | 27690996 | 0.660377       | rs962369   | 27678874 | 60     |
| 11         | rs10767659                  | 0.208333 | 27639887 | 0.628906       | rs12273539 | 27642772 | 60     |
| 11         | rs7103873                   | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27656893 | 60     |
| 11         | rs2049046                   | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27680351 | 60     |
| 11         | rs2030324                   | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27683491 | 60     |
| 11         | rs7934165                   | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27688559 | 60     |
| 11         | rs10767665                  | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27690434 | 60     |
| 11         | rs10219241                  | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27693699 | 60     |
| 11         | rs10835215                  | 0.458333 | 27637320 | 0.612665       | rs11030101 | 27696525 | 60     |
| 11         | rs2883187                   | 0.475    | 27637320 | 0.593487       | rs11030101 | 27697668 | 60     |
| 11         | rs2353487                   | 0.225    | 27639887 | 0.572187       | rs12273539 | 27646975 | 60     |
| 11         | rs10767659                  | 0.208333 | 27650567 | 0.203029       | rs1401635  | 27642772 | 60     |
| 11         | rs10767659                  | 0.208333 | 27690996 | 0.203029       | rs962369   | 27642772 | 60     |
| 11         | rs2353487                   | 0.225    | 27650567 | 0.182455       | rs1401635  | 27646975 | 60     |
| 11         | rs2353487                   | 0.225    | 27690996 | 0.182455       | rs962369   | 27646975 | 60     |
| 11         | rs10767659                  | 0.208333 | 27692783 | 0.175042       | rs11030121 | 27642772 | 60     |
| 11         | rs2353487                   | 0.225    | 27692783 | 0.153618       | rs11030121 | 27646975 | 60     |
| 11         | rs2883187                   | 0.475    | 27639887 | 0.153021       | rs12273539 | 27697668 | 60     |
| 11         | rs7103873                   | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27656893 | 60     |
| 11         | rs2049046                   | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27680351 | 60     |
| 11         | rs2030324                   | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27683491 | 60     |
| 11         | rs7934165                   | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27688559 | 60     |
| 11         | rs10767665                  | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27690434 | 60     |
| 11         | rs10219241                  | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27693699 | 60     |
| 11         | rs10835215                  | 0.458333 | 27639887 | 0.138222       | rs12273539 | 27696525 | 60     |
| 11         | rs2353487                   | 0.225    | 27637320 | 0.092086       | rs11030101 | 27646975 | 60     |
| 11         | rs11030103                  | 0.133333 | 27637320 | 0.070146       | rs11030101 | 27638909 | 60     |
| 11         | rs11030101                  | 0.333333 | 27639887 | 0.070146       | rs12273539 | 27637320 | 60     |
| 11         | rs7940188                   | 0.133333 | 27637320 | 0.070146       | rs11030101 | 27650315 | 60     |
| 11         | rs12291186                  | 0.133333 | 27637320 | 0.070146       | rs11030101 | 27652894 | 60     |
| 11         | rs16917234                  | 0.133333 | 27637320 | 0.070146       | rs11030101 | 27654950 | 60     |
| 11         | rs10767659                  | 0.208333 | 27637320 | 0.064777       | rs11030101 | 27642772 | 60     |
| 11         | rs10835210                  | 0.316667 | 27639887 | 0.060087       | rs12273539 | 27652486 | 60     |
| 11         | rs12291063                  | 0.125    | 27637320 | 0.056604       | rs11030101 | 27650677 | 60     |
| 11         | rs11030103                  | 0.133333 | 27692783 | 0.018656       | rs11030121 | 27638909 | 60     |
| 11         | rs12273539                  | 0.133333 | 27692783 | 0.018656       | rs11030121 | 27639887 | 60     |
| 11         | rs7940188                   | 0.133333 | 27692783 | 0.018656       | rs11030121 | 27650315 | 60     |
| 11         | rs12291186                  | 0.133333 | 27692783 | 0.018656       | rs11030121 | 27652894 | 60     |
| 11         | rs16917234                  | 0.133333 | 27692783 | 0.018656       | rs11030121 | 27654950 | 60     |
| 11         | rs12291063                  | 0.125    | 27692783 | 0.015415       | rs11030121 | 27650677 | 60     |
| 11         | rs2883187                   | 0.475    | 27650567 | 0.011948       | rs1401635  | 27697668 | 60     |
| 11         | rs2883187                   | 0.475    | 27690996 | 0.011948       | rs962369   | 27697668 | 60     |
| 11         | rs28722151                  | 0.35     | 27639887 | 0.011517       | rs12273539 | 27637752 | 60     |
| 11         | rs7103873                   | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27656893 | 60     |
| 11         | rs2049046                   | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27680351 | 60     |
| 11         | rs2030324                   | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27683491 | 60     |
| 11         | rs7934165                   | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27688559 | 60     |
| 11         | rs10767665                  | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27690434 | 60     |
| 11         | rs10219241                  | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27693699 | 60     |
| 11         | rs10835215                  | 0.458333 | 27650567 | 0.008872       | rs1401635  | 27696525 | 60     |
| 11         | rs7103873                   | 0.458333 | 27690996 | 0.008872       | rs962369   | 27656893 | 60     |

(Continued)

TABLE E5. (Continued)

| Chromosome | 1000 Genomes project SNP | MAF      | POS1     | $R^2$    | TagSNP     | POS2     | N_INDV |
|------------|--------------------------|----------|----------|----------|------------|----------|--------|
| 11         | rs2049046                | 0.458333 | 27690996 | 0.008872 | rs962369   | 27680351 | 60     |
| 11         | rs2030324                | 0.458333 | 27690996 | 0.008872 | rs962369   | 27683491 | 60     |
| 11         | rs7934165                | 0.458333 | 27690996 | 0.008872 | rs962369   | 27688559 | 60     |
| 11         | rs10767665               | 0.458333 | 27690996 | 0.008872 | rs962369   | 27690434 | 60     |
| 11         | rs10219241               | 0.458333 | 27690996 | 0.008872 | rs962369   | 27693699 | 60     |
| 11         | rs10835215               | 0.458333 | 27690996 | 0.008872 | rs962369   | 27696525 | 60     |
| 11         | rs7124442                | 0.058333 | 27639887 | 0.008845 | rs12273539 | 27633617 | 60     |
| 11         | rs11030103               | 0.133333 | 27650567 | 0.008845 | rs1401635  | 27638909 | 60     |
| 11         | rs11030103               | 0.133333 | 27690996 | 0.008845 | rs962369   | 27638909 | 60     |
| 11         | rs12273539               | 0.133333 | 27650567 | 0.008845 | rs1401635  | 27639887 | 60     |
| 11         | rs11030108               | 0.058333 | 27639887 | 0.008845 | rs12273539 | 27652040 | 60     |
| 11         | rs1013402                | 0.058333 | 27639887 | 0.008845 | rs12273539 | 27668957 | 60     |
| 11         | rs11030119               | 0.058333 | 27639887 | 0.008845 | rs12273539 | 27684678 | 60     |
| 11         | rs12273539               | 0.133333 | 27690996 | 0.008845 | rs962369   | 27639887 | 60     |
| 11         | rs7940188                | 0.133333 | 27650567 | 0.008845 | rs1401635  | 27650315 | 60     |
| 11         | rs7940188                | 0.133333 | 27690996 | 0.008845 | rs962369   | 27650315 | 60     |
| 11         | rs12291186               | 0.133333 | 27650567 | 0.008845 | rs1401635  | 27652894 | 60     |
| 11         | rs16917234               | 0.133333 | 27650567 | 0.008845 | rs1401635  | 27654950 | 60     |
| 11         | rs12291186               | 0.133333 | 27690996 | 0.008845 | rs962369   | 27652894 | 60     |
| 11         | rs16917234               | 0.133333 | 27690996 | 0.008845 | rs962369   | 27654950 | 60     |
| 11         | rs12291063               | 0.125    | 27650567 | 0.006866 | rs1401635  | 27650677 | 60     |
| 11         | rs12291063               | 0.125    | 27690996 | 0.006866 | rs962369   | 27650677 | 60     |
| 11         | rs28722151               | 0.35     | 27650567 | 0.004281 | rs1401635  | 27637752 | 60     |
| 11         | rs28722151               | 0.35     | 27690996 | 0.004281 | rs962369   | 27637752 | 60     |
| 11         | rs11030101               | 0.333333 | 27692783 | 0.003922 | rs11030121 | 27637320 | 60     |
| 11         | rs7127507                | 0.083333 | 27639887 | 0.003884 | rs12273539 | 27671460 | 60     |
| 11         | rs7944119                | 0.083333 | 27639887 | 0.003884 | rs12273539 | 27678874 | 60     |
| 11         | rs7124442                | 0.058333 | 27637320 | 0.002156 | rs11030101 | 27633617 | 60     |
| 11         | rs11030101               | 0.333333 | 27650567 | 0.002156 | rs1401635  | 27637320 | 60     |
| 11         | rs11030108               | 0.058333 | 27637320 | 0.002156 | rs11030101 | 27652040 | 60     |
| 11         | rs1013402                | 0.058333 | 27637320 | 0.002156 | rs11030101 | 27668957 | 60     |
| 11         | rs11030119               | 0.058333 | 27637320 | 0.002156 | rs11030101 | 27684678 | 60     |
| 11         | rs11030101               | 0.333333 | 27690996 | 0.002156 | rs962369   | 27637320 | 60     |
| 11         | rs7127507                | 0.083333 | 27637320 | 0.0016   | rs11030101 | 27671460 | 60     |
| 11         | rs7944119                | 0.083333 | 27637320 | 0.0016   | rs11030101 | 27678874 | 60     |
| 11         | rs2883187                | 0.475    | 27692783 | 0.001073 | rs11030121 | 27697668 | 60     |
| 11         | rs10835210               | 0.316667 | 27650567 | 0.000951 | rs1401635  | 27652486 | 60     |
| 11         | rs10835210               | 0.316667 | 27690996 | 0.000951 | rs962369   | 27652486 | 60     |
| 11         | rs28722151               | 0.35     | 27692783 | 0.000384 | rs11030121 | 27637752 | 60     |
| 11         | rs10835210               | 0.316667 | 27692783 | 0.000368 | rs11030121 | 27652486 | 60     |
| 11         | rs7103873                | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27656893 | 60     |
| 11         | rs2049046                | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27680351 | 60     |
| 11         | rs2030324                | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27683491 | 60     |
| 11         | rs7934165                | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27688559 | 60     |
| 11         | rs10767665               | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27690434 | 60     |
| 11         | rs10219241               | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27693699 | 60     |
| 11         | rs10835215               | 0.458333 | 27692783 | 0.000223 | rs11030121 | 27696525 | 60     |

MAF, Minor allele frequency of the 1000 Genomes Project SNPs; N\_INDV, number of subjects in the 1000 Genomes Project used to calculate  $R^2$  values (CHB + JPT populations); POS1, chromosomal position of the 1000 Genomes Project SNP; POS2, chromosomal position of the tagSNP;  $R^2$ , linkage disequilibrium calculated between 1000 Genomes Project SNPs and our tagSNPs; TagSNP, SNPs from the current study used calculated linkage ( $R^2$ ).