ONLINE METHODS

Samples. For methylation analysis, blastocysts (54 blastocysts) were collected from E3.5 and oocytes from 5 day (1,883 oocytes), 20 day (germinal vesicle, 3,586 oocytes) and superovulated 4–5-week-old (MII, 443 oocytes) C57BL/6J mice. *Dnmt3a*$^{-/-}$ germinal vesicle oocytes (635) were obtained from crosses with ZP3-Cre transgenic males, and *Dnmt3L*$^{-/-}$ germinal vesicle oocytes (843) were collected from DNMT3L-null homozygous females[18]; the corresponding control oocytes were collected from wild-type littermates (385 *Dnmt3a*$^{+/+}$ oocytes and 809 *Dnmt3L*$^{+/+}$ oocytes). For allelic methylation studies, E3.5 blastocysts were collected from C57BL/6J × CAST/Ei hybrids. For mRNA-Seq, oocytes were collected from 10-day-old (644 growing oocytes) and 35-day-old (913 fully grown germinal vesicle oocytes) C57BL/6J mice. For the H3K4me3 ChIP-Seq, 6,400 day 15 growing oocytes (CD1) were collected.

RRBS. Genomic DNA was purified using the QIAamp DNA Micro kit (QIAGEN) followed by MspI digestion (Fermentas), end-repair/A-Tailing (1 nM dCTPs, 1 nM dGTPs, 10 nM dATPs; Klenow exo-, Fermentas) and 5mC-adaptor ligation (T4 Ligase, Fermentas) performed within the same tube and buffer (Tango 1X, Fermentas) by heat inactivating each enzymatic step followed by adjustment with the reagents required for the next step. Then bisulphite conversion was performed (Imprint DNA Modification, Sigma), and converted DNA was amplified (6 cycles) using a uracil stalling free polymerase (Pfu Turbo Cx, Stratagene) followed by size selection (150–450 bp, QIAGEN) and second-round amplification (10–13 cycles; Platinium Pfx polymerase, Invitrogen). Libraries were purified (SPRI beads, Agencourt) and sequenced on Illumina Genome Analyzer IIx. Because of low complexity at the start of each sequence (MspI fragments), bareback was performed (see URLs). Sequence alignment and methylation calls were performed using Bismark[28]. CpGs with read depth <5 were discarded. For every analysis, all informative CpGs were used. To score CGI methylation, the following cutoffs were applied: methylation level was determined for CGIs with information on ≥10% of their total CpGs (with a minimum of 5 CpGs) and by averaging individual cytosine methylation levels. CGIs with an average methylation level ≥75% and ≤25% were called methylated and unmethylated, respectively. The unmethylated CGIs group corresponds to CGIs called unmethylated in both oocytes and sperm. A false discovery rate for CpG methylation was determined empirically from the calls observed for CpGs within 11 known DMRs and was maximally 0.0439% at the minimum level of observation of five reads. *P* values for all methylated CGIs, based on departure from genome-wide methylation levels, were all <0.05% (χ^2 test with Bonferroni correction for multiple testing). CGIs with contradictory methylation assignments in replicate libraries were excluded from analysis.

mRNA-Seq. Total RNA was extracted (TRIzol, Invitrogen), mRNA was purified using oligo(dT) Dynabeads (Invitrogen) and fragmented (Ambion) followed by first-strand (Superscript III, Invitrogen) and second-strand (DNA Pol.I, NEB) synthesis and reaction purification (Minelute, QIAGEN). One-step A-tailing and adaptor ligation were performed as was done for RRBS followed by size selection (250 bp ± 25 bp, QIAquick, QIAGEN) and library amplification (15 cycles; Platinium Pfx polymerase, Invitrogen). Libraries were purified (SPRI beads, Agencourt) and sequenced on Illumina Genome Analyzer IIx, either single read for expression analysis or paired end for gene structure prediction (day 10 oocytes). Sequences were aligned using TopHat.

ChIP-Seq. H3K4me3 immunoprecipitation (39,159; Active Motif) was performed as described elsewhere[29] with minor modifications. ChIP was performed in duplicate from 3,200 oocytes. Illumina libraries were generated (input and immunoprecipitation) using a NEBNext kit (set 1; NEB), except that adaptor ligation was performed as for RRBS. Sequences were aligned using an ungapped Eland alignment with default stringency parameters. Because of high background results from the limited starting material, reads from duplicates were combined. Technical assessment was made by comparison with embryonic stem cell H3K4me3 ChIP-Seq datasets (GSM594581 and GSM535982)[11,30].

Direct bisulphite sequencing. DNA was purified by proteinase K digestion and phenol-chloroform extraction, spiked with lambda DNA and bisulphite treated (Zymo). Each PCR comprised a minimum of 50 oocytes or 2–3 blastocyst equivalents. Cloning and analysis were performed as described elsewhere[31], with removal of clones with identical patterns of conversion. Primers used for the amplification of specific CGIs from bisulphite modified DNA are given in **Supplementary Table 4**.

Statistical analysis. For categorical data, such as distribution of CpGs or CGIs methylation, χ^2 tests were applied. For quantitative data, Mann-Whitney U tests (between 2 groups) and Kruskall-Wallis tests (between more than 2 groups) were applied.

Additional information. Dataset analysis was based on build NCBIM37/mm9 of the mouse genome and performed using SeqMonk (see URLs). Promoter CGIs were defined as overlapping an annotated transcription start site (TSS) (Ensembl, RefSeq or UCSC), intragenic CGIs were defined as overlapping an annotated gene without its TSS and intergenic CGIs were defined as not overlapping annotated genes or promoters. Promoters were defined as the region 2 kb upstream of annotated TSSs. For repetitive element analysis, positions of individual instances of LINE, SINE, tandem repeats, long terminal repeats (LTR) and low-complexity regions (LCR) were extracted from Ensembl. The overlap between full-length CGIs and individual repeat types was determined as the percentage of the CGI length using a custom Perl script. CpG periodicity was determined as the distribution of inter-CpG distance (from C to C) between all pairs of CpGs in each region, averaged over all of the regions in a particular grouping.

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Five-year study assessing the feasibility and safety of autologous blood transfusion in pregnant Japanese women

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Abstract

Aim: To assess the feasibility and safety of autologous blood donation during pregnancy in Japanese women.

Material and Methods: We enrolled patients who were either at high risk for massive blood loss during delivery or had blood that was difficult to match for transfusion between March 2005 and February 2010. After delivery, we reviewed hospital records of these patients to collect data on blood donation procedures, obstetric outcome and blood transfusions received.

Results: We enrolled 314 patients during the study period and performed 809 blood donations. The median volume of donated blood was 1200 mL (range, 400–2000 mL). Vasovagal reflex as an adverse donor reaction occurred in 10 of the 314 patients (3.2%) during 11 of the 809 donations (1.4%). There were no cases of non-reassuring fetal heart rate patterns during blood donations. Twenty-five (7.8%) of the 322 neonates were admitted to the neonatal intensive care unit. All 322 infants were healthy 1 month after delivery. Among 314 patients, autologous blood re-transfusion was performed for 56 (17.8%) and homologous blood transfusion was performed concurrently for 5 (1.6%). Placenta previa was the indication with the highest re-transfusion rate (42.4%). All re-transfusions were performed without side-effects.

Conclusion: Autologous blood donation is feasible and safe for pregnant women and their infants. Although indications of autologous blood donation are controversial, it should be considered for cases of placenta previa.

Key words: adverse effect, autologous, blood, pregnancy, transfusion.

Introduction

Obstetric hemorrhage is a major cause of maternal death,[1] and its incidence has been increasing.[2–4] Transfusion therapy is one of the most important procedures for improving maternal outcome. Autologous blood donation (donation, storage and re-transfusion) may be effective for patients at risk for massive bleeding during delivery or are difficult to transfuse because of a rare blood type. The Royal College of Obstetricians and Gynecologists (RCOG) in the UK does not recommend autologous blood donation during pregnancy because of concerns regarding placental insufficiency, whether the woman can replace her hemoglobin before delivery, and whether the collected units will be sufficient in the event of major obstetric hemorrhage.[5] The American College of Obstetricians and Gynecologists (ACOG) also does not recommend this procedure.[6] However, autologous blood transfusion has two merits: the immediate availability of blood without cross-matched testing and reducing the need for homologous blood transfusion. For this reason, we suggest that autologous blood donation should be included as an option for pregnant women, especially

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for high-risk patients and those with rare blood types. A series of studies have demonstrated that autologous transfusion is safe in pregnancy.[7–12] Among these studies, two had a large sample size. McVay *et al.* reported 272 patients who donated in the third trimester of pregnancy. However, they were only able to analyze outcomes in 199 given the retrospective study design. Recently, Obed *et al.* reported the feasibility of autologous blood donation during pregnancy with 625 obstetric patients using a prospective study design. However, they did not evaluate the incidence of adverse effects (e.g. maternal vasovagal reflex), incidence of autologous blood use, quantity of blood loss at delivery, or outcomes of deliveries and neonates. Accordingly, the study by Obed *et al.* does not sufficiently address the feasibility and safety of autologous blood donation during pregnancy. The aim of this study is to prospectively assess the feasibility and safety of autologous blood donation and transfusion during pregnancy in Japanese women.

Material and Methods

This study was approved by the ethics committee of our institution. Candidates for enrollment were pregnant women scheduled to deliver at our center during the study period (between March 2005 and February 2010). The inclusion criterion was to be a patient at high risk for massive blood loss at delivery or a patient for which securing cross-matched blood for transfusion would be difficult.

At approximately 32 weeks of gestation, we explained the study to these patients and determined whether they met eligibility criteria. Patients with any maternal medical complication, fetal growth restriction or fetal structural anomalies were excluded from this study. We obtained written informed consent and initiated iron supplementation (100 mg/day) at the time of enrollment.

Patients who were scheduled to deliver by planned cesarean section began donating their blood approximately 5 weeks before the day of the operation and patients who were scheduled to deliver by spontaneous onset of labor began donating at approximately 36 weeks of gestation, because donated blood could be stored for up to 35 days. We scheduled patients at low risk for obstetric hemorrhage to donate 400 or 800 mL of autologous blood, and aimed for high-risk patients to donate 1200 mL or more.

The schedule on the day of blood donation was as follows:

1 Blood was sampled to examine whether the patient was anemic. Blood donation was postponed if the hemoglobin concentration was below 10 g/dL.
2 Lidocaine tape (Penles tape; Nitto Denko Corporation, Tokyo, Japan) was applied to lessen pain during donation.
3 Patients were hydrated orally (about 500 mL of isotonic water).
4 Patients donated approximately 400 mL of blood by gravity with fetal heart rate (FHR) monitoring.

All blood donations were performed by obstetricians. Donation was stopped and proper medical care was given if patients had an abnormal donor reaction or abnormal FHR patterns.

The policy of autologous blood transfusion was the same as for homologous blood transfusion. We did not determine the criteria for transfusion using patient hematocrit or hemoglobin levels because a blood test of an obstetric patient suffering from massive bleeding might not accurately reflect her condition. We performed autologous blood transfusion when a patient had unstable vital signs or low urinary output. If vital signs were stable, autologous transfusion was not performed. After delivery, we reviewed hospital records of these patients to collect data on blood donation procedures, obstetric outcome and blood transfusions received.

Results

A total of 314 patients (mean age ± SD, 34.8 ± 4.7 years) were enrolled during the study period; a total of 809 blood donations were performed. Gravidity and parity were 0, 0–5 and 0, 0–5 (median, range), respectively. Mean height and body weight before pregnancy were 159.0 ± 9.0 cm and 52.9 ± 7.1 kg, respectively.

Among the 314 patients, it would have been difficult to prepare matched blood for transfusion for 86 (56 with Rh negative blood type, 30 with positive irregular antibodies), and 228 patients were at high risk for massive bleeding during delivery (66 with total placenta previa, 62 with leiomyoma, 61 with low-lying placenta, 12 with previous cesarean section or past history of uterine surgery, 8 with twin pregnancy, and 21 with a history of massive bleeding during a previous delivery).

Data for autologous blood donations from pregnant women are shown in Table 1. Median donated blood volume and number of blood donations were 1200 mL

Table 1 Data of autologous blood donation during pregnancy

Volume of donated blood (mL)	1200, 400–2000	(median, range)
No. blood donations (times)	3, 1–5	(median, range)
Duration of blood donation (min)	7.3 ± 3.7	(mean ± SD)
Hemoglobin concentration at first donation (g/dL)	11.4 ± 0.8	(mean ± SD)
Hemoglobin concentration at delivery (g/dL)	10.8 ± 1.1	(mean ± SD)
Vasovagal reflex (per patient)	10/314 (3.2%)	
Vasovagal reflex (per donation)	11/809 (1.4%)	
Non-reassuring FHR during blood donation	0/809 donations	

FHR, fetal heart rate.

Table 2 Delivery and neonatal outcomes of patients who donated blood during pregnancy

Gestational age at delivery (weeks)	37.8 ± 1.6	(mean ± SD)
Mode of delivery		
Normal vaginal delivery	106 (33.8%)	
Cesarean section	181 (57.6%)	
Instrumental vaginal delivery	27 (8.6%)	
Blood loss during delivery ($n = 314$)	1039 ± 942	(mean ± SD)
Transfused patients ($n = 56$)	2243 ± 1090	(mean ± SD)]*
Non-transfused patients ($n = 258$)	777 ± 664	(mean ± SD)]*
Birth weight (g)	2845 ± 430	(mean ± SD)
Apgar scores at 1 min	8	(median)
Apgar scores at 5 min	9	(median)
Cord blood pH of umbilical artery	7.28 ± 0.41	(mean ± SD)
NICU admission	25/322 infants (7.8%)	

*Student's t-test, $P < 0.05$. NICU, neonatal intensive care unit.

(range, 400–2000 mL) and three times (range, 1–5 times), respectively. Twenty-nine patients donated once, 109 donated twice, 150 donated three times, 18 donated four times, and 8 donated five times. Mean hemoglobin concentrations at the first donation and at delivery were 11.4 ± 0.8 g/dL and 10.8 ± 1.1 g/dL, respectively. Vasovagal reflex (VVR) as an adverse donor reaction was observed in 10 of the 314 patients (3.2%) during 11 of 809 donations (1.4%). No cases of non-reassuring fetal heart rate pattern occurred during the 809 blood donations.

The delivery and neonatal outcomes of patients who donated blood during pregnancy are shown in Table 2. Mean gestational age at delivery was 37.8 ± 1.6 weeks. One hundred and six patients (33.8%) delivered vaginally, 181 (57.6%) underwent cesarean section, and 27 (8.6%) delivered by forceps or vacuum extractor. Mean blood loss during delivery was 1039 ± 942 mL. There was a significant difference in the volume of blood loss between transfused (2243 ± 1090 mL) and non-transfused patients (777 ± 664 mL) using Student's t-test ($P < 0.05$). The mean birth weight of 322 infants was 2845 ± 430 g. Median Apgar scores at 1 and 5 min were 8 and 9, respectively. Mean blood pH of the umbilical artery at delivery was 7.28 ± 0.41. Twenty-five infants (7.8%) were admitted to the NICU. Of these, 18 infants had low birth weight, 4 had transient tachypnea, 1 had a Group B streptococcal infection, and 1 was affected by maternal general anesthesia. None of the infants had abnormal findings 1 month after delivery.

The numbers of transfusions performed grouped by primary indication for study enrollment are shown in Table 3. Autologous blood transfusion was performed for 56 of the 314 patients (17.8%), and homologous blood transfusion was performed concurrently for 5 patients (1.6%). Three of 56 patients with Rh negative blood type (5.4%) and none of the 30 patients with positive irregular antibodies received an autologous transfusion. Of 66 patients with total placenta previa, 28 (42.4%) received autologous blood transfusions and 4 also received transfusions of homologous blood. Nine patients (14.5%) with leiomyoma, 12 (19.7%) with low-lying placenta, 1 (8.3%) with previous cesarean section, and 3 (14.3%) with a past history of massive bleeding during a previous delivery received autologous blood transfusions. One patient with a low-lying

Table 3 Transfusions grouped by primary indication for study enrollment

Indication	*n*	Autologous blood transfusion	Homologous blood transfusion
Patients with blood difficult to match for transfusion			
Rh-negative blood type	56	3	0
Positive irregular antibodies	30	0	0
Patients at high risk for massive bleeding at delivery			
Total placenta previa	66	28	4
Complicated with leiomyoma	62	9	0
Low-lying placenta	61	12	1
Previous cesarean section or past history of uterine surgery	12	1	0
Twin pregnancy	8	0	0
History of massive bleeding during previous delivery	21	3	0
	314	56 (17.8%)	5 (1.6%)

Table 4 Five cases that required transfusion of homologous and autologous blood

Diagnosis	Blood loss (mL)	Autologous blood transfusion (mL)	Homologous blood transfusion (red cells) (units†)	Homologous blood transfusion (fresh frozen plasma) (units†)
Placenta previa	5100	400	8	0
Placenta previa	2732	400	2	0
Placenta previa	4500	1200	9	6
Placenta previa	4340	1200	5	0
Low-lying placenta	3800	1200	5	5

†One unit of blood product originates from 200 mL of whole blood.

placenta also received homologous blood. None of the transfused patients experienced side-effects from bacterial contamination in the donated blood.

Table 4 shows data of 5 patients who were transfused with homologous and autologous blood. Four patients had placenta previa and 1 had a low-lying placenta. Blood loss for each patient was 5100, 2732, 4500, 3800 and 4340 (mL), respectively. The 2 patients with 4500 and 3800 mL blood loss and placenta previa were transfused fresh frozen plasma (FFP) with red blood cells.

Discussion

Some reports have pointed out that the volume of blood donated during pregnancy is insufficient for transfusion during obstetric hemorrhage because most pregnant women are anemic. However, in this study, 285 of the 314 patients could donate at least twice (800 mL). Patients at low risk for obstetric hemorrhage were scheduled to donate 400 or 800 mL, and high-risk patients were scheduled to donate at least 1200 mL. Almost all patients were able to donate as scheduled.

The incidence of vasovagal reflex as an adverse donor reaction is reported to be about 1%.[13,14] The incidence of VVR for donations from pregnant women in this study (1.4%) was consistent with these reports. In addition, the 10 patients who suffered from VVR all had mild symptoms and easily recovered by stopping donation and undergoing bed rest. In other words, pregnant patients were not at increased risk for VVR, and if it happened, the symptoms could be managed.

In the present study, FHR monitoring was performed for all patients and no abnormal findings were observed during donations. Previous reports have shown similar findings.[7–11] In the case of maternal VVR, FHR monitoring showed a reassuring fetal status. The 400 mL of blood donated by the mother scarcely influenced feto-placental circulation. The infants were all in good condition when examined 1 month after birth. The long-term cohort studies seem to increase the reassurance of autologous blood donation during pregnancy.

There was a significant difference between transfused and non-transfused patients because we decided that the indication for re-transfusion of autologous blood was the same as for homologous blood. Given the risk of side-effects, such as bacterial contamination in the autologous blood, we believed that

re-transfusion of autologous blood should not be performed when vital signs were stable.

Re-transfusion of autologous blood was performed for 42.4% of patients with placenta previa, 19.7% with low-lying placenta, 14.5% with leiomyoma, 14.3% with a past history of massive bleeding during a previous delivery, 5.4% with an Rh negative blood type, and 8.3% with previous cesarean section. In the present study, we did not assess the cost-benefit analysis of autologous blood donation during pregnancy. However, as reported previously,[15] patients with placenta previa seem to be the best candidates for autologous blood donation during pregnancy because of the high frequency of re-transfusion. Other indications are accompanied by the problem of wasted blood. If autologous blood had not been prepared, banked blood suitable for high-risk patients would generally be stocked for deliveries. In these situations, homologous blood products as a social resource would have been wasted. Reducing the demand on homologous blood supply is very important because the number of blood donors in Japan has been decreasing (Ministry of Health, Labor and Welfare, Japan 2009). It is also important to note that there was the benefit of being prepared for emergent massive bleeding.

In the present study, we were able to manage cases requiring transfusion using only autologous blood for almost all patients with placenta previa (24/28 patients). Although the UK guidelines[1] point out that autologous blood is insufficient in cases of massive blood loss during delivery, our study showed that donated blood could generally provide adequate transfusion in cases of placenta previa. We speculate that the observed differences between patients may be due to autologous blood containing various levels of clotting factors and fibrinogen.[16] In addition, a recent study[17] reported that the transfusion of whole blood might decrease adverse side-effects of transfusion for obstetric hemorrhage. As autologous blood transfusion uses whole blood, fewer side-effects are expected.

In conclusion, autologous blood donation is feasible and safe for pregnant Japanese women and their infants. Although indications of autologous blood donation are controversial, it should be considered in cases of placenta previa. For other indications, the circumstances of blood supply at the institution should be considered.

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ORIGINAL

GATA3 abnormalities in six patients with HDR syndrome

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Abstract. *GATA3* mutations cause HDR (*h*ypoparathyroidism, sensorineural *d*eafness, and *r*enal dysplasia) syndrome and, consistent with the presence of the second DiGeorge syndrome locus (*DGS2*) proximal to *GATA3*, distal 10p deletions often leads to HDR and DiGeorge syndromes. Here, we report on six Japanese patients with *GATA3* abnormalities. Cases 1–5 had a normal karyotype, and case 6 had a 46,XX,del(10)(p15) karyotype. Cases 1–6 had two or three of the HDR triad features. Case 6 had no DiGeorge syndrome phenotype except for hypoparathyroidism common to HDR and DiGeorge syndromes. Mutation analysis showed heterozygous *GATA3* mutations in cases 1–5, i.e., c.404–405insC (p.P135fsX303) in case 1, c.700T>C & c.708–709insC (p.F234L & p.S237fsX303) on the same allele in case 2, c.737-738insG (p.G246fsX303) in case 3, c.824G>T (p.W275L) in case 4, and IVS5+1G>C (splice error) in case 5. Deletion analysis of chromosome 10p revealed loss of *GATA3* and preservation of *D10S547* in case 6. The results are consistent with the previous finding that *GATA3* mutations are usually identified in patients with two or three of the HDR triad features, and provide supportive data for the mapping of *DGS2* in the region proximal to *D10S547*.

Key words: HDR syndrome, *GATA3*, DiGeorge syndrome, *DGS2*, Phenotypic spectrum

HDR (*h*ypoparathyroidism, sensorineural *d*eafness, and *r*enal dysplasia) syndrome is an autosomal dominant disorder first reported by Bilous *et al.* [1]. This condition is primarily caused by haploinsufficiency of *GATA3* on chromosome 10p15, although *GATA3* mutations have not been identified in a small portion of patients with clinical features compatible with HDR syndrome [2, 3]. *GATA3* consists of six exons, and encodes a transcription factor with two transactivating domains and two zinc finger domains on exons 2–6 [2]. *GATA3* is expressed in the developing parathyroid glands, inner ears, and kidneys, together with thymus and central nervous system (CNS) [4, 5].

Distal 10p deletions involving *GATA3* often lead to DiGeorge syndrome associated with hypoplastic thymus, T-cell immunodeficiency, hypoparathyroidism, congenital cardiac defects, and facial dysmorphism, in addition to HDR syndrome [6, 7]. Thus, deletion mappings have been performed, localizing the second DiGeorge syndrome locus (*DGS2*) to a ~1 cM region proximal to *D10S547* (the locus order: 10pter–*GATA3*–*D10S547*–*DGS2*–10cen) [6, 7].

Here, we report clinical and molecular findings in five patients with intragenic *GATA3* mutations and one patient with distal 10p deletion involving *GATA3*, and discuss the clinical features in *GATA3* mutation posi-

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Table 1 Summary of six patients with *GATA3* mutation or deletion

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Present age	40 years	39 years	4 years	31 years	17 years	4 years
Sex	Female	Female	Male	Female	Male	Female
Karyotype	46,XX	46,XX	46,XY	46,XX	46,XY	46,XX,del(10)(p15)
Hypoparathyroidism	Yes	Yes	Yes	Yes	Yes	Yes
Symptom	Convulsion	Tetany	No[b]	Convulsion	Convulsion	Convulsion
Ca (mg/dL)	3.4	3.4	2.7	4.3	3.0	4.7
P (mg/dL)	8.0	7.9	8.1	7.9	8.7	8.6
Intact PTH (pg/mL)	Undetected	Undetected	14	Undetected	Undetected	15
Age at diagnosis	10 years	13 years	17 months	3 years	17 months	2 weeks
Sensorineural deafness	Yes	Yes	No	Yes	Yes	Yes
Hearing level (dB)[a]	50 (B)	>70 (B)	Normal	60 (B)	50 (B)	90 (B)
Age at diagnosis	13 years	6 years		11 years	12 months	6 months
Renal lesion	Yes	Yes	Yes	Equivocal[c]	Yes	Yes
Malformation	RH (L)	PCD (B)	PD (R)	Absent	RH (L)	VUR (B)
Age at diagnosis	9 years	27 years	17 months		17 months	2 months

Abbreviations: PTH, parathyroid hormone; dB, decibel; B, bilateral; L, left; R, right; RH, renal hypoplasia; PCD, pelvicalyceal deformity; PD, pelvic duplication; and VUR, vesicoureteral reflux.
[a] Degree of hearing loss: normal, <25 dB; mild 26–40 dB; moderate 41–55 dB; moderately severe, 56–70 dB; and profound, >90 dB.
[b] Hypocalcemia was revealed by routine biochemical studies, when this boy was admitted because of bronchopneumonia.
[c] Renal malformation was absent, but renal dysfunction with increased serum creatinine was noticed during pregnancy.
Normal reference data: Ca: 8.84–10.44 mg/dL; P: 4.5–6.5 mg/dL; and intact PTH: 10–65 pg/mL.

tive patients and the chromosomal location of *DGS2*.

Patients and Methods

Patients

We studied six hitherto unreported Japanese patients (cases 1–6) with two or three HDR triad features. Cases 1–5 had a normal karyotype, and case 6 had a 46,XX,del(10)(p15) karyotype. Cases 1–4 and 6 were apparently sporadic cases, whereas case 5 was a possible familial case: the father received renal dialysis due to chronic renal failure from his twenties, and the paternal grandmother had unilateral renal hypoplasia, although they lacked clinical features suggestive of hypoparathyroidism and hearing difficulty.

Clinical phenotypes of the HDR triad features are summarized in Table 1. Hypoparathyroidism was noticed by convulsion in cases 1 and 4–6 and by tetany in case 2; in case 3, it was incidentally found by biochemical examinations at the time of admission due to bronchopneumonia. After confirming parathyroid hormone deficiency, 1α(OH) vitamin D therapy was started, successfully normalizing serum calcium and phosphate values in cases 1–6. Sensorineural deafness was demonstrated in cases 1, 2, and 4–6 by auditory brainstem response or audiometry, and they required hearing aids in their daily life. Case 3 had no hearing difficulty with normal auditory brainstem response. Renal lesion was radiologically confirmed in cases 1–3, 5, and 6. Although case 4 had no discernible renal malformation, she manifested renal dysfunction during pregnancy. In addition, case 6 exhibited developmental delay but lacked hypoplastic thymus, T-cell immunodeficiency, congenital cardiac defects, and facial dysmorphism characteristic of DiGeorge syndrome.

Mutation analysis of GATA3

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. After obtaining informed consent, leukocyte genomic DNA samples of cases 1–6 were amplified by PCR for the coding regions on exons 2–6 and their flanking splice sites, and the PCR products were subjected to direct sequencing from both directions on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). The primer sequences and the PCR conditions were as described previously [2, 3]. To confirm a heterozygous mutation, the corresponding PCR products were subcloned with a TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA), and normal and mutant alleles were sequenced separately.

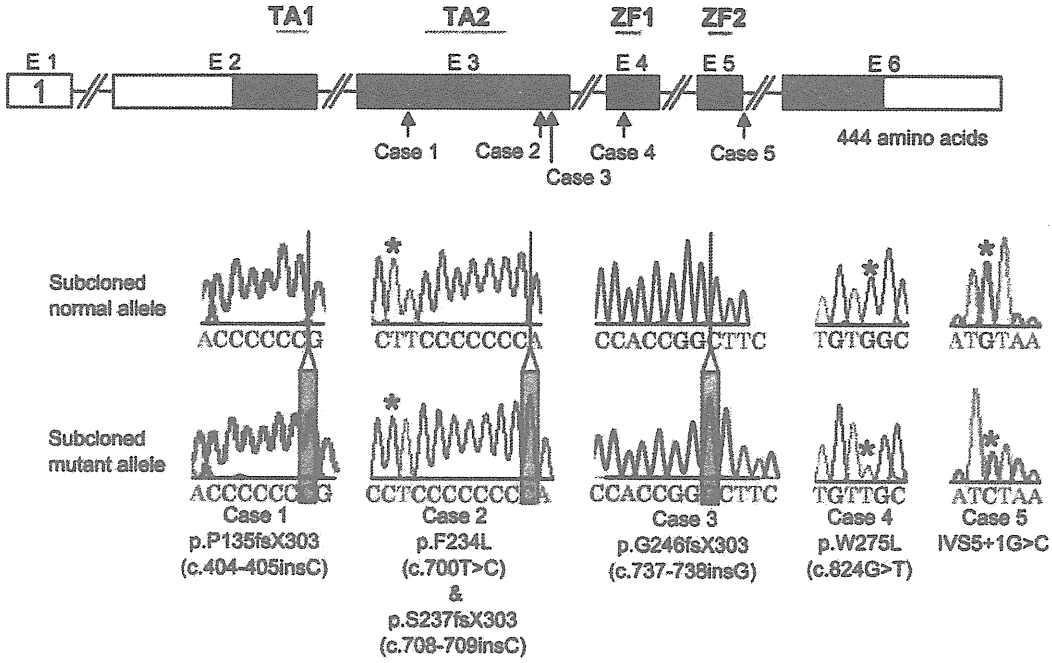

Fig. 1 Mutation analysis of *GATA3*.
Upper part: The structure of *GATA3* and the position of the mutations identified in cases 1–5. *GATA3* consists of exons 1–6 (E1–E6) and encodes two transactivating domains (TA1 and TA2) and two zinc finger domains (ZF1 and ZF2). The black and white boxes denote the coding regions and the untranslated regions, respectively.
Lower part: Electrochromatograms showing the subcloned normal and mutant sequences in cases 1–5.

Deletion analysis of 10p

To indicate an extent of the 10p deletion in case 6, oligoarray comparative genomic hybridization (CGH) was carried out with 1x244K Human Genome Array (catalog No. G4411B) (Agilent Technologies, Palo Alto, CA), according to the manufacturer's protocol. Furthermore, fluorescence *in situ* hybridization (FISH) was performed with an RP11-554F11 BAC probe containing the whole *GATA3* gene [3] and an RP11-17E09 BAC probe containing *D10S547* (BACPAC Resources Center, Oakland, CA), together with a CEP 10 probe for *D10Z1* (Abbott, Chicago, IL) utilized as an internal control. The two BAC probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the control probe was detected according to the manufacturer's protocol.

Results

Mutation analysis of GATA3

Direct sequencing identified heterozygous *GATA3* mutations in cases 1–5, i.e., a frameshift mutation (c.404–405insC, p.P135fsX303) in case 1, a missense mutation (c.700T>C, p.F234L) and a frameshift mutation (c.708–709insC, p.S237fsX303) on the same allele in case 2, a frameshift mutation (c.737-738insG, p.G246fsX303) in case 3, a missense mutation (c.824G>T, p.W275L) in case 4, and a splice donor site mutation (IVS5+1G>C) in case 5 (Fig. 1). Unfortunately, the renal phenotype positive father and paternal grandmother of case 5 were not examined. These mutations were absent from 200 control subjects. No intragenic mutation was identified in case 6 with distal 10p deletion.

Deletion analysis of 10p

CGH revealed a ~10 Mb terminal deletion from chromosome 10p of case 6 (Fig. 2). FISH analysis showed that the 10p deletion chromosome was missing *GATA3* and retained *D10S547*.

Discussion

Cases 1–6 had two or three of the HDR triad features and heterozygous *GATA3* abnormalities. This is consistent with the previous notion that *GATA3* mutations

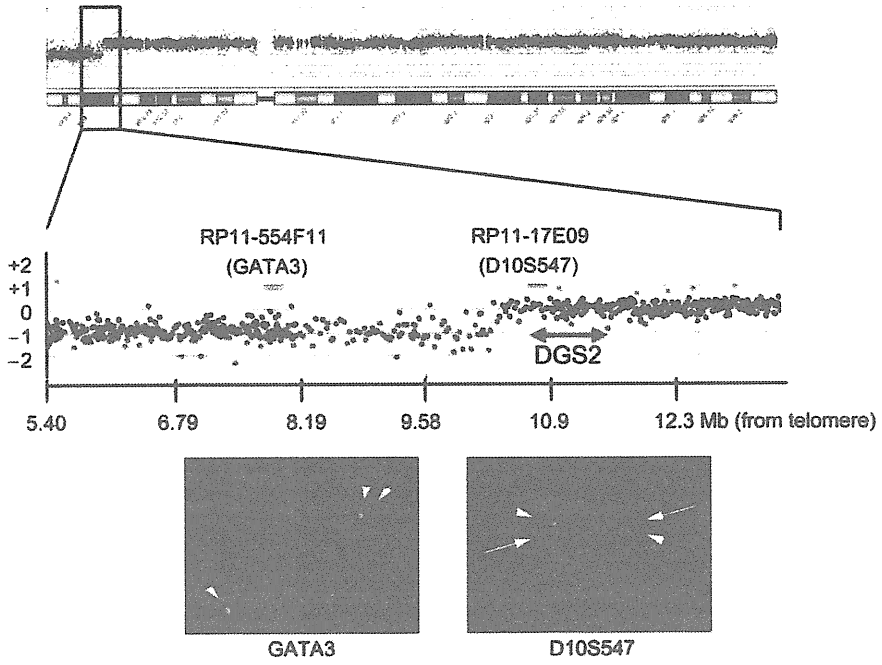

Fig. 2 Deletion analysis of 10p. The green and black signals in CGH indicate the deleted and preserved regions on the 10p deleted chromosome, respectively. The critical region for *DGS2* is indicated. The RP11-554F11 probe containing *GATA3* detects only a single signal (an arrow), whereas the RP11-17E09 probe containing *D10S547* identifies two signals (arrows). The arrowheads indicate *D10Z1* detected by a control CEP 10 probe.

are usually identified in patients with two or three of the HDR triad features [8, 9]. However, this would more or less be due to an ascertainment bias that *GATA3* are usually examined in patients diagnosed as having HDR syndrome. Indeed, familial studies of probands with typical HDR syndrome have identified *GATA3* mutations in subjects with apparently deafness only phenotype [3, 10], although there has been no report documenting apparently normal phenotype in individuals with *GATA3* mutations. It is possible, therefore, that *GATA3* mutations are associated with a relatively wide penetrance and expressivity of the HDR triad features. In this context, it is notable that the father and the paternal grandmother of case 5 had renal abnormalities as the sole discernible clinical phenotype. This suggests that *GATA3* mutations may cause renal abnormalities alone in exceptional patients, although mutations analysis could not be performed for the father and the grandmother.

Case 6 lacked T-cell immunodeficiency, congenital cardiac defects, and abnormal facial appearance characteristic of DiGeorge syndrome. While case 6 had hypoparathyroidism, this is explained by loss of *GATA3*. In addition, developmental delay is ascribed to chromosome aberration. Thus, genotype-phenotype correlation in case 6 is consistent with the previous mapping of *DGS2* to a region proximal to *D10S547* [6, 7].

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Genome-Wide Association Study Identifies HLA-DP as a Susceptibility Gene for Pediatric Asthma in Asian Populations

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Abstract

Asthma is a complex phenotype influenced by genetic and environmental factors. We conducted a genome-wide association study (GWAS) with 938 Japanese pediatric asthma patients and 2,376 controls. Single-nucleotide polymorphisms (SNPs) showing strong associations ($P<1\times10^{-8}$) in GWAS were further genotyped in an independent Japanese samples (818 cases and 1,032 controls) and in Korean samples (835 cases and 421 controls). SNP rs987870, located between *HLA-DPA1* and *HLA-DPB1*, was consistently associated with pediatric asthma in 3 independent populations ($P_{combined}=2.3\times10^{-10}$, odds ratio [OR] = 1.40). *HLA-DP* allele analysis showed that *DPA1*0201* and *DPB1*0901*, which were in strong linkage disequilibrium, were strongly associated with pediatric asthma (*DPA1*0201*: $P=5.5\times10^{-10}$, OR = 1.52, and *DPB1**0901: $P=2.0\times10^{-7}$, OR = 1.49). Our findings show that genetic variants in the *HLA-DP* locus are associated with the risk of pediatric asthma in Asian populations.

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Introduction

Asthma is the most common chronic disorder in children, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. The prevalence of childhood asthma in Japan is 5.0% among school children in 2006 [1], and an estimated 300 million people worldwide have asthma [2]. Asthma is characterized by airway hyperresponsiveness and inflammation, tissue remodeling, and airflow obstruction. Infiltration of lymphocytes, mast cells, and eosinophils in the airways cause airway inflammation, and T helper (Th) type 2 cytokines play crucial roles in orchestrating the inflammatory responses; thus, asthma is considered a Th2-type immune disease.

Previously conducted genome-wide association studies (GWAS) for asthma identified association with the loci on chromosomes 17q21 (*ORMDL3* for Caucasian pediatric asthma, odds ratio (OR) = 1.45, $P=1\times10^{-10}$) [3], 5q21 (*PDE4D* for pediatric asthma, OR = 0.6, $P=4.7\times10^{-7}$) [4], 9q21.31 (*TLE4* for Hispanic pediatric asthma, OR = 0.6, $P=6.8\times10^{-7}$) [5], and 1q31 (*DENND1B* for Europeans and African ancestries [6], OR = 0.77 and 1.41, respectively; combined $P=1.7\times10^{-13}$). A GWAS for severe asthma identified association with the region between

Author Summary

Asthma is the most common chronic disorder in children, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. Here, taking advantage of recent technological advances in human genetics, we performed a genome-wide association study and follow-up validation studies to identify genetic variants for asthma. By examining 6,428 Asians, we found rs987870 and *HLA-DPA1*0201/DPB1*0901* were associated with pediatric asthma. The association signal was stretched in the region of *HLA-DPB2*, collagen, type XI, alpha 2 (*COL11A2*), and Retinoid X receptor beta (*RXRB*), but strong linkage disequilibrium in this region made it difficult to specifically identify causative variants. Interestingly, the SNP (or the HLA-DP allele) associated with pediatric asthma (Th-2 type immune diseases) in the present study confers protection against Th-1 type immune diseases, such as type 1 diabetes and rheumatoid arthritis. Therefore, the association results obtained in the present study could partially explain the inverse relationship between asthma and Th-1 type immune diseases and may lead to better understanding of Th-1/Th-2 immune diseases.

RAD50 and *IL5* on chromosome 5q (OR = 1.64, $P = 3.0 \times 10^{-7}$) and *HLA-DR/DQ* (OR = 0.68, $P = 9.6 \times 10^{-6}$), but they did not include a replication dataset [7]. Recently, Moffatt *et al.* conducted a large-scale GWAS in Caucasian populations and identified 6 loci (*IL18R1*, *HLA-DQ*, *IL33*, *SMAD3*, *GSDMB/GSDMA*, and *IL2RB*) associated with asthma [8].

In the present study, we conducted the first GWAS in Asian population for pediatric asthma by using Illumina Human-Hap550/610-Quad BeadChip (Illumina, San Diego, USA).

Results

GWAS analysis

The GWAS flow chart is shown in Figure 1. We analyzed 450,326 SNPs in 938 cases and 2,376 controls, using standard quality control practices (Table S1). The genotypes in cases and controls were compared using the Cochran–Armitage trend test (Figure 2). There was only minor inflation of the genome-wide statistical results owing to population stratification (genomic control $\lambda_{GC} = 1.048$; Figure 3). Five SNPs (rs3019885, rs987870, rs2281389, rs2064478, and rs3117230) showed strong association with pediatric asthma with $P < 1 \times 10^{-8}$. Of these, rs2064478 and rs3117230 were in complete linkage disequilibrium (LD) ($r^2 = 1$) with rs2281389. In order to validate the results of the GWAS, we tested the remaining 3 SNPs (rs3019885, rs987870, and rs2281389) in 2 independent replication cohorts comprising Asians (Japanese and Koreans), considering $P < 0.05$ as significant replication.

Of these 3 SNPs, significant associations were noted at rs987870 in both cohorts (Table 1). To merge the findings of these studies, we conducted meta-analysis with a fixed-effects model by using the Mantel–Haenszel method. As shown in Table 1, the Mantel–Haenszel *P* value of 2.3×10^{-10} was noted for rs987870 (OR = 1.40, confidence interval (CI) = 1.26–1.55).

HLA-DP association with pediatric asthma

The rs987870 is located between *HLA-DPA1* and *HLA-DPB1*. Genotype imputation using MACH [9] revealed association between asthma and the SNPs that were in strong LD with rs987870 (Figure 4, Table S2). Moreover, rs987870 C allele was in complete LD with *DPA1*0201* ($r^2 = 1$). We determined *HLA-DPA1* genotypes by using direct sequencing and MACH imputation of the data from 1135 cases and 2376 controls and found that *DPA1*0201* was strongly associated with pediatric asthma ($P = 5.2 \times 10^{-10}$, OR = 1.52, Table 2). Then, we determined the *HLA-DPB1* genotypes in 1135 cases and 2296 controls and found that *DPB1*0901* was associated with pediatric asthma ($P = 2.0 \times 10^{-7}$, OR = 1.49, Table 3). *DPB1*0901* was in strong LD with *DPA1*0201* and rs987870 C allele (*D* prime = 0.93). Because more than 90% of pediatric asthma patients were allergic to house dust mites, it is possible that the association was due to IgE reactivity (sensitization) against mites. We performed an association study for mite sensitization using independent adult subjects without allergic respiratory diseases such as asthma and perennial allergic rhinitis (367 subjects with house dust mite-specific IgE and 1633 subjects without mite-specific IgE). Subjects with house dust mite-specific IgE were non-allergic in terms of symptoms but possessed mite-specific IgE. Subjects without mite-specific IgE did not exhibit allergic symptoms. We did not find an association between rs987870 and mite sensitization ($P = 0.54$, OR = 1.07, Table S3).

Discussion

Our GWAS in Asian populations found HLA-DP as susceptibility gene for pediatric asthma. Majority of pediatric asthmas are atopic (i.e., familial tendency to produce IgE antibodies against common environmental allergens) and possess specific IgE against the house dust mite. Mite sensitization is more prevalent in Asia than in Europe and is observed in 39% of the general adult population in Japan [10]. High prevalence of mite sensitization in asthmatic children has also been reported in Taiwan, where 94.2% of children with asthma are sensitized against *Dermatophagoides pteronyssinus* [11]. However, only a small subset of subjects with house dust allergy develop asthma [12].

We performed an independent association study for mite sensitization in adult subjects without allergic respiratory diseases and did not find an association between rs987870 and mite sensitization without symptoms. If the relative risk for mite sensitization in the individuals carrying a putative risk allele was 1.4 and the allele frequency was 0.15 compared to that in individuals without the allele, the statistical power of the sample size for mite sensitization study was 0.92 at an alpha level of 0.05. These results suggested that *DPA1*0201* and *DPB1*0901* may be associated with asthma rather than IgE production against house dust mite.

The association signal was stretched in the region of *HLA-DPB2*, collagen, type XI, alpha 2 (*COL11A2*), and Retinoid X receptor beta (*RXRB*) (Figure 4). Because of LD in this region, it is difficult to specifically identify causative variants. *HLA-DPB2* is a pseudogene. *COL11A2* encodes a component of type XI collagen called the pro-alpha2(XI) chain. Mutations in *COL11A2* have been associated with non-syndromic deafness, otospondylomegaepiphyseal dysplasia, Weissenbacher-Zweymüller syndrome, and Stickler syndrome (OMIM ID *120290). RXRB belongs to the RXR family and is involved in mediating the effects of retinoic acid. RXRB forms a heterodimer with the retinoic acid receptor and thus preferentially increases its DNA binding and transcriptional activity at promoters containing retinoic acid [13]. All SNPs showing strong association with asthma ($P < 1 \times 10^{-10}$) were located in introns or intergenic regions. LD of these associated SNPs with rs987870 was not strong; therefore, it is likely that the functional effect is due to *DPA1*0201* and *DPB1*0901*.

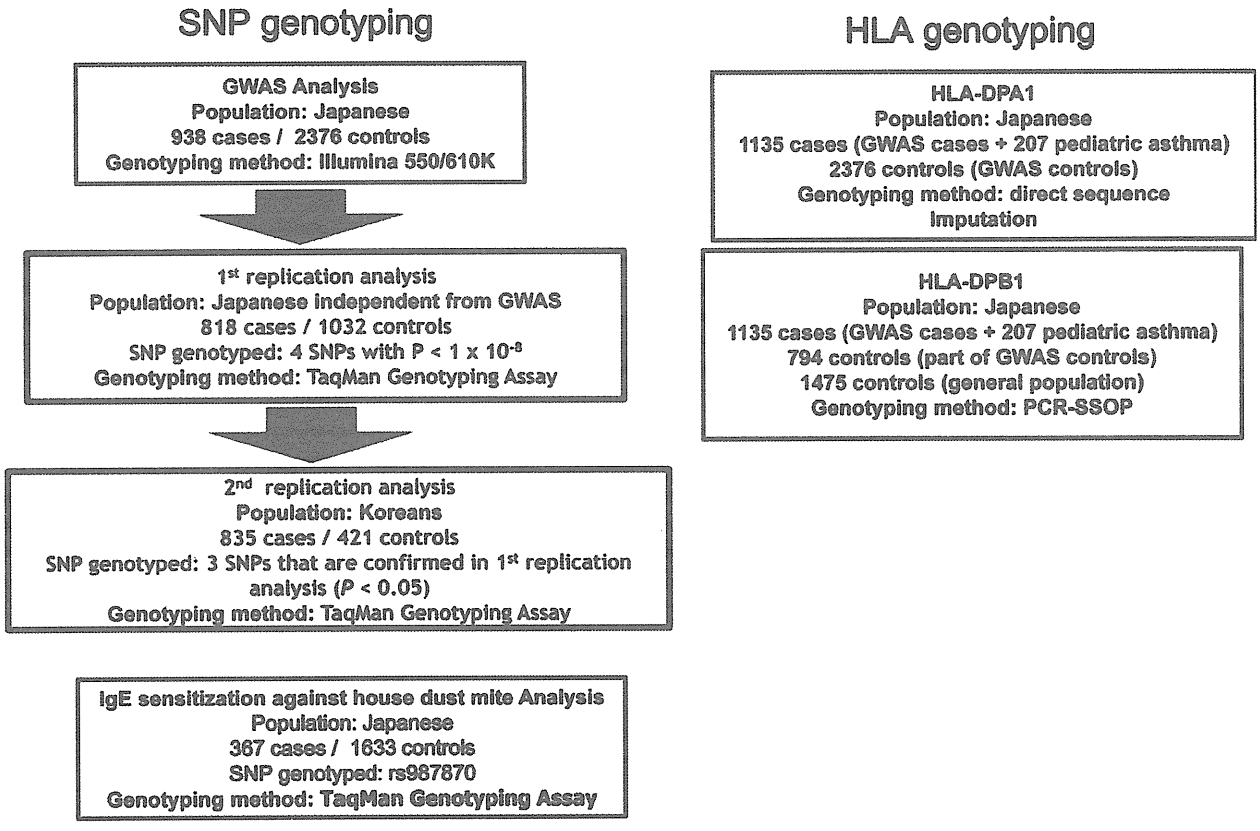

Figure 1. Flow chart of the present study.
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In HLA-DP, Caraballo *et al.* reported that *DPB1*0401* is significantly decreased in patients with allergic asthma in Mulatto population (an admixture population of European and African ancestries) [14]. Apart from the study of Caraballo *et al.*, the association between *HLA-DP* alleles and asthma was restricted to occupational [15] or aspirin-induced asthma [16]. Howell *et al.* reported associations between HLA-DR genotype and asthma and between *HLA-DPA1*0201* and IgE specific to grass pollen mix and the pollen allergen Phl p 5 [17]. Grass pollen allergy is not a major cause of asthma in Japan [18]; therefore, the *HLA-DPA1*0201* association in the present study was less likely to be due to sensitization to grass pollen.

*DPA1*0201* has also been reported to be positively associated with lower levels of rubella-induced antibodies [19], cytokine immune responses against measles vaccine [20], and ulcerative colitis [21], and negatively associated with type 1 diabetes [22].

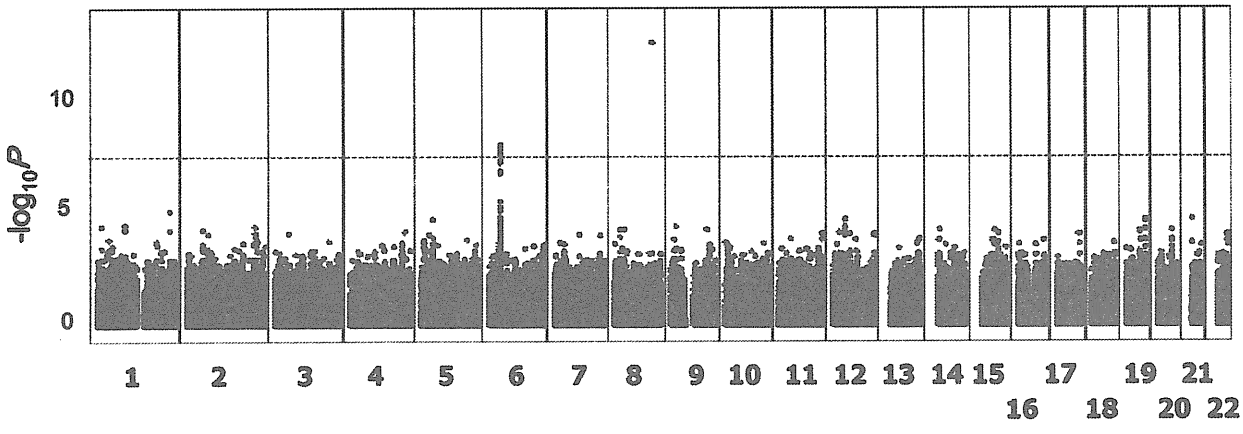

Figure 2. P values of GWAS. The Manhattan plot shows the Cochran–Armitage trend test *P* values for 938 cases of asthma and 2,376 controls; 450,326 autosomal SNPs were considered in the study. The dashed line indicates the genome-wide significance level ($P<5\times10^{-8}$).
doi:10.1371/journal.pgen.1002170.g002

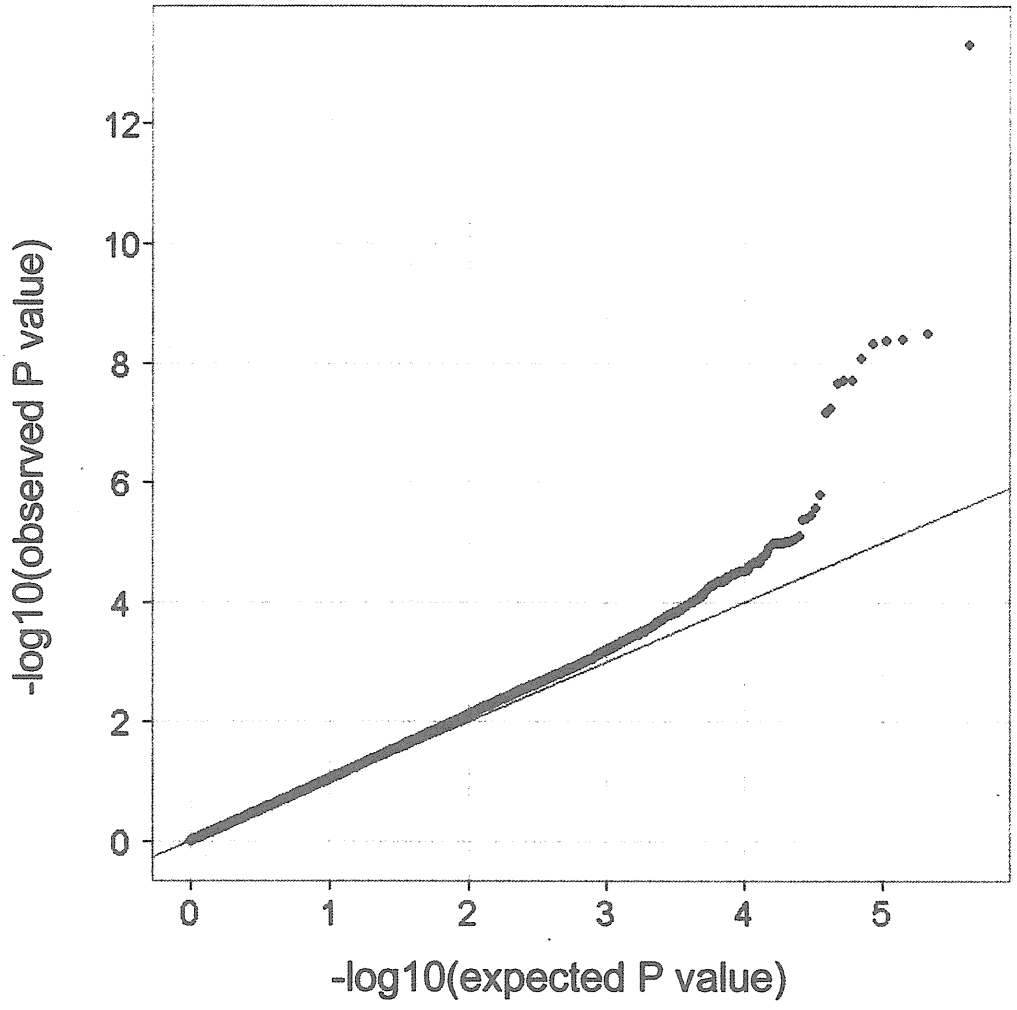

Figure 3. Quantile–quantile (Q–Q) plot of GWAS for pediatric asthma. The results of the Cochrane–Armitage trend *P* are plotted as dots and the line y = x is in red. The horizontal and vertical lines represent expected *P* values under null distribution and observed *P* values, respectively.
doi:10.1371/journal.pgen.1002170.g003

*DPB1*0901* was shown to be associated with systemic sclerosis [23], non-permissive mismatches for hematologic stem cell transplantation [24], ulcerative colitis [21], and Takayasu's arteritis [25]. *HLA-DP* molecules present short peptides of largely exogenous origin to CD4-positive helper T cells and other T cells, leading to subsequent immunological responses. T cells recognize complex formation between a specific HLA type and a particular antigen-derived epitope. Therefore, HLA molecules capable of binding a particular epitope can restrict T cell induced-immune responses, leading to association between particular HLA types and immune-related diseases. Type 1 diabetes is a Th-1 type immune disease. Varney *et al.* studied 1,771 type 1 diabetes multiplex families, analyzing them by the affected family-based control method [26], and found that *DPA1*0201* has a protective effect on the development of type 1 diabetes (adjusted $P = 5 \times 10^{-4}$, OR 0.7) [22]. Epidemiologic studies have associated type 1 diabetes with lower prevalence of asthma and other allergic diseases [26,27]. Also, the previous GWAS of rheumatoid arthritis, other Th-1 type immune disease, has shown that rs987870 C allele confers protection against rheumatoid arthritis [28]. These findings suggest that *HLA-DPA1*0201* could determine Th1/Th2 dominance and could partially explain the inverse relationship between asthma and Th-1 type immune diseases.

Previous GWAS involving European, Mexican, and African populations showed association of asthma with SNPs located in several newly discovered genes. Our GWAS dataset supported an association between identical SNPs reported in *ORMDL3/GSDMB/GSDMA*, *IL5/RAD50/IL13*, *HLA-DR/DQ*, and *SMAD3* and pediatric asthma ($P<0.05$, Table S4). Two asthma GWA studies revealed an association of HLA-DQ with pediatric/adult asthma in Caucasians [7,8]. HLA-DQ, like HLA-DP, is an αβ heterodimer of the MHC Class II type. Like HLA-DP, HLA-DQ recognizes and presents foreign antigens, but is also involved in recognizing common self-antigens and presenting those antigens to the immune system.

We failed to replicate the top SNPs of *PDE4D*, *TLE4*, *DENND1B*, *IL18R1*, and *IL2RB* that were reported in the original articles, but several SNPs in the regions surrounding *PDE4D* and *IL2RB* showed significant association when we set the significance level at $P = 0.05$ (Table S4). The different LD patterns/allele frequencies observed in *PDE4D* and *IL2RB* in Asians and Caucasians may explain the different SNP associations observed

Table 1. Results of GWAS and replication studies for 4 SNPs.

SNP	Nearest Gene	Allele[a]	Samples	MAF (asthma)	MAF (control)	OR (95%CI)[b]	P[c]	P[e]
rs3019885	SLC30A8	T/G	GWAS	0.41	0.31	1.55(1.39–1.73)	1.3×10^{-14}	
			First replication (Japanese)	0.34	0.30	1.21(1.05–1.39)	8.7×10^{-3}	
			Second replication (Koreans)	0.27	0.26	1.075(0.88–1.31)	4.7×10^{-1}	
			Meta analysis (HM)[d]			1.34(1.24–1.45)	5.0×10^{-13}	0.0011
rs987870	HLA-DPB1	T/C	GWAS	0.19	0.14	1.51(1.31–1.74)	7.5×10^{-9}	
			First replication (Japanese)	0.17	0.14	1.26(1.05–1.50)	1.2×10^{-2}	
			Second replication (Koreans)	0.12	0.10	1.34(1.01–1.76)	4.1×10^{-2}	
			Meta analysis (HM)[d]			1.40(1.26–1.55)	2.3×10^{-10}	0.33
rs2281389	HLA-DPB1	T/C	GWAS	0.23	0.17	1.47(1.29–1.68)	8.5×10^{-9}	
			First replication (Japanese)	0.20	0.17	1.20(1.02–1.42)	2.9×10^{-2}	
			Second replication (Koreans)	0.08	0.08	1.085(0.80–1.48)	6.1×10^{-1}	
			Meta analysis (HM)[d]			1.33(1.20–1.47)	1.4×10^{-8}	0.076

[a]The former allele represents the major allele.
[b]Odds ratio and 95% confidence interval (CI) of minor allele.
[c]*P* values of allelic model.
[d]Meta-analysis using Mantel-Haenszel approach.
[e]*P* values for heterogeneity test.
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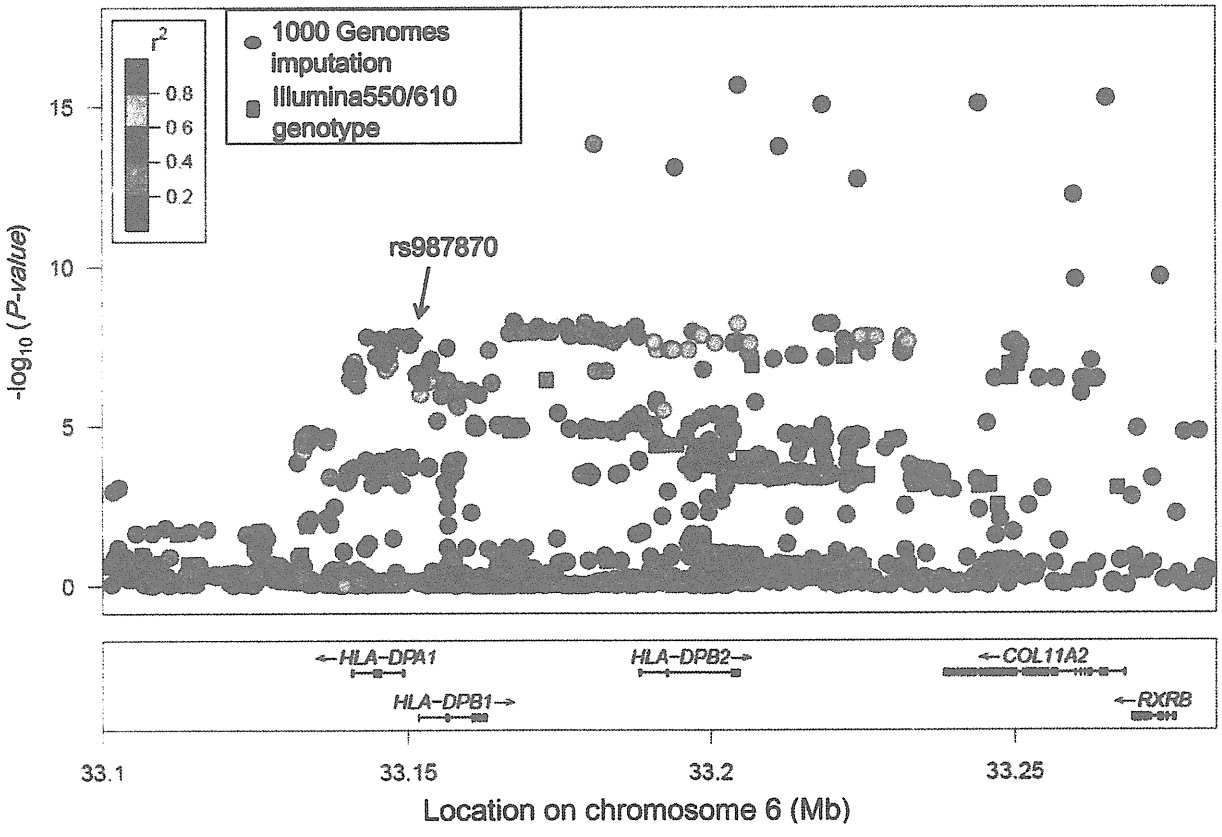

Figure 4. Association findings of genotyped (squares) and imputed (circles) SNPs in the HLA-DP region. SNP rs987870, which consistently showed an association with pediatric asthma in 3 independent populations, is located in the LD block between *HLA-DPA1* and *HLA-DPB1*. The color intensity of each symbol reflects the extent of LD with rs987870: from red ($r^2>0.8$) to blue ($r^2<0.2$). The physical positions are based on NCBI build 36 of the human genome.
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Table 2. HLA-DPA1-rs987870 Haplotype analysis of pediatric asthma.

DPA1	rs987870	asthma	control	Odds ratio (95%CI)	P values
*DPA1*0103*	T	858(38%)	1866(39%)	0.98 (0.85–1.05)	0.29
*DPA1*0201*	C	439 (19%)	650 (14%)	1.52 (1.33–1.74)	5.5×10^{-10}
*DPA1*0202*	T	957(42%)	2223(47%)	0.83 (0.75–0.92)	0.00046
*DPA1*0401*	T	3 (0.1%)	5 (0.1%)	1.26 (0.30–5.28)	0.75

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in different ethnic populations. rs1342326 in *IL33* was not polymorphic in the Asian population.

There were several limitations of the present GWAS. The controls for the GWAS and 1st replication samples were from adult populations. Information regarding history of asthma in early childhood or other asthma-related information (i.e., status of allergic sensitization and lung function) was not collected for these controls. Therefore, we cannot exclude the possibility that our control samples may include subjects who outgrew asthma. The prevalence of pediatric asthma in Japan is around 5%: therefore, our GWAS samples have reduced power compared with that of selected controls. In the 1st replication Japanese controls, subjects with present and past history of allergic diseases were excluded, and Korean controls in the 2nd replication were non-allergic pediatric controls (Table S5).

The genomic control value in the present study was 1.053, indicating minor population stratification. The Japanese population comprises 2 clusters (Hondo and Ryukyu; Hondo is the mainland of Japan and Ryukyu is the name of the island south of Japan). We performed principal component analysis using EIGENSTRAT software [29] to identify subjects belonging to Ryukyu. Because 2nd or 3rd generation Chinese live in Japan, and the genetic population structure in Chinese differs from that in Japanese, we also performed principal component analysis to exclude Chinese subjects. Although hidden population stratification may exist, its influence on the final results is not expected to be significant.

rs3019885 is located in intron 2 of solute carrier family 30 (SLC30A8), and showed strong association in the GWAS population. The association was replicated in the independent Japanese samples, but not in the Korean population. SLC30A8 is a zinc efflux transporter expressed at high levels only in the pancreas; the GWAS revealed that variants of *SLC30A8* are associated with type 2 diabetes [30]. Japanese and Koreans are genetically close but we cannot exclude the possibility that the association of rs3019885 with pediatric asthma is population specific.

In conclusion, we performed the first GWAS in Asian population for pediatric asthma and found that *DPA*0201/DPB1*0901* is strongly associated with pediatric asthma. The association with the HLA-DP locus emphasizes the importance of the HLA-class II molecules on the biological pathways involved in the etiology of pediatric asthma, and suggests that HLA-DP can be a therapeutic target for asthma.

Materials and Methods

Ethical statement

The study was approved by the institutional review board and the ethics committee of each institution. Written informed consent was obtained from each participant in accordance with institutional requirements and the Declaration of Helsinki Principles.

Subject participants

Characteristics of pediatric asthma cases and controls are summarized in Table S5.

GWAS population. All subjects with asthma were child or child-onset (<15 years old) asthmatics in Japan. Patients were recruited from 3 pediatric hospitals and 1 pediatric clinic, and the diagnosis of the asthma in all patients was confirmed by specialists in pediatric allergology on the basis of the criteria of the National Institutes of Health, USA, with minor modifications.

The control cases for the GWAS were healthy Japanese adult subjects from Osaka (n = 964), Tokyo (n = 660), and Ibaraki

Table 3. HLA-DPB1 allele frequency in pediatric asthma and controls.

Allele	Asthma	Control 1	Control 2	Asthma vs Control 1		Asthma vs Control 2		Asthma vs Control 1+2	
	n = 1135	n = 794	n = 1475	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)
DPB1*05:01	34.4%	36.5%	38.0%	0.18	0.91(0.80–1.04)	0.007	0.85(0.76–0.96)	0.013	0.87(0.79–0.97)
DPB1*02:01	22.4%	24.2%	24.3%	0.19	0.90(0.78–1.05)	0.11	0.89(0.79–1.02)	0.09	0.90(0.80–1.02)
DPB1*09:01	14.5%	10.1%	10.3%	5.5×10^{-5}	1.51(1.23–1.84)	3.4×10^{-6}	1.48(1.25–1.75)	2.0×10^{-7}	1.49(1.28–1.74)
DPB1*04:02	10.0%	9.2%	9.6%	0.38	1.10(0.89–1.37)	0.57	1.05(0.88–1.27)	0.43	1.07(0.90–1.27)
DPB1*04:01	4.8%	7.1%	5.0%	0.0019	0.65(0.50–0.86)	0.64	0.94(0.73–1.21)	0.08	0.82(0.65–1.03)
DPB1*03:01	4.9%	4.7%	4.0%	0.76	1.05(0.78–1.41)	0.11	1.24(0.95–1.62)	0.21	1.17(0.92–1.48)
DPB1*02:02	3.7%	3.2%	3.4%	0.46	1.14(0.80–1.63)	0.61	1.08(0.80–1.45)	0.49	1.10(0.84–1.45)
DPB1*13:01	1.6%	1.5%	2.1%	0.85	1.05(0.62–1.77)	0.18	0.75(0.50–1.14)	0.38	0.84(0.57–1.24)
DPB1*14:01	1.8%	1.7%	1.4%	0.88	1.04(0.63–1.70)	0.258	1.28(0.83–2.01)	0.39	1.19(0.80–1.76)

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(n = 778) who had no current history of asthma [31]. In the GWAS, we genotyped 978 cases with pediatric asthma and 2402 controls using Illumina HumanHap550v3/610-Quad Genotyping BeadChip (Illumina, San Diego, USA). Subjects from Osaka and Ibaraki were randomly selected from residents of Suita city and Tone town, respectively. Subjects from Tokyo were hospital workers from Keio University Hospital, Tokyo. We excluded samples considered duplicated, related (first- or second-degree relatives), or belonging to Han Chinese or Ryukyu. In total, 938 cases and 2376 controls were considered for further analysis.

First replication population (Japanese). We recruited 818 subjects with childhood atopic asthma from the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Dokkyo University School of Medicine, National Research Institute for Child Health & Development, National Sagamihara Hospital, and Chiba University Hospital. All subjects with bronchial asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute, National Institutes of Health, 1991) by physicians who were asthma specialists [32,33]. After the exclusion of individuals who had been diagnosed with asthma, atopic dermatitis, or nasal allergies by physicians' interviews, 825 healthy individuals were recruited from the Midousuji Rotary Club [32,33]. Two hundred and seven control subjects who never had the symptoms of allergic rhinitis/asthma and did not show any sensitization to 7 common aeroallergens were recruited from Fukui [34].

Second replication population (Korean). Patients with pediatric asthma were enrolled at Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea. The control subjects were age-matched children with no history of asthma or other allergic diseases, negative skin prick test, and normal total IgE values (<100 IU/mL) recruited from the same district (Seoul). Total of 835 cases and 421 controls participated in this study. The details of the patients and controls were described in a previous study [35].

Subjects for IgE sensitization against house dust mite. General populations for mite sensitivity study were recruited from Fukui [10] and Tsukuba in Japan. Total and specific IgE levels (produced in response to Japanese cedar, *Dermatophagoides*, *Dactylis glomerata*, *Ambrosia artemisiifolia*, *Candida albicans*, and *Aspergillus*) were measured using the CAP-RAST method (for Fukui samples; Pharmacia Diagnostics AB, Uppsala, Sweden) or MAST-26 (for Tsukuba samples; Hitachi Chemical Co. Ltd., Tokyo, Japan). Positive sensitization against house dust mite was defined as specific IgE levels against the house dust mite (*Dermatophagoides farinae* or *Dermatophagoides pteronyssinus*) greater than or equal to 0.70 IU/ml (class 2) or lumicount greater than 2.76 (class 2). Subjects with asthma (current or past) or perennial allergic rhinitis were excluded from the analysis. Sensitized subjects (Mite-positive) were non-allergic in terms of symptoms but possessed mite-specific IgE. Non-sensitized subjects (Mite-negative) did not show any allergic symptoms and did not have mite-specific IgE.

Subjects for *HLA-DPA1* typing. Cases with asthma included 938 subjects used in GWAS analysis and 207 Japanese subjects with child- or child-onset (<15 years) asthmatics recruited in Tsukuba. The diagnosis of asthma in all patients was confirmed by specialists in pediatric allergology on the basis of the criteria of the National Institutes of Health, USA, with minor modifications. The control subjects were 2378 subjects that were used in GWAS analysis. Because most of the DNA from the GWAS controls was not available for genotyping, and we found that imputation of the *HLA-DPA1* allele using GWAS results was highly accurate (error rate, 0.003), we decided to genotype the *HLA-DPA1* allele by direct sequencing and imputation. Among the subjects for *HLA-DPA1* genotyping (1135 cases and 2376 controls), genotyping of 383 subjects was performed by direct sequencing and genotyping of the remaining 3128 samples was performed by imputation.

Subjects for *HLA-DPB1* typing. Cases with asthma included 938 subjects used in GWAS analysis and 207 Japanese subjects with child- or child-onset (<15 years) asthmatics; the same as those used in *HLA-DPA1* typing. The control 1 subjects for *HLA-DPB1* typing were 794 healthy adult subjects from Tokyo and 399 subjects were the same as those in GWAS. The control 2 subjects (n = 1475) were general datasets from Japanese population samples publically available at http://www.hla.or.jp/hapro/top.html. Because most of the DNA from the GWAS controls was not available for genotyping, and the imputation of the DPB1 allele using the GWAS results was not possible, we used 794 healthy adult subjects from Tokyo and 399 subjects from the GWAS for *DPB1* genotyping (Control 1). The control 2 subjects (n = 1475) were general datasets from Japanese population samples publically available at http://www.hla.or.jp/hapro/top.html. The status of asthma or other allergic diseases for these samples is not available.

Genotyping

Genotyping for GWAS was performed using the Illumina HumanHap550v3/610-Quad Genotyping BeadChip (Illumina), as per manufacturer's instruction.

In replication analyses, genotyping of each individual was performed with the TaqMan genotyping system (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). PCR was performed on a 384-well format, and automatic allele calling was performed using ABI PRISM 7900HT data collection and analysis software, version 2.2.2 (Applied Biosystems).

HLA-DPB1 genotyping of 1135 cases, 794 controls (control 1) and 1475 controls (control 2) were performed with the WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan), as per manufacturer's instruction. First, the target DNA was amplified by polymerase chain reaction (PCR) with biotinylated primers specifically designed for each HLA-DPB1 locus. Then, the PCR product was denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescent-coded microsphere beads. Concurrently, the biotinylated PCR product was labeled with phycoerythrin-conjugated streptavidin and immediately examined with the Luminex 100 system (Luminex, Austin, TX). Genotype determination and data analysis were performed with the WAKFlow typing software (Wakunaga).

HLA-DPA1 genotyping was performed with direct sequencing of exon 2 with forward primer 5′-TCAGGATGCCCAGACTTTCAA-3′ and reverse primer 5′-CAGGGGGCACTTAGGCTTCC-3′, and with the sequencing primer 5′-TCAGGATGCCCAGACTTTCAA-3′ using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Statistical analysis

In the GWAS, we examined the potential genetic relatedness on the basis of pairwise identity by state for all of the successfully genotyped samples using the EIGENSTRAT software [29]. In the GWAS, we genotyped 978 cases with pediatric asthma and 2402 controls using Illumina HumanHap550v3/610-Quad Genotyping BeadChip (Illumina, San Diego, USA). Samples of duplicated (identical individual or monozygotic twin), first-, second-, and third-degree pairs were detected, and the individual with a lower call rate was excluded from further analysis. PCA was performed, and the results were combined with those obtained for our in-

house Ryukyu and Han Chinese reference samples. Yamaguchi-Kabata *et al.* characterized the Japanese population structure using the genotypes for 140,387 SNPs in 7003 Japanese individuals, along with 60 European, 60 African, and 90 East-Asian individuals, in the HapMap project and found that the Japanese population is composed of 2 clusters (Hondo and Ryukyu) [36]. Hondo is the biggest island of Japan, and the island of Ryukyu is located in southern Japan. Also, we have 2nd or 3rd generation Chinese living in Japan, and Chinese present a different genetic population structure from Japanese. Therefore, we excluded samples belonging to Han Chinese or Ryukyu, and 938 cases and 2376 controls were considered for further analysis.

Cluster plots of SNPs were checked by visual inspection and SNPs with ambiguous calls were excluded. We excluded SNPs with a low genotyping rate (<90%), minor allele frequency less than 0.01 in either pediatric asthma cases or controls, or with Hardy-Weinberg equilibrium P value$<10^{-4}$ in controls. Finally, 450,326 SNPs were used for the GWAS. Details regarding the exact number of remaining SNPs after applying each quality control criterion are available in Table S1.

The genomic control inflation factor (λ_{GC}), defined as the median association test statistic across all SNPs divided by its expected value, was calculated by the method proposed by Devlin *et al.* [37]. GWAS and replication analyses were performed using the Cochran–Armitage trend test and χ^2 test. The meta-analysis was performed with the Mantel–Haenszel approach as a fixed-effects model [38]. All statistical findings were reported without correction. The results of GWAS were plotted with GWAS GUI v0.0.2 [39]. HLA-DP region was plotted with LocusZoom [40]. The power calculation was performed with Genetic Power Calculator [41]. Quantile-quantile (Q-Q) plot was plotted with ggplot2 package [42] in R version 2.10.0 (http://www.r-project.org/).

Imputation of genotypes in the DP region was performed with MACH version 1.0 [9] with 1000 Genome Project data (1000G 2010-6 release, http://www.sph.umich.edu/csg/yli/mach/download/1000G-2010-06.html).

HLA-DPA1 allele imputation

The HLA-DP region was in strong linkage disequilibrium and some DPB1 alleles were known to be linked with particular DPA1 alleles. First, we imputed HLA-DPA1 alleles by using the actual genotype data of samples obtained from Illumina HumanHap550v3/610-Quad (Illumina) and 1000 Genome Project data of Asian origin (JPT+CHB) (http://www.sph.umich.edu/csg/abecasis/MaCH/download/1000G-2010-06.html). The accuracy of the imputated data was confirmed by direct sequencing. The error rate of imputation was 1/352 (0.003).

Supporting Information

Table S1 Number of remaining SNPs after applying each quality control criterion.
(XLS)

Table S2 SNPs that are strong linkage disequilibrium ($r^2>0.9$) with rs987870.
(XLS)

Table S3 Association analysis for mite IgE sensitization.
(XLS)

Table S4 Genotyping data of the Japanese pediatric asthma GWAS.
(XLS)

Table S5 Characteristics of cases and controls.
(XLS)

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Author Contributions

Conceived and designed the experiments: E Noguchi, K Matsumoto. Performed the experiments: S Yoshihara, S-J Hong, Y Goto, T Asada, S Fujieda, N Hizawa, Y Nakamura, M Tamari, T Arinami, T Yoshida, Y Suzuki, H Sakamoto. Analyzed the data: H Saito, T Hirota, K Ochiai, M Sakashita. Contributed reagents/materials/analysis tools: Y Imoto, F Kurosaka, A Akasawa, N Shimojo, Y Kohno, N Kanno, Y Yamada, M-J Kang, J-W Kwon, F Yamashita, K Inoue, H Hirose, I Saito, T Sakamoto, H Masuko, I Nomura. Wrote the paper: E Noguchi, K Matsumoto.

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