博士論文

Identification of Genetic Factors of Idiopathic Membranous Nephropathy

(特発性膜性腎症における遺伝的解析)

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Table of Contents **Page**

Abstract

Idiopathic membranous nephropathy (IMN) is one of the major causes of adult nephrotic syndrome. Associations of phospholipase A_2 receptor 1 (*PLA2R1)* and *HLA-DQA1* with IMN have been reported in European and Asian populations. However, high-density association mapping covering the whole region of *PLA2R1* and *HLA* regions for the association with IMN has not been performed yet in the East Asian populations. In the first stage of the study, I performed genotyping of 15 SNPs in *PLA2R1* and *HLA* typing of *HLA-A*, *B*, *C*, *DRB1*, *DQB1* and *DPB1* in patients with 53 Japanese IMN patients and 419 healthy controls. In the second stage, I performed replication study with 130 Japanese IMN cases and 392 controls. I also analyzed the associations in the combined data set including both first and second sample sets. Moreover, interaction analysis of *HLA* and *PLA2R1* was conducted. In the first stage, single point analysis on *PLA2R1* identified 7 significant SNPs, and in the replication stage, 5 of which were confirmed. For *HLA* genes, strong associations were observed with *HLA-DRB1*15:01* and *HLA-DQB1*06:02*, and both were successfully replicated in the second stage. In the interaction analysis, more than additive effect was detected in patients carrying both risk alleles of *HLA-DRB1-DQB1* and *PLA2R1*. The present study identified the primary associations of *HLA* and *PLA2R1* polymorphisms with IMN in the Japanese population. Furthermore, the increased risk of IMN by combination of *PLA2R1* and *HLA* risk alleles confirmed the importance of the interaction of these two genes in the development of IMN.

Abbreviation

CKD	Chronic Kidney Disease
CI	Confidence Interval
ESRD	End-stage Renal Disease
GWAS	Genome Wide Association Study
HLA	Human Leukocyte Antigen
HWE	Hardy-Weinberg equilibrium
IgG4	Immunoglobulin G4
IMN	Idiopathic Membranous Nephropathy
JPT	Japanese population
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency
Mb	Megabase
MN	Membranous Nephropathy
OR	Odds Ratio
Pc	P-value corrected
PCR	Polymerase Chain Reaction
PLA2R	Phospholipase A2 Receptor
PLA2R1	Phospholipase A2 Receptor 1

RERI	Relative Excess Risk due to Interaction
RPEs	Rapid Predispositional Effects
SNPs	Single Nucleotide Polymorphisms
5’ UTR	5’ Untranslated Region

INTRODUCTION

General Introduction of Nephrotic Syndrome

Chronic kidney disease (CKD) describes a progressive loss of renal function over a period of months or years. According to the survey of Japanese Society of Nephrology, more than 13 million people have CKD, and treatment is preferable in 40% of them. Furthermore, end-stage renal disease (ESRD) is increasing together with aging society. In Japan, More than three hundred thousand people receive dialysis in 2011 [1]. CKD including ESRD has multiple complications such as hypertension, cardiovascular disease, anemia and mineral-bone disorder. It worsens quality of life and increases financial burden.

In many CKD cases, proteinuria is present. Nephrotic syndrome is an advanced type of kidney diseases in terms of massive proteinuria. Nephrotic syndrome accompanies hypoalbuminemia, edema, and occasionally pleural effusion, ascites, and thrombotic tendency. Treatment duration generally is as long as several years in this disease. Thus, comprehension of nephrotic syndrome is an urgent issue in a nephrology field.

Idiopathic Membranous Nephropathy

Membranous nephropathy (MN) is one of the most common causes of adult nephrotic syndrome. MN is a rare disease with the incidence rate of approximately 1 case in 100,000 persons per year [2]. Eighty percent of MN patients are classified as “idiopathic” while the remaining twenty percent are known as “secondary” for being associated with other medical conditions including systemic lupus erythematous (SLE), chronic infections including hepatitis B, graft vs host disease, drugs and toxins including penicillamine and mecury [3-5]. Idiopathic membranous nephropathy (IMN) is characterized by subepithelial immune complex deposition of glomerular basement membrane, which is regarded as the

landmark of the diagnosis of MN.

The immune deposition of IgG and complement cause the functional impairment of glomerular epithelial wall which results in proteinuria [6]. A demographic study published in 2012 reported the mean age of IMN patients in Japan was 62.2 (2-88) years old [7], with male to female ratio of 1.3, which was lower than that of 2.0 in North Americans, Australians and Asians [8, 9]. IMN is a chronic disease that is known for its clinical course of spontaneous remission and relapses. It was reported that 20-40% of IMN patients developed to ESRD after 10 to 15 years in Caucasian population [10-12]. In contrast, IMN is considered to run a more benign clinical course and good prognosis in Japanese than in European population [10, 12, 13]. Better prognosis was observed in Japanese IMN patients than in European patients despite more advanced histological findings [14].

In Japan, Shiiki *et al* reported that the overall renal survival rates were 95.8%, 90.3%, 81.1%, and 60.5% at 5, 10, 15, and 20 years after diagnosis, respectively. Male gender, old age (greater than or equal to 60 years), high serum creatinine concentration (greater than or equal to 1.5 mg/dL), and the development of tubulointerstitial lesions (greater than or equal to 20% of the biopsy sample area) were significant predictors of progression to ESRD [15]. What determines the difference of outcome among populations has been unclear. Therefore, it is worthwhile to investigate Human Leukocyte Antigen (HLA) involvement in IMN.

PLA2R1 is a 185-kD transmembrane glycoprotein that is a member of the mannose receptor family [16, 17]. Anti-PLA2R (M-type phospholipase A2 receptor) antibodies were detected in 70% of patients with IMN, but not secondary membranous nephropathy indicating PLA2R as a major target antigen in this disease and establishing IMN as an autoimmune disease [8]. PLA2R antibodies are not detected in healthy individuals or patients with other causes of nephrotic syndrome [18, 19]. According to several studies, anti-PLA2R1 antibodies

show correlation with disease activity; disappearing during a spontaneous or treatment-inducted remission and reappearing during relapse [20-22]. PLA2R is reported to being predominantly expressed in human kidney tissues [23], and also found to be present in the lungs and on leukocytes [24, 25]. Despite being expressed in other body tissues, the disease is limited only to kidneys and the exact role of *PLA2R1* in the pathogenesis of IMN is still unknown.

Recent genome-wide association study (GWAS) including independent cohorts of three European populations has identified *PLA2R1* on chromosome 2 and *HLA-DQA1* on chromosome 6 to be susceptible genes to IMN [26]. Interestingly, Stanescu *et al*. also reported a strong genetic interaction of risk alleles at both *HLA* (rs2187668) and *PLA2R1* (rs4664308), although relatively modest risk of IMN was identified at each locus. It is assumed that genetic variation in immune response gene (*HLA*) and podocyte gene (*PLA2R1*) may lead to the production of autoantibodies resulting in development of IMN [27]. However, this assumption has not been proved experimentally yet. In a subsequent study, Coenen *et al*. performed sequencing of *PLA2R1* exons in IMN patients in order to study whether rare variants are responsible for the development of IMN, but the result did not prove the hypothesis [28]. *PLA2R1* association with IMN was also reported by case-control candidate gene studies in European, African Americans and Asians including Chinese, Taiwanese and Koreans [29-32].

IMN association with certain *HLA* class II genes has long been known [33-35]. Strong association of *HLA-B8DR3* with IMN was reported in European Caucasoids [36, 37]. In recent years, association of a single nucleotide polymorphism (SNP) in *HLA-DQA1* (rs2187668) with IMN has also been reported [26, 32, 38].

Human leucocyte antigen (*HLA*) genes are known as the most polymorphic genes in

human genome stretching around 6 megabase pairs (Mb) on chromosome 6 [39]. *HLA* super-locus encodes six classical genes, which have important roles in the regulation of the immune system. *HLA* locus is considered to be one of the most gene dense and complex genes, and reported to be associated with more than 100 different diseases, including autoimmune diseases and some other complex diseases [40].

SNPs have been widely used as markers in genetic studies for detecting disease susceptibility genes by using standard single-locus test. Nowadays, the interest has been moving to the detection of effects by gene-gene interactions (epistasis) and gene-environment interactions which will be helpful in understanding the biological pathways of diseases [41]. Bateson in 1901 used the term epistasis to describe the masking effect of one gene upon another gene. In 1918, Fisher expanded the term to include any statistical deviations from additivity of the two loci.

In Japan, IMN was reported to account for 77.9% of total membranous nephropathy patients while membranous nephropathy was present in 36.8% of primary nephrotic syndrome patients [7]. Limited number of genetic studies of IMN has been reported so far. Despite previous studies have reported the effect of single locus and the genetic interaction of SNPs in *PLA2R1* and *HLA-DQA1* regions, no study to date, has performed the high-density association mapping of both *PLA2R1* and *HLA* regions for identifying the primary polymorphisms and it is still not known the interaction effect of *PLA2R1* risk variants and classical *HLA* alleles. Thus, the objective of this study is to fill a gap in the understanding of genetics of IMN by high-resolution study of *PLA2R1* and *HLA* regions. Besides the single point associations, interaction analysis between *HLA* and *PLA2R1* risk alleles was performed.

MATERIALS AND METHODS

Human subjects and sample collection

This study included a total of 989 subjects comprised of healthy controls and cases with (biopsy-proven) idiopathic membranous nephropathy. The study was approved by the Ethical Committees at The Faculty of Medicine at The University of Tokyo, BioBank Japan and Pharma SNP Consortium (Tokyo, Japan) (http://www.jpma.or.jp/information/research/psc/e02psc/about.html). Written informed consent was obtained from each participant before sample collection. In the first set of study, I recruited 53 biopsy-proven IMN patients and 419 healthy controls residing around Tokyo area. IMN samples were collected by the department of Hemodialysis and Apheresis of the University of Tokyo Hospital, and control samples were collected by the Department of Human Genetics of the University of Tokyo. Diagnosis of the IMN patients was established by renal biopsy together with other routine clinical procedures. Patients with any evidence of secondary memebranous nephropathy were excluded from the study. Either needle renal biopsy or open renal biopsy was performed. Control samples were collected from volunteer healthy individuals. In the second set of study, 130 IMN cases collected by BioBank Japan and 392 healthy controls from the Department of Hemodialysis and Apheresis of The University Tokyo Hospital and Pharma SNP Consortium (Tokyo, Japan) were analyzed for replication purposes. Among control samples used in replication study, 200 samples were collected from healthy individuals visiting the health centers for routine medical check-up, and 192 samples were part of the samples from Pharma SNP Consortium. Control samples were not collected from healthy individuals who were not being diagnosed with any kidney diseases. Both the discovery and replication studies included the Japanese individuals.

PART 1: Fine mapping of *PLA2R1* gene

Study overflow of fine mapping of *PLA2R1* was shown in Figure 1.

Tag Single Nucleotide Polymorphism (SNP) Selection

SNP genotype information of *PLA2R1* was downloaded from the HapMap JPT population database (http://hapmap.ncbi. nlm.nih.gov/). HapMap data was analyzed using Haploview software (ver 4.1) and I selected Tag SNPs by using Tagger algorithm implemented in Haploview [42]. I selected SNPs by applying the following selection criteria: (i) minor allele frequency (MAF) threshold of > 0.10 in the HapMap JPT population, (ii) r^2 threshold of greater than or equal to 0.8 (Figure 2). Finally, I selected a total of twelve tag SNPs meeting the criteria together with additional three reported SNPs (rs35771982, rs4665143 and rs3749119) from previous literatures [28], to validate their association with idiopathic membranous nephropathy in the Japanese population. The previously reported SNP, rs3828323, in European and Korean studies [28, 29], was known to be in LD with the tag SNP rs1511223 in 3' UTR region, and thus excluded from this study.

SNP Genotyping

The genomic DNA was extracted from the peripheral blood at each institution following the standard protocol. The concentration of genomic DNA was determined using spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies). I performed genotyping of the total 15 SNPs using discovery sample set of 472 individuals by using the TaqMan SNP Genotyping Assay (ABI: Applied Biosystems Inc. Foster City, CA, USA) to determine the genotypes according to the manufacturer's protocol. For TaqMan Genotyping Assay, 10 ng of genomic DNA was used per reaction well. The mixture for every reaction was prepared with

2.5 μl of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA), and 0.125 μl of TaqMan SNP Genotyping Assay primer/probe (40x) from Applied Biosystem and 1.375 μl of Milli Q water. Then, 4 μl of reaction mixture was added to the 1 μl of DNA template. The polymerase chain reaction (PCR) was performed using Light cycler 480II (Roche, Germany) with cycling parameters of 95 °C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

I also performed TaqMan SNP genotyping of significant variants (rs1511223, rs35771982, rs10196882, rs877635, rs2715928, rs16844715 and rs3749119) in the replication study including 522 IMN cases and healthy controls.

Statistical analysis

Single locus association analysis

To compare the allele and genotype frequencies between case and control groups, chi-square test or Fisher exact test was applied as appropriate. Departure from Hardy-Weinberg equilibrium (HWE) was tested in all SNPs by a chi-squared goodness-of-fit test. Association analyses of all SNPs in *PLA2R1* gene were performed by using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) [43].

Haplotype association analysis

Haplotype association test was conducted using the combined data set including 183 IMN cases and 811 healthy controls. Out of 13 SNPs selected in this study, 5 SNPs with significant associations in all data sets were included in haplotype analysis. HaploView software [44] and the sliding window analysis implemented in PLINK were used to display

the linkage disequilibrium structure of *PLA2R1* SNPs in JPT HapMap samples and to perform a haplotype analysis of *PLA2R1* gene.

PART 2: Associations of *HLA* genes with IMN

Study overflow of *HLA* association with IMN was shown in Figure 3.

HLA genotyping

Genotyping for six *HLA* genes (*HLA-A*, *-B*, *-C*, *-DRB1*, *-DQB1* and *-DPB1*) was performed in 53 Japanese idiopathic membranous nephropathy patients by the polymerase chain reaction (PCR)-Luminex typing method using the WAKFlow HLA typing kit (Wakunaga, Hirohsima, Japan) (Figure 4). Briefly, target DNA was amplified by PCR (polymerase chain reaction) with biotinylated primers specifically designed for each HLA locus. The PCR product was then denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescent coded microsphere beads. In the meanwhile, biotinylated PCR products were labeled with phycoerythrin-conjugated streptavidin and finally examined with Luminex 100. Genotype determination and data analysis were performed automatically, using the WAKFlow typing software. For healthy control samples, I utilized *HLA* data published previously [45]. HLA typing of significant *HLA* regions, *HLA-DRB1* and *DQB1*, was performed for the second set of samples for the replication study.

Statistical analysis

HLA single locus association analysis

To compare *HLA* allele frequency and carrier frequency between IMN patients and controls, I used Chi-square test when the sample number was 10 or more than 10 and used Fisher's exact test when the sample number was less than 10. I also conducted Relative

predispositional effects (RPEs) tests to identify the associations sequentially according to their strengths [46]. When any of the obtained value was zero, the odds ratio was calculated using Woolf's correction. The odds ratio (OR) with 95% CI was also calculated. Bonferroni correction for multiple testing was applied and corrected P values (Pc) were calculated by multiplying the P values by the number of alleles tested for each locus.

HLA haplotype association analysis

I also performed *HLA* haplotype association analysis applying Arlequin algorithm [47]. Two-locus haplotypes (*DRB1-DQB1*) were calculated between IMN cases and controls by maximum likelihood methods.

PART 3: Genetic interaction between *PLA2R1* and *HLA* risk alleles

Interaction analysis was performed using combined data set including a total of 981 samples (182 IMN cases and 799 healthy controls). Samples with failed genotyping in either *PLA2R1* or *HLA* region were excluded from analysis. Five *PLA2R1* SNPs (rs1511223, rs35771982, rs2715928, rs16844715 and rs3749119) significantly associated in both first and second stages, and significantly associated *HLA* haplotype, *HLA-DRB1*15:01—DQB1*06:02* were included. Individuals carrying risk alleles of *PLA2R1* and *HLA* were compared with those with non-risk alleles using chi-square test or Fisher's exact test. Additive interactions were also tested using RERI, Relative Excess Risk due to Interaction [48]. RERI is equal to 0 means there is no biological interaction or exactly additivity and RERI greater than 0 means positive interaction or more than additivity [49, 50].

RESULTS

PART 1: Fine mapping of *PLA2R1* gene

Association analysis of *PLA2R1* SNPs in the first set of IMN patients and controls

Fine mapping of *PLA2R1* SNPs was performed in the first stage of the study including 53 IMN patients and 419 healthy controls. Of the 15 SNPs genotyped, two SNPs failed in genotyping and were therefore excluded from the study. Significant deviations from Hardy-Weinberg equilibrium ($P<0.05$) were not observed for any of the 13 SNPs included in the study. Of 13 SNPs included in the association analysis, I found 9 SNPs significantly associated with IMN ($P<0.05$) (Table 1). When I corrected for the multiple testing, 7 SNPs survived to be significant (Table 2).

Replication in the second data set and combined analysis

Seven significant SNPs in the first set were attempted to replicate in a total of 130 IMN patients and 392 healthy controls. Five SNPs (rs1511223: $P=8.88\times10^{-3}$; OR=1.57, rs35771982: $P=1.52\times10^{-8}$; OR=2.57, rs2715928: $P=2.43\times10^{-8}$; OR=2.36, rs16844715: $P=7.96\times10^{-8}$; OR=2.23, and rs3749119: $P=1.21\times10^{-8}$; OR=2.61) were successfully replicated, while rs10196882 and rs877635 did not reach significant level in the second set.

When the association analysis was conducted in the combined data sets, rs35771982 and rs3749119 (which are in high linkage disequilibrium with each other) exhibited the strongest associations ($P=3.09\times10^{-15}$, OR=2.93 and $P=2.16\times10^{-15}$, OR=3.02, respectively) (Table 2). Two intronic SNPs, rs2715928 ($P=2.10\times10^{-11}$, OR=2.30) and rs16844715 ($P=3.77\times10^{-14}$, OR=2.51), also showed significant associations with IMN.

Haplotype association analysis

As part of the association analysis, I also performed haplotype association tests in order to determine whether haplotypes from combination of common variants increased the risk of IMN. I performed haplotype analysis including SNPs, which were consistently significant in all data sets, using the HaploView software and PLINK sliding window analysis. Analysis of LD pattern showed high LD (r^2=0.81) between rs35771982, missense SNP located in exon 5, and rs3749119 located in 5' untranslated region (5' UTR) of *PLA2R1* and more significant SNP (rs35771982) was included in the haplotype analysis. Haplotypes with low frequency (<1%) in either cases or controls were excluded. Compared to the risk and strength of association by single variant analysis, sliding window haplotype analysis in PLINK including two, three and four markers did not exhibit stronger association nor higher risk with IMN (Table 3).

PART 2: Associations of *HLA* genes with IMN

Allelic association

Association analysis of HLA genes in the first set of IMN patients and controls

In the discovery stage, *HLA-A*, *-B*, *-C*, *-DRB1*, *-DQB1* and *–DPB1* genotypes were determined in a total of 53 IMN patients and 419 healthy controls. Regarding *HLA* class I genes, *HLA-A*33:03* showed a marginal association (P=0.03, OR=0.4) with IMN (Table 4). Tendency of negative association was observed in *HLA-B*07:02* and *HLA-B*4403* with IMN while positive association was found with *HLA-B*35:01* (Table 5). *HLA-C*0704* exhibited a high odds ratio for IMN (P=5.79E-03, OR=5.89) although it remained not significant when the p-value was corrected for the number of alleles tested. Little evidence of negative association was observed in two other *HLA-C* alleles, *HLA-C*01:02* and *HLA-C*14:03* with IMN (Table 6). No alleles mentioned above remained significant after correction for multiple comparisons.

Regarding *HLA* class II genes, *HLA-DRB1*15:01* was the most strongly associated allele ($P=7.72\times10^{-5}$, OR=2.85), which remained to be significant when P-value was corrected for the number of alleles tested (Table 7). Negative association was also observed in *HLA-DRB1*01:01*, *HLA-DRB1*04:05* and *HLA-DRB1*13:02*. Among four *HLA-DQB*1 alleles that showed significant associations with IMN, *HLA-DQB1*06:02* ($P=5.12\times10^{-4}$, OR=2.60) only survived to be significant after correction for multiple comparisons (Table 8). No association was observed between *HLA-DPB1* alleles and IMN (Table 9).

Replication in the second sample set and combined set

The replication study of two *HLA* class II genes, *HLA-DRB1* and *HLA-DQB1*, was performed in an independent set of 130 IMN patients and 392 healthy controls. A significant positive association was also observed for *HLA-DRB1*15:01* and *HLA-DQB1*06:02* with IMN in the replication stage with $P=1.71\times10^{-9}$, OR=3.36 and $P=5.14\times10^{-10}$, OR=3.56, respectively (Tables 10 and 11). Except for *HLA-DRB1*13:02* and HLA-*DQB1*06:04* which showed marginal association with IMN, none of the significant *HLA-DRB1* and *HLA-DQB1* alleles in first set was found to have P-value<0.05.

Relative Predispositional Effects (RPEs) with IMN

In the combined data analysis, both *HLA-DRB1*15:01* and *HLA-DQB1*06:02* emerged with stronger evidence of associations with IMN holding $P=3.94\times10^{-13}$, OR=3.09 and $P=8.90\times10^{-13}$, OR=3.10, respectively (Tables 10 and 11). I also performed RPEs testing to further explore the associations between *HLA-DRB1* and *HLA-DQB1* alleles with IMN in the combined analysis. In RPEs testing, sequential elimination of carriers of each allele with the most significant association was performed to identify the associations sequentially according to their strength. In the analysis of *HLA-DRB1*, the most significant association was between *DRB1*15:01* and IMN ($P=3.94\times10^{-13}$, OR=3.09) in the combined analysis. The analysis was conducted again after elimination of *DRB1*15:01* carriers. The next most significant positive association was observed between *DRB1*14:54* and IMN ($P=1.00\times10^{-4}$, OR=2.05). When the third turn of analysis was performed after elimination of both *DRB1*15:01* and *DRB1*14:54* carriers, the next most significant association was found at *DRB1*11:01* ($P=8.54\times10^{-4}$, OR=2.15). Further rounds of association analysis after eliminating the carriers of *DRB1*15:01*, *DRB1*14:54* and *DRB1*11:01* did not show any *DRB1* alleles which survived to remain significant after multiple correction (Table 10).

Regarding to *HLA-DQB1* analysis, the most significant association was found between *DQB1*06:02* and IMN ($P=8.90\times10^{-13}$, OR=3.1). Analysis after elimination of *DQB1*06:02* carrier showed the next most significant association to be *DQB1*05:02* with IMN ($P=9.86\times10^{-5}$, OR=2.34). The next most significant association after elimination of both *DQB1*06:02* and *DQB1*05:02* carriers was found to be *DQB1*03:01* with IMN ($P=3.11\times10^{-3}$, OR=1.48). After elimination of carriers of all *DQB1*06:*02, *DQB1*05:*02 and *DQB1*03:01*, the next most significant association was observed to be *DQB1*05:03* with IMN ($P=2.96\times10^{-3}$, OR=1.68). Further analysis after sequential elimination of all

*DQB1*06:02*, *DQB1*05:02*, *DQB1*03:01* and *DQB1*05:03* did not reveal any *DQB1* alleles that passed significant level after multiple correction (Table 11).

*DRB1*15:01-DQB1*06:02* haplotype distribution

Two-locus haplotype frequencies were calculated in the combined data set (182 IMN cases and 799 controls) by Arlequin software. Haplotypes with frequencies less than 1% in both cases and controls were excluded from the study and major haplotypes are shown in Table 7. In control group, the most common haplotype was found to be *DRB1*09:01-DQB1*03:03* present in 222 of 799 controls (27.8%) and the second most common haplotype was *DRB1*04:05-DQB1*04:01* observed in 194 of 799 controls (24.3%). Among IMN patients, *DRB1*15:01-DQB1*06:02* present in 69 of 182 IMN cases (37.9%) was observed to be the most common haplotype and *DRB1*15:02-DQB1*06:01* found in 42 of 182 cases (23.1%) was found to be the second most common haplotype.

*DRB1*15:01-DQB1*06:02* Haplotype association

The results of *HLA-DRB1-DQB1* haplotype association analysis were shown in Table 12. The most susceptible haplotype observed in this study was *DRB1*15:01-DQB1*06:02* haplotype (P=1.89×10^{-12}, OR=3.07). The other susceptible haplotypes were *DRB1*14:54-DQB1*05:02* haplotype (P=2.67×10^{-3}, OR=3.1), *DRB1*11:01-DQB1*03:01* haplotype (P=3.21×10^{-3}, OR=2.34) and *DRB1*04:01-DQB1*03:01* haplotype (P=5.16×10^{-3}, OR=2.71). The most protective haplotype was observed to be *DRB1*13:02-DQB1*06:04* haplotype (P=6.10×10^{-3}, OR=0.44).

PART 3: Genetic interaction between *HLA* and *PLA2R1* risk alleles

In the single point analysis of the fine mapping study of *PLA2R1* gene, I found 5 significant SNPs (rs1511223, rs35771982, rs2715928, rs16844715, and rs3749119) in the first stage were successfully replicated in the second stage. Regarding to *HLA* study of IMN, *HLA-DRB1*15:01* and *DQB1*06:02* were significant in both the first and the second stages of the study. Sixty-nine (37.9%) of IMN patients were found to carry *HLA-DRB1*15:01-DQB1*06:02* haplotype (P=1.89×10^{-12}, OR=3.07). I, therefore, analyzed the total of 182 IMN cases and 799 healthy controls (both HLA-matched and non-HLA-matched controls with *HLA-DRB1*15:01-DQB1*06:02*) to investigate the interactions between *HLA-DRB1*15:01-DQB1*06:02* haplotype and *PLA2R1* risk alleles.

Interaction analysis exhibited positive interaction or more than additive effects with IMN (Table 13). I observed that the evidence for the interaction was strongest between *HLA-DRB1*15:01 - HLA-DQB1*06:02* and the intronic SNP rs2715928 (OR=17.53, P=4.26×10^{-26}, and RERI=13.72). It was found that 18.6% of patients carried both rs2715928 and *HLA-DRB1*15:01-DQB1*06:02* while 1.9% of controls possessed them.

In addition, positive interaction was also observed between *HLA-DRB1*15:01 - HLA-DQB1*06:02* and the missense SNP rs35771982 (OR= 15.91, P=2.76×10^{-29}, and RERI=10.88) that is in strong LD with 5'UTR SNP rs3749119, and intronic SNP rs16844715 (OR=15.91, P=2.30×10^{-26}, and RERI=11.34) for IMN. The data showed that 23.2% of IMN cases and 3.4% of controls carried both rs35771982 and *HLA-DRB1*15:01 - HLA-DQB1*06:02*, and rs3749119 and *HLA-DRB1*15:01 - HLA-DQB1*06:02* risk allele combinations.

These results suggest that risk allele of PLA2R1 risk variants causes an increased risk of IMN in individuals carrying *HLA-DRB1*15:01-DQB1*06:02* haplotype.

DISCUSSION

General discussion

The genetic association of *PLA2R1* and *HLA-DQA1* risk alleles with IMN in the European populations was reported by a GWAS study that included three independent genome-wide association studies in three populations [26]. Coenen *et al*. subsequently investigated the effect of rare variants on IMN by sequencing *PLA2R1* exons in European ancestry patients, and the results did not prove the positive association of rare variants with IMN [28]. So far, associations of *PLA2R1* with IMN have not been investigated in the Japanese population. In this study, I aimed to identify the primary risk SNPs of *PLA2R1*, and *HLA* risk alleles and their haplotypes, the interaction between the risk alleles of *PLA2R1* and *HLA* genes on the development of IMN through high-resolution association study.

PART 1: Fine mapping of *PLA2R1* gene

This study identified significant association of PLA2R1 with IMN in Japanese. I found 7 SNPs within *PLA2R1* gene confirmed to be significantly associated with IMN, including a non-synonymous SNP (H [His] ⇒ D [Asp]) and a 5' UTR SNPs reported in previous studies [28-30, 32], and additional 5 SNPs. In the Japanese population, rs35771982 of *PLA2R1* was the most strongly associated with IMN. In agreement with the reports of Coenen *et al*. and Liu *et al* [28, 30]. I found that G allele of non-synonymous SNP, rs35771982, which is in strong LD with 5' UTR SNP, rs3749119 had significantly increased the risk of developing IMN. Contrary to our finding and to those by Coenen *et al*. and Liu *et al*., Lv *et al*. and Kim *et al*. reported that C allele of rs35771982 elevated the risk of IMN [29, 31]. Consistent with our result, Coenen *et al*. also reported that C allele of rs3749119 raised the risk of IMN. This finding provided the first independent replication of positive association of rs3749119 with IMN susceptibility. Additionally, I observed a significant association with A allele of rs1511223, located in 3' UTR with IMN, which is in agreement with the result reported by Saeed *et al* [32]. The SNP (rs1511223) is located in high LD with rs3828323 that was reported to be associated with IMN in European and Korean populations [28, 29].

Moreover, the current study identified the new significant associations of two intronic SNPs, both located in the first intron of *PLA2R1*. Our results also suggest that rare haplotypes from combination of common variants within *PLA2R1* region may not explain the rare occurrence of IMN in general population.

It was reported that the prevalence of idiopathic membranous nephropathy was lower in Japanese than in the European and other Asian populations [51]. The results of fine mapping study show a stronger association of IMN with *PLA2R1* (Table 2) than *HLA*, which was in agreement with the Chinese study [31] and opposite to the findings from European

GWAS study [26]. Stanescu *et al*. reported the stronger association of IMN with *HLA* than *PLA2R1*. These findings suggest that the risk of idiopathic membranous nephropathy is higher with *PLA2R1* than with *HLA*, supporting the finding reported by Beck and Colleagues [8]. However, the process of antibody formation and the pathologic role of *PLA2R1* in IMN are still not clearly understood. It is evident from the data that risk alleles of *PLA2R1* are common in general population, and yet idiopathic membranous nephropathy is still rare disease, suggesting the contributions of other risk factors (genetic and environmental factors) to the development of IMN.

PART 2: Associations of *HLA* genes with IMN

With respect to HLA association with IMN, recent studies have reported the strong association of *HLA-DQA1* SNP rs2187668 with IMN in European and Chinese populations [26, 31]. It has been reported that rs2187668 is a tag SNP for *HLA-DRB1*03:01* in northern European populations, and the haplotype including *DRB1*03:01* was associated with membranous nephropathy [12, 52, 53].

This part of the study aimed to identify the most important *HLA* alleles for IMN through HLA typing approach. This study identified the strong positive associations of *HLA-DRB1*15:01* and *DQB1*06:02* with IMN in Japanese population that were replicated in the second sample set and combined dataset. Similar association with IMN was not found in other populations. However, these individual or haplotype associations with different immune disorders have been reported in different populations [54-57].

In a French population, increased frequency *HLA-DR3* and decreased frequency of *HLA-DMA*01:02* was associated with IMN [33]. Taken together, the discrepancy of *HLA* associations with IMN in different populations may explain the population difference in the genetic contribution to the development of IMN and difference of disease severity of IMN as a phenotype. In British IMN patients, Vaughan *et al*. reported the associations of *HLA-DRB1*03:01*, *HLA-DQA1*05:01* and *HLA-DQB1*02:01* with IMN [35]. Both *DRB1*03:01* and *DQB1*02:01* alleles are less common in the Japanese population. Not many studies regarding the *HLA* associations of IMN have been reported worldwide yet.

IgG4 antibodies were detected in 26 of 37 patients with IMN [8]. IgG4 co-localized with PLA2R in the immune complex deposition on the glomerular basement membrane in patients with IMN, but not in those with secondary MN. Significantly higher frequency of

*HLA-DRB1*15* was reported in primary sclerosing cholangitis patients with increased levels of IgG4 inserum [58]. Elevated serum concentrations of IgG4 are found in 60 to 70 percent of patients with IgG4-related disease. Hence, *HLA* may be associated with IgG4-related IMN.

With respect to HLA class I, no association of HLA class I alleles with IMN has been reported to date. In our analysis of the discovery set including 472 samples of IMN cases and healthy controls, I also observed the weak association of *HLA-B*07:02* with IMN before correction appearing in 1 of 53 IMN patients (1.9%) and 53 of 419 healthy individuals (12.6%). Additionally, our results showed the weak association of *HLA-C*01:02* with IMN before correction although the association ceased to be significant when I corrected P-value for multiple correction. Our study also showed the potential risk of *HLA-C*07:04* with Japanese IMN patients. *HLA-C*07:04* was reported to increase relative risk for carbamazepine-induced cutaneous adverse reactions in Japanese [59]. The shared *HLA* allelic associations might have a pathological role because some kidney diseases such as vasculitis also develop skin symptoms.

*HLA-DRB1-DQB1*haplotype analysis identified a strong positive association between *HLA-DRB1*15:01-DQB-06:02* haplotype and IMN in Japanese population. It is known that 12% of general Japanese population carries the haplotype. *DRB1*15:01-DQB1*06:02* haplotype was also found to be associated with Narcolepsy [60, 61] and Essential Hypersomnia [62] in Japanese population. Although, this study also identified potential susceptible and protective haplotypes to be associated with IMN, *DRB1*15:01-DQB1*06:02* haplotype exhibited the strongest association with IMN in Japanese.

PART 3: Genetic interaction between *PLA2R1* and *HLA* risk alleles

European GWAS study and a number of recent studies have reported the elevated risk of susceptibility to IMN in the patients carrying both *HLA* SNP (rs2187668 which is known as a tag SNP for *HLA-DRB1*03:01*) and *PLA2R1* variants [26, 31, 32]; however, no study has reported the interaction between classic *HLA* alleles or *HLA* haplotypes and *PLA2R1* risk variants with IMN until today.

Therefore, the present study was designed to examine the risk of combined effect of *HLA* and *PLA2R*1 in the Japanese IMN patients. I carried out the case-control association study including 182 IMN patients and 799 healthy control individuals to investigate the interaction effect between *PLA2R1* and *HLA* risk alleles which were significant in both discovery and replication stages in this study. *HLA-DRB1*15:01* and *DQB1*06:02* are in tight LD in Japanese population and known to be in haplotype showing significant associations in both single point and haplotype analyses.

Our results from the interaction analysis confirmed the increased risk of IMN by *PLA2R1* and *HLA* together, and additionally proved that IMN patients with risk haplotype *HLA-DRB1*15:01-DQB*06:02* and risk alleles of *PLA2R1* conferred the elevated risk (highest OR = 17.53) for the development of IMN. The present study identified positive interaction or more than additive effects on the risk of IMN among the individuals with risk alleles of five *PLA2R1* SNPs (rs1511223, rs35771982, rs2715928, rs16844715 and rs3749119) and *HLA-DRB1*15:01-DQB1*06:02*. Little is known about the mechanism involved in the interaction between HLA and *PLA2R1*, and disease development. It may be possible that PLA2R1 risk variants may introduce a unique conformation in the protein, which lead to preferential antigenic peptide recognition by the predisposing HLA haplotype, *HLA-DRB1*15:01-DQB1*06:02*, and result in the production of autoantibodies. These

findings of the increased risk of IMN by interaction between HLA haplotypes and PLA2R1 SNPs further supports the concept that genetic interaction between *HLA* and *PLA2R1* may interfere with the immune regulation resulting in predisposition to the development of idiopathic membranous nephropathy, and provide the clues to the better understanding of pathogenesis of IMN. To date, out study is the first to report the increased risk of interaction effect between *PLA2R1* risk variants and *HLA* risk haplotype in IMN.

CONCLUSIONS

The present study was aimed to investigate the genetic risk factors of IMN by high-density mapping approach. This study provides the evidences of *PLA2R1* and *HLA* associations with IMN in Japanese population. In fine mapping study of *PLA2R1*, I observed 7 SNPs to be strongly associated with IMN. In the *HLA* association study, I identified 2 highly significant *HLA* alleles together with several potential alleles. The most interesting finding to emerge from the present study is that the risk of IMN is highly increased by the combination of *PLA2R2* and *HLA*. In contrast to European GWAS study, this study identified different HLA risk alleles and interaction between *HLA* and *PLA2R1* suggesting the racial difference in the genetic contribution of disease susceptibility and severity of IMN. The current data also highlight the importance of interaction effect of *PLA2R1* and *HLA* in the development of IMN. Furthermore, this is the first study to investigate the positive interaction or more than additive effects between *HLA* risk haplotype and *PLA2R1* risk variants. The key strengths of this study are the identifications of classic *HLA* alleles by advanced *HLA* typing technology and the interactions between *HLA-DRB1*15:01-HQB1*06:02* haplotype and *PLA2R1* risk variants. The findings in this study provide a new understanding to the development of IMN. Due to the little understanding of the pathological mechanism of the interaction of *PLA2R1* and *HLA* in IMN, further studies (genetic and functional) are needed for the better understanding of IMN development.

This study has several limitations. Firstly, sample size was relatively small for genetic association study. However, given the strength of the associations of both *PLA2R1* and *HLA* alleles, this study shows that the relatively small size of cases is sufficient enough to identify the significant alleles in rare diseases like idiopathic membranous nephropathy. Secondly, the comparison among pathological findings and clinical courses and genotyping

data was impossible because all the samples were anonymised. Impact of genetic risk alleles on responsiveness to treatment as well as histological and clinical severity may be clinically useful.

In summary, this three-staged study of 182 IMN cases and 811 healthy controls identified the genetic risk alleles of *PLA2R1* and *HLA* in Japanese and confirmed the increased risk conferred by the combination of *PLA2R1* and *HLA* risk alleles.

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TABLES

Table 1. Association analysis of PLA2R1 SNPs with IMN in the first sample set

SNP	Position	Allele	Case (2n=106)		Control (2n=838)		OR (95% CI)[a]	P-value[b]
			No	%	No	%		
rs1511223	3' UTR	A	88	83.0	569	68.6	2.24(1.32-3.8)	2.17E-03
		C	18	17.0	261	31.4		
rs2715931	intronic	A	18	17.0	94	11.2	1.61(0.93-2.8)	0.09
		C	88	83.0	742	88.8		
rs35771982	missense	G	87	82.1	469	56.1	3.58(2.14-6)	3.01E-07
		C	19	17.9	367	43.9		
rs10929964	intronic	T	27	26.0	201	24.3	1.09(0.69-1.74)	0.71
		A	77	74.0	627	75.7		
rs2203053	intronic	G	55	52.9	345	41.5	1.58(1.05-2.39)	0.03
		A	49	47.1	487	58.5		
rs10196882	intronic	T	30	28.8	126	15.3	2.25(1.42-3.58)	4.72E-04
		C	74	71.2	700	84.7		
rs6751162	intronic	A	24	23.1	181	22	1.07(0.66-1.73)	0.80
		C	80	76.9	643	78		
rs16844706	intronic	C	42	43.7	275	33.4	1.55(1.01-2.38)	0.04
		T	54	56.3	549	66.6		
rs4665143	synonymous	A	43	42.2	308	37.1	1.24(0.81-1.88)	0.32
		G	59	57.8	522	62.9		
rs877635	intronic	A	52	49.1	199	23.9	3.07(2.03-4.64)	3.33E-08
		C	54	50.9	635	76.1		
rs2715928	intronic	A	66	67.3	390	49.4	2.12(1.36-3.3)	7.83E-04
		G	32	32.7	400	50.6		
rs16844715	intronic	C	75	72.1	378	45.3	3.12(1.99-4.89)	2.53E-07
		T	29	27.9	456	54.7		
rs3749119	5' UTR	C	86	84.3	476	57.2	4.02(2.32-6.97)	1.31E-07
		T	16	15.7	356	42.8		

[a]OR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[b]P-values for allele frequency comparisons between cases and controls using the chi-square test.

Table 2. Replication and Combined analysis of significant *PLA2R1* SNPs

SNP	Risk allele	Replication set						Combined analysis					
		Case (2n=260)		Control (2n=772)		OR (95% CI)[a]	P-value[b]	Case (2n=366)		Control (2n=1610)		OR (95% CI)[a]	P-value[b]
		No	%	No	%			No	%	No	%		
rs1511223	A	203	79.3	536	70.9	1.57(1.12-2.21)	8.88E-03	293	80.5	1105	69.7	1.80(1.36-2.38)	3.57E-05
rs35771982	G	200	78.7	450	59.1	2.57(1.84-3.58)	1.52E-08	289	79.8	919	57.5	2.93(2.22-3.85)	3.09E-15
rs10196882	T	52	20.8	119	15.7	1.41(0.98-2.02)	6.49E-02	82	23.0	245	15.5	1.63(1.23-2.16)	5.92E-04
rs877635	A	69	27.2	200	26.5	1.03(0.75-1.42)	8.42E-01	123	34.0	403	25.3	1.52(1.19-1.94)	7.95E-04
rs2715928	A	185	71.7	385	51.7	2.36(1.74-3.21)	2.43E-08	251	70.1	775	50.5	2.30(1.79-2.94)	2.10E-11
rs16844715	C	168	66.1	350	46.7	2.23(1.66-3.00)	7.96E-08	245	68.1	728	46.0	2.51(1.97-3.19)	3.77E-14
rs3749119	C	198	79.2	453	59.3	2.61(1.86-3.66)	1.21E-08	286	80.8	929	58.2	3.02(2.28-4.01)	2.16E-15

[a]OR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.

[b]P-values for allele frequency comparisons between cases and controls using the chi-square test.

Table 3. Haplotype association analysis of *PLA2R1* SNPs in the combined sample set

Haplotype	Effect	rs35771982	rs2715928	rs16844715	Case freq (n=183)	Control freq (n=805)	OR (95% CI)	P-value
H1	Risk	G	A	C	0.573	0.368	2.31 (1.82; 2.94)	7.30E-13
H2	Protective	C	G	T	0.191	0.406	0.34 (0.26; 0.45)	1.84E-14
H4	Neutral	G	G	C	0.106	0.076	–	0.06
H5	Neutral	G	A	T	0.121	0.121	–	1.00

Table 4. Association analysis of *HLA-A* alleles with IMN in the first set

HLA-A alleles[a]	Allele frequency						
	IMN (2n=106)		Control (2n=838)		OR (95% CI)[b]	P-value[c]	Pc
	No	%	No	%			
*A*01:01*	0	0.0	10	1.2	0.37(0.02-6.38)	0.30	4.85
*A*02:01*	17	16.0	85	10.1	1.70(0.96-2.99)	0.06	1.03
*A*02:06*	8	7.5	61	7.3	1.04(0.48-2.24)	0.15	2.46
*A*02:07*	3	2.8	23	2.7	1.03(0.31-3.51)	0.24	3.86
*A*11:01*	11	10.4	80	9.5	1.10(0.57-2.14)	0.78	12.46
*A*24:02*	36	34.0	316	37.7	0.85(0.56-1.31)	0.46	7.41
*A*24:20*	0	0.0	10	1.2	0.37(0.02-6.38)	0.30	4.85
*A*26:01*	7	6.6	67	8.0	0.82(0.36-1.83)	0.14	2.28
*A*26:02*	3	2.8	12	1.4	2.01(0.56-7.24)	0.15	2.47
*A*26:03*	5	4.7	22	2.6	1.84(0.68-4.97)	0.10	1.67
*A*31:01*	12	11.3	67	8.0	1.47(0.77-2.82)	0.24	3.85
*A*33:03*	4	3.8	76	9.1	0.39(0.14-1.10)	0.03	0.42

[a]alleles: risk alleles with frequencies of less than 1% in both cases and controls are omitted.
[b]OR : Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[c]P-values for allele frequency comparisons between cases and controls using the chi-square test.
Pc: Corrected P-value for the number of alleles tested.

Table 5. Association analysis of HLA-B alleles with IMN in the first set

HLA-B alleles[a]	Allele frequency						
	IMN (2n=106)		Control (2n=838)		OR (95% CI)[b]	P-value[c]	Pc
	No	%	No	%			
*B*07:02*	1	0.9	57	6.8	0.13(0.02-0.95)	6.33E-03	0.11
*B*13:01*	2	1.9	13	1.6	1.22(0.27-5.50)	0.28	5.11
*B*15:01*	15	14.2	71	8.5	1.79(0.98-3.25)	0.05	0.98
*B*15:18*	4	3.8	14	1.7	2.31(0.75-7.16)	0.09	1.64
*B*35:01*	15	14.2	67	8.0	1.90(1.04-3.47)	0.03	0.60
*B*39:01*	3	2.8	34	4.1	0.69(0.21-2.29)	0.19	3.49
*B*40:01*	5	4.7	46	5.5	0.85(0.33-2.20)	0.18	3.22
*B*40:02*	12	11.3	57	6.8	1.75(0.91-3.39)	0.09	1.63
*B*40:06*	4	3.8	34	4.1	0.93(0.32-2.67)	0.21	3.75
*B*44:03*	3	2.8	68	8.1	0.33(0.10-1.07)	0.02	0.40
*B*46:01*	4	3.8	38	4.5	0.83(0.29-2.37)	0.20	3.53
*B*48:01*	3	2.8	22	2.6	1.08(0.32-3.68)	0.24	4.32
*B*51:01*	8	7.5	71	8.5	0.88(0.41-1.89)	0.15	2.62
*B*52:01*	12	11.3	80	9.5	1.21(0.64-2.31)	0.56	10.01
*B*54:01*	5	4.7	65	7.8	0.59(0.23-1.50)	0.09	1.67
*B*55:02*	0	0.0	20	2.4	0.19(0.01-3.13)	0.09	1.63
*B*59:01*	1	0.9	16	1.9	0.49(0.06-3.74)	0.28	5.13
*B*67:01*	3	2.8	11	1.3	2.20(0.60-8.00)	0.14	2.50

[a]alleles: risk alleles with frequencies of less than 1% in both cases and controls are omitted.
[b]OR : Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[c]P-values for allele frequency comparisons between cases and controls using the chi-square test.
Pc: Corrected P-value for the number of alleles tested.

Table 6. Association analysis of *HLA-C* alleles with IMN in the first set

HLA-C alleles[a]	Allele frequency						
	IMN (2n=106)		Control (2n=838)		OR (95% CI)[b]	P-value[c]	Pc
	No	%	No	%			
*Cw*01:02*	9	8.5	139	16.6	0.47(0.23-0.95)	0.01	0.12
*Cw*03:03*	22	20.8	113	13.5	1.68(1.01-2.80)	0.04	0.51
*Cw*03:04*	16	15.1	105	12.5	1.24(0.70-2.20)	0.45	5.41
*Cw*04:01*	6	5.7	42	5.0	1.14(0.47-2.75)	0.17	2.03
*Cw*07:02*	10	9.4	121	14.4	0.62(0.31-1.22)	0.16	1.95
*Cw*07:04*	5	4.7	7	0.8	5.89(1.84-18.91)	5.79E-03	0.07
*Cw*08:01*	8	7.5	48	5.7	1.35(0.62-2.93)	0.12	1.44
*Cw*08:03*	3	2.8	12	1.4	2.01(0.56-7.24)	0.15	1.85
*Cw*12:02*	12	11.3	81	9.7	1.20(0.63-2.28)	0.58	7.01
*Cw*14:02*	5	4.7	50	6.0	0.78(0.30-2.01)	0.16	1.97
*Cw*14:03*	3	2.8	69	8.2	0.33(0.10-1.05)	0.02	0.24
*Cw*15:02*	5	4.7	31	3.7	1.29(0.49-3.40)	0.17	2.04

[a]alleles: risk alleles with frequencies of less than 1% in both cases and controls are omitted.
[b]OR : Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[c]P-values for allele frequency comparisons between cases and controls using the chi-square test.
Pc: Corrected P-value for the number of alleles tested.

Table 7. Association analysis of *HLA-DRB1* alleles with IMN in the first set

HLA-DRB1 alleles[a]	Allele frequency						
	IMN (2n=106)		Control (2n=838)		OR (95% CI)[b]	P-value[c]	Pc
	No	%	No	%			
*DRB1*01:01*	2	1.9	57	6.8	0.26(0.06-1.10)	0.02	0.46
*DRB1*04:01*	3	2.8	10	1.2	2.42(0.65-8.93)	0.12	2.58
*DRB1*04:03*	2	1.9	24	2.9	0.65(0.15-2.81)	0.24	4.97
*DRB1*04:05*	7	6.6	122	14.6	0.42(0.19-0.92)	0.01	0.18
*DRB1*04:06*	5	4.7	28	3.3	1.44(0.54-3.80)	0.15	3.20
*DRB1*04:10*	1	0.9	12	1.4	0.66(0.08-5.11)	0.35	7.38
*DRB1*08:02*	5	4.7	32	3.8	1.25(0.48-3.28)	0.18	3.68
*DRB1*08:03*	6	5.7	63	7.5	0.74(0.31-1.75)	0.13	2.83
*DRB1*09:01*	13	12.3	128	15.3	0.78(0.42-1.43)	0.42	8.77
*DRB1*11:01*	6	5.7	23	2.7	2.13(0.85-5.36)	0.06	1.27
*DRB1*12:01*	2	1.9	30	3.6	0.52(0.12-2.20)	0.18	3.69
*DRB1*12:02*	1	0.9	18	2.1	0.43(0.06-3.29)	0.25	5.26
*DRB1*13:01*	2	1.9	6	0.7	2.67(0.53-13.42)	0.17	3.63
*DRB1*13:02*	3	2.8	65	7.8	0.35(0.11-1.13)	0.03	0.59
*DRB1*14:03*	0	0.0	11	1.3	0.34(0.02-5.79)	0.27	5.64
*DRB1*14:05*	2	1.9	17	2.0	0.93(0.21-4.09)	0.29	6.04
*DRB1*14:06*	3	2.8	13	1.6	1.85(0.52-6.61)	0.17	3.55
*DRB1*14:54*	6	5.7	26	3.1	1.88(0.75-4.67)	0.08	1.71
*DRB1*15:01*	21	19.8	67	8.0	2.85(1.66-4.89)	7.72E-05	1.62E-03
*DRB1*15:02*	11	10.4	70	8.4	1.27(0.65-2.49)	0.48	10.05
*DRB1*16:02*	3	2.8	2	0.2	12.2(2.02-73.9)	0.01	0.23

[a]alleles: risk alleles with frequencies of less than 1% in both cases and controls are omitted.
[b]OR : Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[c]P-values for allele frequency comparisons between cases and controls using the chi-square test.
Pc: Corrected P-value for the number of alleles tested.

Table 8. Association analysis of *HLA-DQB1* alleles with IMN in the first set

HLA-DQB1 alleles[a]	Allele frequency						
	IMN (2n=106)		Control (2n=838)		OR (95% CI)[b]	P-value[c]	Pc
	No	%	No	%			
*DQB1*03:01*	17	16.0	99	11.8	1.43(0.82-2.50)	0.21	2.50
*DQB1*03:02*	12	11.3	77	9.2	1.26(0.66-2.41)	0.47	5.69
*DQB1*03:03*	13	12.3	134	16.0	0.74(0.40-1.35)	0.32	3.88
*DQB1*04:01*	7	6.6	122	14.6	0.42(0.19-0.92)	0.01	0.10
*DQB1*04:02*	1	0.9	26	3.1	0.30(0.04-2.22)	0.14	1.63
*DQB1*05:01*	3	2.8	63	7.5	0.36(0.11-1.16)	0.03	0.39
*DQB1*05:02*	5	4.7	17	2.0	2.40(0.87-6.64)	0.06	0.73
*DQB1*05:03*	7	6.6	30	3.6	1.91(0.82-4.46)	0.06	0.77
*DQB1*06:01*	17	16.0	133	15.9	1.02(0.59-1.76)	0.96	11.48
*DQB1*06:02*	19	17.9	65	7.8	2.60(1.49-4.55)	5.12E-04	6.15E-03
*DQB1*06:03*	2	1.9	6	0.7	2.67(0.53-13.42)	0.17	2.07
*DQB1*06:04*	3	2.8	63	7.5	0.36(0.11-1.16)	0.03	0.39

[a]alleles: risk alleles with frequencies of less than 1% in both cases and controls are omitted.
[b]OR : Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[c]P-values for allele frequency comparisons between cases and controls using the chi-square test.
Pc: Corrected P-value for the number of alleles tested.

Table 9. Association analysis of *HLA-DPB1* alleles with IMN in the first set

HLA-DPB1 alleles[a]	Allele frequency						
	IMN (2n=106)		Control (2n=838)		OR (95% CI)[b]	P-value[c]	Pc
	No	%	No	%			
*DPB1*02:01*	26	24.5	211	25.2	0.97(0.61-1.55)	0.89	9.84
*DPB1*02:02*	2	1.9	35	4.2	0.44(0.10-1.87)	0.13	1.42
*DPB1*03:01*	6	5.7	36	4.3	1.34(0.55-3.26)	0.15	1.61
*DPB1*04:01*	2	1.9	51	6.1	0.30(0.07-1.24)	0.04	0.41
*DPB1*04:02*	7	6.6	83	9.9	0.64(0.29-1.43)	0.08	0.93
*DPB1*05:01*	45	42.5	322	38.4	1.19(0.79-1.79)	0.41	4.53
*DPB1*06:01*	2	1.9	5	0.6	3.21(0.62-16.76)	0.15	1.60
*DPB1*09:01*	12	11.3	66	7.9	1.50(0.78-2.87)	0.22	2.44
*DPB1*13:01*	0	0.0	12	1.4	0.31(0.02-5.29)	0.24	2.62
*DPB1*14:01*	2	1.9	10	1.2	1.60(0.34-7.38)	0.25	2.80
*DPB1*19:01*	2	1.9	5	0.6	3.21(0.62-16.76)	0.15	1.60

[a]alleles: risk alleles with frequencies of less than 1% in both cases and controls are omitted.
[b]OR : Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.
[c]P-values for allele frequency comparisons between cases and controls using the chi-square test.
Pc: Corrected P-value for the number of alleles tested.

Table 10. Replication and Combined analysis of significant *HLA-DRB1* alleles

	Replication analysis							Combined analysis						
HLA-DRB1	IMN (2n=258)		Control (2n=772)		OR (95% CI)[a]	P-value[b]	P c	IMN (2n=364)		Control (2n=1610)		OR (95% CI)[a]	P-value[b]	P c	P (RPE)
alleles	No	%	No	%				No	%	No	%				
*DRB1*01:01*	11	4.3	59	7.6	0.54(0.28-1.04)	0.06	ns	13	3.6	116	7.2	0.48(0.27-0.86)	0.01	0.33	0.03
*DRB1*04:01*	9	3.5	11	1.4	2.50(1.02-6.10)	0.03	0.77	12	3.3	21	1.3	2.58(1.26-5.29)	7.42E-03	0.22	5.07E-03
*DRB1*04:05*	24	9.3	79	10.2	0.90(0.56-1.45)	0.67	ns	31	8.5	201	12.5	0.65(0.44-0.97)	0.03	0.98	
*DRB1*11:01*	12	4.7	15	1.9	2.46(1.14-5.33)	0.02	0.53	18	4.9	38	2.4	2.15(1.21-3.82)	7.31E-03	0.21	8.54E-04
*DRB1*13:02*	9	3.5	63	8.2	0.41(0.20-0.83)	3.49E-03	0.10	12	3.3	128	8.0	0.39(0.22-0.72)	1.79E-03	0.05	0.02
*DRB1*14:54*	16	6.2	23	3.0	2.15(1.12-4.14)	0.02	0.55	22	6.0	49	3.0	2.05(1.22-3.43)	5.50E-03	0.16	1.00E-04
*DRB1*15:01*	52	20.2	54	7.0	3.36(2.22-5.06)	1.71E-09	4.97E-08	73	20.1	121	7.5	3.09(2.25-4.24)	3.94E-13	1.14E-11	

[a]OR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.

[b]P-value for allele or genotype frequency comparisons between cases and controls using the chi-square test.

P c: Corrected P-value for the number of alleles tested.

RPE: Relative predispositional effects.

RPE were tested by sequential elimination of carriers of each of the alleles *DRB1*15:01*, *DRB1*14:54* and *DRB1*11:01*.

Table 11. Replication and Combined analysis of significant *HLA-DQB1* alleles

	Replication analysis							Combined analysis							
HLA-DQB1	IMN (n=258)		Control (n=772)		OR (95% CI)[a]	P-value[b]	P c	IMN (n=364)		Control (n=1610)		OR (95% CI)[a]	P-value[b]	P c	P (RPE)
alleles	No	%	No	%				No	%	No	%				
*DQB1*03:01*	41	15.9	84	10.9	1.55(1.03-2.32)	0.03	0.46	58	15.9	183	11.4	1.48(1.07-2.04)	0.02	0.23	3.11E-03
*DQB1*04:01*	23	8.9	76	9.8	0.90(0.55-1.46)	0.66	ns	30	8.2	198	12.3	0.64(0.43-0.96)	0.03	0.40	
*DQB1*05:01*	11	4.3	60	7.8	0.53(0.27-1.02)	0.05	ns	14	3.8	123	7.6	0.48(0.27-0.85)	0.01	0.14	
*DQB1*05:02*	11	4.3	14	1.8	2.41(1.08-5.38)	0.03	0.38	16	4.4	31	1.9	2.34(1.27-4.33)	5.24E-03	0.07	9.86E-05
*DQB1*05:03*	16	6.2	32	4.1	1.53(0.82-2.83)	0.17	ns	23	6.3	62	3.9	1.68(1.03-2.76)	0.04	ns	2.96E-03
*DQB1*06:02*	51	19.8	50	6.5	3.56(2.34-5.41)	5.14E-10	7.20E-09	70	19.2	115	7.1	3.10(2.24-4.27)	8.90E-13	1.25E-11	
*DQB1*06:04*	9	3.5	54	7.0	0.48(0.23-0.99)	0.01	0.20	12	3.3	117	7.3	0.44(0.24-0.80)	5.64E-03	0.08	0.04

[a]OR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%.

[b]P-value for allele or genotype frequency comparisons between cases and controls using the chi-square test.

P c: Corrected P-value for the number of alleles tested.

RPE: Relative predispositional effects.

RPE were tested by sequential elimination of carriers of each of the alleles *DQB1*06:02*, *DQB1*05:02*, *DQB1*03:01* and *DQB1*05:03*.

Table 12. Frequency disribution of DRB1-DQB1haplotypes in Japanese IMN patients and controls

DRB1-DQB1 haplotypes	IMN (n=182)		Control (n=799)		OR (95% CI)[a]	P-value[b]
	No	%	No	%		
*DRB1*01:01-DQB1*05:01*	13	7.1%	115	14.4%	0.48(0.27-0.86)	0.01
*DRB1*04:01-DQB1*03:01*	12	6.6%	20	2.5%	2.71(1.31-5.59)	5.16E-03
*DRB1*04:03-DQB1*03:02*	6	3.3%	46	5.8%	0.57(0.24-1.34)	0.07
*DRB1*04:05-DQB1*04:01*	30	16.5%	194	24.3%	0.65(0.44-0.98)	0.04
*DRB1*04:06-DQB1*03:02*	14	7.7%	61	7.6%	1.01(0.56-1.83)	0.96
*DRB1*04:10-DQB1*04:02*	2	1.1%	21	2.6%	0.42(0.1-1.79)	0.12
*DRB1*08:02-DQB1*03:02*	7	3.8%	33	4.1%	0.94(0.41-2.13)	0.16
*DRB1*08:02-DQB1*04:02*	5	2.7%	28	3.5%	0.79(0.3-2.05)	0.17
*DRB1*08:03-DQB1*06:01*	18	9.9%	124	15.5%	0.62(0.37-1.03)	0.06
*DRB1*09:01 DQB1*03:03*	35	19.2%	222	27.8%	0.66(0.46-0.97)	0.03
*DRB1*10:01 DQB1*05:01*	1	0.5%	8	1.0%	0.55(0.07-4.42)	0.32
*DRB1*11:01 DQB1*03:01*	18	9.9%	35	4.4%	2.34(1.31-4.17)	3.21E-03
*DRB1*12:01 DQB1*03:01*	7	3.8%	37	4.6%	0.83(0.37-1.88)	0.15
*DRB1*12:01 DQB1*03:03*	4	2.2%	10	1.3%	1.77(0.55-5.69)	0.15
*DRB1*12:02 DQB1*03:01*	3	1.6%	27	3.4%	0.49(0.15-1.61)	0.10
*DRB1*13:01 DQB1*06:03*	3	1.6%	8	1.0%	1.66(0.44-6.29)	0.20
*DRB1*13:02 DQB1*06:04*	12	6.6%	116	14.5%	0.44(0.24-0.8)	6.10E-03
*DRB1*13:02 DQB1*06:09*	0	0.0%	11	1.4%	0.19(0.01-3.24)	0.11
*DRB1*14:03 DQB1*03:01*	4	2.2%	27	3.4%	0.65(0.23-1.87)	0.15
*DRB1*14:05 DQB1*05:03*	11	6.0%	29	3.6%	1.7(0.84-3.43)	0.14
*DRB1*14:06 DQB1*03:01*	9	4.9%	25	3.1%	1.6(0.74-3.47)	0.08
*DRB1*14:54 DQB1*05:02*	11	6.0%	16	2.0%	3.1(1.43-6.73)	2.67E-03
*DRB1*14:54 DQB1*05:03*	11	6.0%	32	4.0%	1.53(0.77-3.07)	0.22
*DRB1*15:01 DQB1*03:01*	4	2.2%	2	0.3%	8.92(1.63-48.87)	0.01
*DRB1*15:01 DQB1*06:02*	69	37.9%	114	14.3%	3.07(2.22-4.24)	1.89E-12
*DRB1*15:02 DQB1*06:01*	42	23.1%	172	21.5%	1.09(0.76-1.56)	0.64
*DRB1*16:02 DQB1*05:02*	5	2.7%	9	1.1%	2.47(0.82-7.42)	0.07

CI: lower and upper limits of confidence interval at 95%.

[b]P-value for allele or genotype frequency comparisons between cases and controls using the chi-square test. Haplotypes with frequencies of < 1% in both cases and controls are omitted.

Table 13. Interaction analysis between *HLA-DRB1*15:01-DQB1*06:02* haplotype and *PLA2R1* risk variants

HLA	*PLA2R1*	IMN (N=182)		Controls (N=799)		OR (95%CI)	P-value	RERI
		No	%	No	%			
*DRB1*15:01-DQB1*06:02*	rs1511223: A/A							
+	+	45	25.4	44	5.5	9(5.32-15.24)	1.25E-19	5.68
+	-	17	9.6	65	8.2	2.3(1.23-4.3)	7.44E-03	
-	+	74	41.8	323	40.7	2.02(1.34-3.04)	6.78E-04	
-	-	41	23.2	361	45.5	1		
*DRB1*15:01-DQB1*06:02*	rs35771982: G/G							
+	+	41	23.2	27	3.4	15.91(8.94-28.3)	2.76E-29	10.88
+	-	21	11.9	82	10.4	2.68(1.52-4.75)	4.74E-04	
-	+	71	40.1	222	28.0	3.35(2.23-5.04)	1.78E-09	
-	-	44	24.9	461	58.2	1		
*DRB1*15:01-DQB1*06:02*	rs2715928: A/A							
+	+	33	18.6	15	1.9	17.53(9.03-34.03)	4.26E-26	13.72
+	-	29	16.4	94	11.9	2.46(1.5-4.02)	2.35E-04	
-	+	51	28.8	173	21.8	2.35(1.56-3.53)	2.68E-05	
-	-	64	36.2	510	64.4	1		
*DRB1*15:01-DQB1*06:02*	rs16844715: C/C							
+	+	34	19.2	18	2.3	15.91(8.49-29.79)	2.30E-26	11.34
+	-	28	15.8	91	11.5	2.59(1.58-4.26)	1.13E-04	
-	+	51	28.8	144	18.2	2.98(1.98-4.5)	1.98E-07	
-	-	64	36.2	539	68.1	1		
*DRB1*15:01-DQB1*06:02*	rs3749119: C/C							
+	+	41	23.2	27	3.4	15.86(8.9-28.26)	6.42E-29	10.98
+	-	21	11.9	82	10.4	2.67(1.51-4.74)	5.21E-04	
-	+	72	40.7	234	29.5	3.21(2.13-4.84)	7.29E-09	
-	-	43	24.3	449	56.7	1		

RERI: Relative Excess Risk due to Interaction.

FIGURES

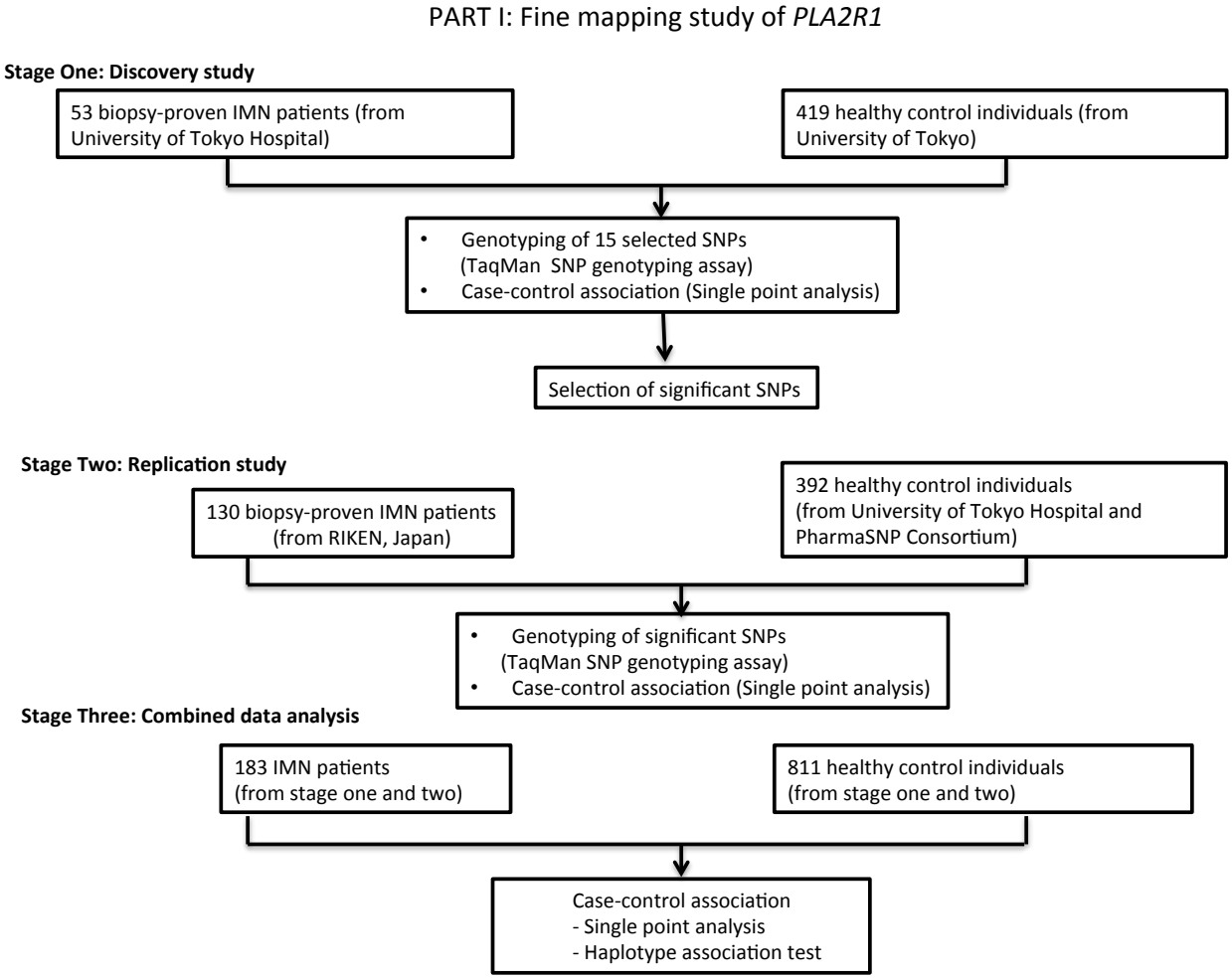

Figure 1. Schematic presentation of overflow of fine mapping study of PLA2R1 in Japanese IMN patients and controls

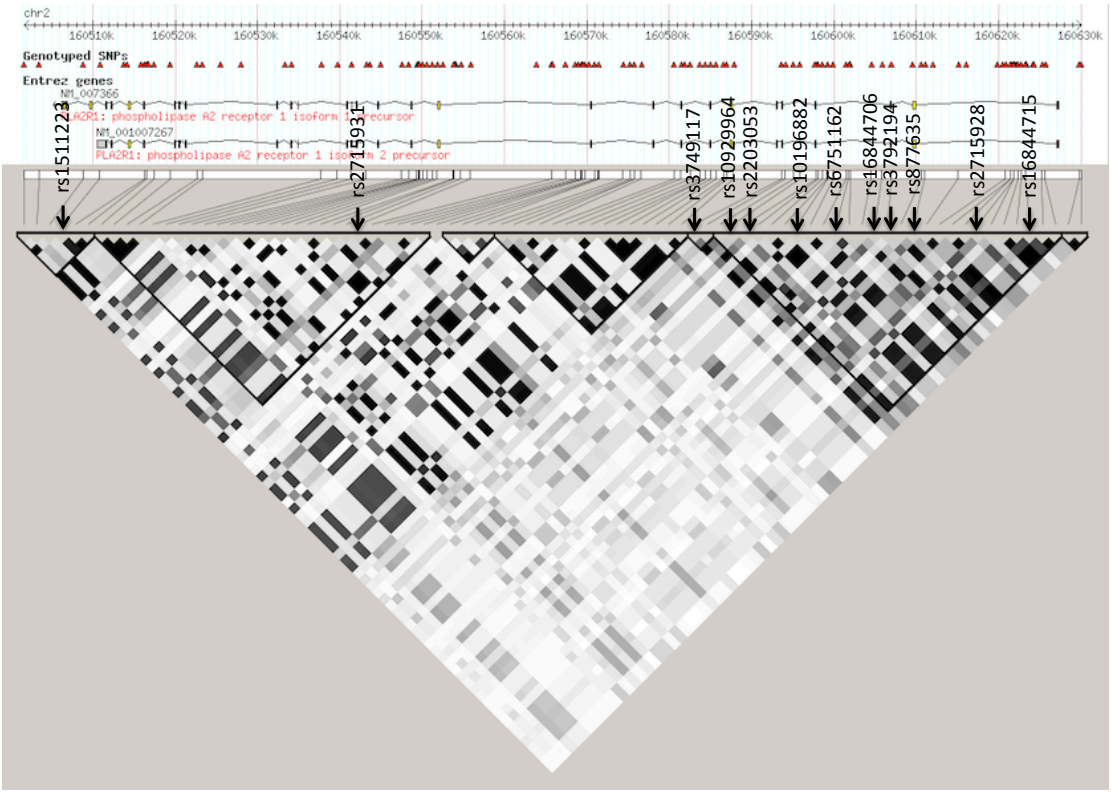

Figure 2: Location of tag SNPs at PLA2R1 locus. LD block images with approximate locations of the 12 SNP markers selected in this study, recombination spots and LD patterns within JPT LD plot of the PLA2R1 gene.

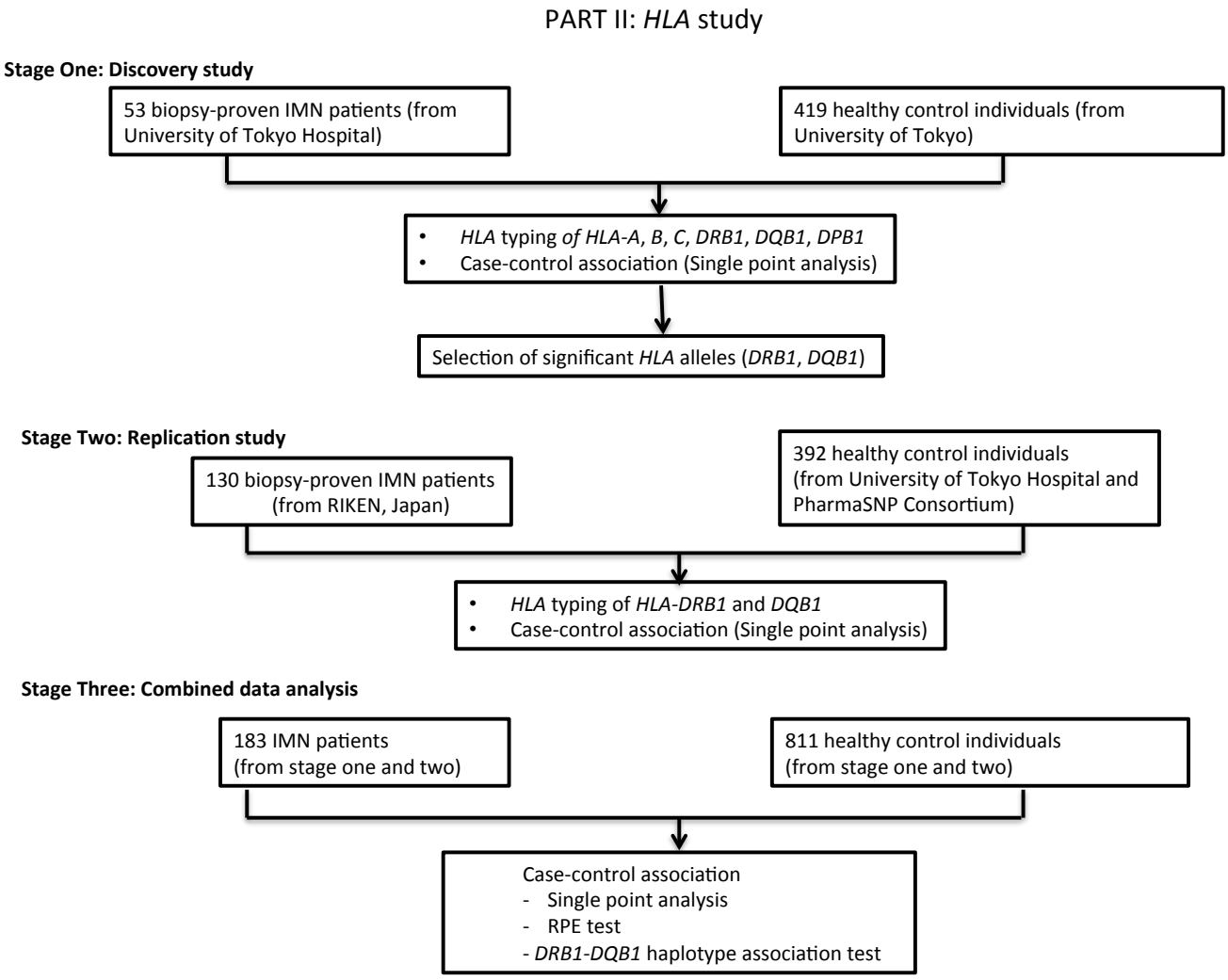

Figure 3. Schematic presentation of overflow of HLA study of Japanese IMN patients and controls

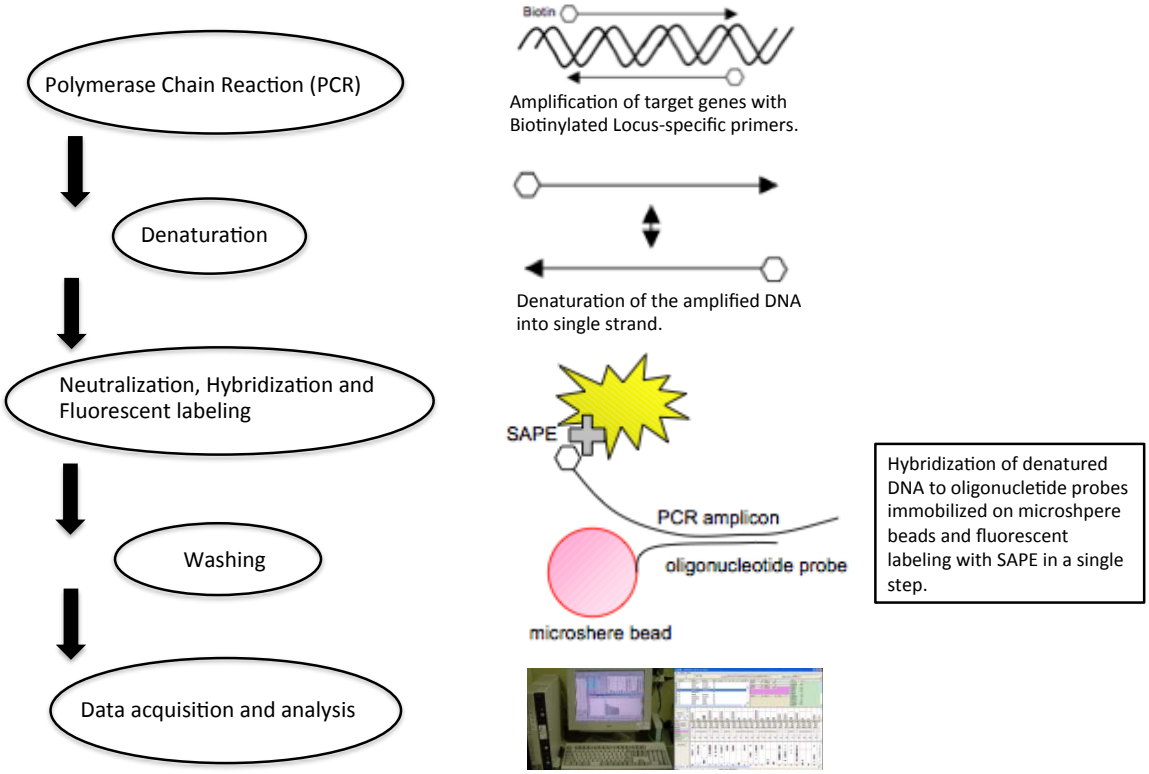

Figure 4. Schematic presentation of overview process in HLA typing using WAKFlow HLA typing kit (Figure modified from http://www.wakunagahla.jp/english/products/wakflow/index.html)