High-density Association Mapping and Interaction Analysis of PLA2R1 and HLA Regions with Idiopathic Membranous Nephropathy in Japanese

Myo Thiri1, Kenjiro Honda2, Koichi Kashiwase3, Akihiko Mabuchi1, Hodaka Suzuki4, Kimio Watanabe4, Masaaki Nakayama4, Tsuyoshi Watanabe1, Kent Doi2, Katsushi Tokunaga1 & Eisei Noiri2,5

Although recent studies showed anti-PLA2R antibody plays a crucial role in idiopathic membranous nephropathy (IMN), detailed HLA mapping and interaction between the HLA genes and PLA2R1 have not been investigated in IMN. We genotyped across the PLA2R1 gene and the HLA region, using 183 IMN patients and 811 healthy controls. Five SNPs around the PLA2R1 gene were significantly associated with IMN. In addition to the two SNPs previously reported to be strongly associated with IMN, rs3749119 and rs35771982 (OR 3.02 and 2.93, P = 3.24E-14 and 4.64E-14, respectively), two novel intronic SNPs (rs2715928 and rs16844715) were also identified as IMN-associated SNPs (OR = 2.30 and 2.51, P = 3.15E-10 and 5.66E-13, respectively). In the HLA gene analysis, DRB1*1501 and DQB1*0602 were strongly associated with IMN (P = 1.14E-11 and 1.25E-11, respectively). The interaction was strongest between HLA-DRB1*15:01 - HLA-DQB1*06:02 and the intronic SNP rs2715928 (OR = 17.53, P = 4.26E-26). Furthermore, positive interaction was also observed between HLA-DRB1*15:01 - HLA-DQB1*06:02 and the missense SNP rs35771982 (OR = 15.91, P = 2.76E-29), which is in strong linkage disequilibrium with 5′UTR SNP rs3749119, and intronic SNP rs16844715 (OR = 15.91, P = 2.30E-26) for IMN. Neither HLA-DRB1*15:01 nor HLA-DQB1*06:02 was associated with steroid responsiveness, overall survival and renal survival during the observation period of mean 11 years though limited number of analysis.

Idiopathic membranous nephropathy (IMN) is one of the most common causes of adult nephrotic syndrome. It is characterized by subepithelial immune complex deposition of glomerular basement membrane. The first antigen neural endopeptidase, type II transmembrane glycoprotein, was identified in neonatal membranous nephropathy (MN) cases with neural endopeptidase-deficient mothers. The second autoantigen is the M-type phospholipase A2 receptor (PLA2R), identified in the adult patients with IMN. PLA2R is found in the sera of 75% of IMN patients, but not in that of secondary MN or any other disorders of renal and autoimmune.

Recent genome-wide association studies (GWAS) including three studies in European populations have identified that the variations in PLA2R1 on chromosome 2 and HLA-DQA1 on chromosome 6 show susceptibility to IMN. Interestingly, Stanescu et al. also reported a strong genetic interaction of risk alleles at both the HLA and PLA2R1 regions, although relatively modest risk of IMN was identified at each locus. Association of HLA-DQA1 and PLA2R1 with IMN was also reported by case-control candidate gene studies in Korea, Taiwan and China.

1Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan. 2Department of Nephrology and Endocrinology, The University of Tokyo Hospital, Tokyo, Japan. 3Japanese Red Cross Kanto-Koshinetsu Block Blood Center, Tokyo, Japan. 4Department of Nephrology, Hypertension, Diabetology, Endocrinology and Metabolism, Fukushima Medical University School of Medicine, Fukushima, Japan. 5Department of Hemodialysis and Apheresis, The University of Tokyo Hospital, Tokyo, Japan. Correspondence and requests for materials should be addressed to E.N. (email: noiri-tky@umin.ac.jp)
In Japan, IMN was reported to account for 77.9% of total MN patients, while MN was present in 36.8% of primary nephrotic syndrome patients. However, not many genetic studies of IMN in Japanese population have been conducted. Although previous studies have reported the effect of single locus and the genetic interaction of SNPs in \( \text{PLA2R1} \) and \( \text{HLA-DQA1} \), no study has performed the high-density association mapping of both the \( \text{PLA2R1} \) gene and \( \text{HLA} \) genes to date for identifying the primary polymorphisms and it is still not known the interaction effect of \( \text{PLA2R1} \) risk variants and fully detailed analysis of \( \text{HLA} \)-gene alleles.

**Results**

**\( \text{PLA2R1} \) association with IMN.** Association analysis of \( \text{PLA2R1} \) SNPs in the first set of IMN patients and healthy controls. Fine mapping of \( \text{PLA2R1} \) SNPs was performed in the first stage of the study including 53 IMN patients and 419 healthy controls. The characteristics and clinical information of 53 IMN cases were described in Table 1. Of the 15 SNPs genotyped, two SNPs failed in genotyping and were excluded from the study. Significant deviations from Hardy-Weinberg equilibrium (\( P < 0.05 \)) were not observed for any of the 13 SNPs. Of the 13 SNPs included in the association analysis, we found 9 SNPs significantly associated with IMN (\( P < 0.05 \)) (bold in Supplementary Table 1). When we 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 were successfully replicated 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 (\( P = 4.51 \times 10^{-15}, \ OR = 2.93 \) and \( P = 1.99 \times 10^{-15}, \ OR = 3.02 \), respectively) (Table 2). Two intronic SNPs, rs2715928 (\( P = 1.14 \times 10^{-10}, \ OR = 2.30 \)) and rs16844715 (\( P = 7.90 \times 10^{-13}, \ OR = 2.51 \)), also showed significant associations with IMN.

All the SNPs that were consistently significant in all data sets were selected in haplotype analysis. Analysis of linkage disequilibrium (LD) pattern showed high LD (\( r^2 = 0.8 \)) between rs35771982, missense SNP located in exon 5, and rs3749119 located in 5' UTR region of \( \text{PLA2R1} \). Risk haplotype composed of risk alleles (G for rs35771982, A for rs2715928 and C for rs16844715) exhibited strong association with IMN (\( P = 7.30 \times 10^{-13}, \ OR = 2.29 \)), while protective haplotype including protective alleles (C for rs35771982, G for rs2715928 and T for rs16844715) was found to be protective to IMN (\( P = 1.84 \times 10^{-14}, \ OR = 0.34 \)). (see Supplementary Table S2).

**Association of \( \text{HLA} \) genes with IMN.** Association analysis of \( \text{HLA} \) genes in the first set of IMN patients and healthy controls. In the discovery stage by full detail analysis, \( \text{HLA-A}, \ -\text{B}, \ -\text{C}, \ -\text{DRB1}, \ -\text{DQB1} \) and \( -\text{DPB1} \) genotypes were determined in a total of 53 IMN patients and 419 healthy controls. Regarding \( \text{HLA} \) class I genes, \( \text{HLA-A}^{*33:03} \) showed marginal association (\( P = 0.03, \ OR = 0.40 \)) with IMN (see Supplementary Table S3).

| Age (yr) | 61 ± 15 |
|---------|---------|
| Sex (M:F) | 30:23 |
| sCre at KBx | 0.92 ± 0.36 |
| sCre after 1 yr | 1.06 ± 0.74 |
| sAlb at KBx | 2.33 ± 0.44 |
| sAlb after 1 yr | 3.23 ± 0.95 |
| Upro at KBx | 6.28 ± 3.35 |
| Upro after 1 yr | 2.12 ± 2.66 |

Table 1. Patient Characteristics and clinical course of the discovery set (\( N = 53 \)). Age (yr): Age at kidney biopsy. sCre at KBx: serum creatinine at kidney biopsy; sCre after 1 yr: serum creatinine one year after kidney biopsy; sAlb at KBx: serum albumin at kidney biopsy; sAlb after 1 yr: serum albumin one year after kidney biopsy; Upro at KBx: ratio of urine protein to creatinine (g/g) at kidney biopsy; Upro after 1 yr: ratio of urine protein to creatinine (g/g) one year after kidney biopsy; Development to ESRD: development to end stage renal disease during observation period; 50% increase of sCre: 50% increase of serum creatinine during observation period; Improvement to Upro < 1 g/gCre: improvement to ratio of urine protein to creatinine (g/g) less than 1.
Table 2. Replication and Combined analysis of significant PLA2R1 SNPs. ^aOR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%. ^bP-values after Bonferroni correction and age/sex adjustment for allele frequency comparisons between cases and controls using the chi-square test.

| SNP            | Position | Risk allele | Replication analysis | Combined analysis |
|----------------|----------|-------------|----------------------|-------------------|
|                | Case (n=130) | Control (n=386) | OR (95% CI) ^a | P-value ^a corrected | Case (n=183) | Control (n=805) | OR (95% CI) ^a | P-value ^a corrected |
|                | No % | No % | OR (95% CI) ^a | P-value ^a corrected | No % | No % | OR (95% CI) ^a | P-value ^a corrected |
| rs1511223 | 3’ UTR | A | 203 79.3 | 536 70.9 | 1.57 (1.12-2.21) | 1.57E-01 | 293 80.5 | 1105 69.7 | 1.80 (1.36-2.38) | 1.08E-03 |
| rs35771982 | H [His] ⇒ D [Asp] | G | 200 78.7 | 450 59.1 | 2.57 (1.84-3.58) | 1.88E-08 | 289 79.8 | 919 57.5 | 2.93 (2.22-3.85) | 4.51E-15 |
| rs10196882 | intronic | T | 52 20.8 | 119 15.7 | 1.41 (0.98-2.02) | N.S. | 82 23.0 | 245 15.5 | 1.63 (1.23-2.16) | 2.95E-02 |
| rs877635 | intronic | A | 69 27.2 | 200 26.5 | 1.03 (0.75-1.42) | N.S. | 123 34.0 | 403 25.3 | 1.52 (1.19-1.94) | 4.78E-02 |
| rs2715928 | intronic | A | 185 71.7 | 385 51.7 | 2.36 (1.74-3.21) | 1.63E-08 | 251 70.1 | 775 50.5 | 2.30 (1.79-2.94) | 1.14E-10 |

Tendency of negative association was observed in HLA-B*07:02 with IMN while positive association was found with HLA-B*35:01 (see Supplementary Table S4). HLA-C*0704 exhibited a high odds ratio for IMN (P = 5.79E-03, OR = 5.89; see Supplementary Table S5). However, 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 = 7.72E-5, OR = 2.85), and it remained to be significant when P-value was corrected for the number of alleles tested (see Supplementary Table S6). Negative association was also observed in HLA-DRB1*04:05. On the other hand, four HLA-DQB1 alleles showed significant associations with IMN. Among them, HLA-DQB1*06:02 survived to be significant after correction for multiple comparisons (P = 5.12E-4, OR = 2.60) (see Supplementary Table S7). No association was observed between HLA-DRB1 alleles and IMN (see Supplementary Table S8).

Replication in the second sample set and the 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 observed for HLA-DRB1*15:01 and HLA-DQB1*06:02 with IMN in the replication stage with P = 1.71E-9, OR = 3.36 and P = 5.14E-10, OR = 3.56, respectively (Tables 3 and 4). 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.

In the combined data analysis, both HLA-DRB1*15:01 and HLA-DQB1*06:02 were confirmed to have strong associations with IMN holding P = 3.94E-13, OR = 3.09 and P = 8.90E-13, OR = 3.1, respectively (Tables 3 and 4). Both HLA-DRB1*15:01 and HLA-DQB1*06:02 remained significant after Bonferroni correction (P = 1.14E-11 and 1.25E-11, respectively).

HLA DRB1-DQB1 haplotype association in the combined data set. The results of HLA-DRB1-DQB1 haplotype association analysis were shown in Table 5. Only those haplotypes with frequencies >1% in either IMN patients or healthy controls were included in the analysis. The most susceptible haplotype was DRB1*15:01-DQB1*06:02 haplotype (P = 1.89E-12, OR = 3.07). Other susceptible haplotypes were DRB1*14:54-DQB1*05:02 haplotype (P = 2.67E-3, OR = 3.1), DRB1*11:01-DQB1*03:01 haplotype (P = 3.21E-3, OR = 2.34) and DRB1*04:01-DQB1*03:01 haplotype (P = 5.16E-3, OR = 2.71). The most protective haplotype was DRB1*13:02-DQB1*06:04 haplotype (P = 6.10E-3, OR = 0.44).

Genetic interaction analysis between PLA2R1 and HLA risk alleles. We analyzed the total of 177 IMN cases and 792 healthy controls to investigate the interactions between HLA-DRB1*15:01-HLA-DQB1*06:02 and PLA2R1 risk alleles.

Interaction analysis exhibited more than additive effects with IMN (Table 6). We observed that the evidence for the interaction was strongest between HLA-DRB1*15:01 - HLA-DQB1*06:02 and the intronic SNP rs2715928 (P = 4.26E-26, OR = 17.53). In addition, positive interaction was also observed between HLA-DRB1*15:01 - HLA-DQB1*06:02 and the missense SNP rs35771982 (P = 2.76E-29, OR = 15.91) that is in strong LD with 5’UTR SNP rs3749119, and intronic SNP rs16844715 (P = 2.30E-26, OR = 15.91) for IMN.

HLA and clinical outcome. During the observation period [median 11 years, interquartile range (IQR) 8.5–13 years], 50% increase of serum creatinine was found in eight patients. Among them, four patients developed to end stage renal disease. Ten patients died of heart failure, infection, and traffic accident. Supplementary Tables S9 and S10 shows overall survival and renal survival according to risk HLA alleles, HLA DRB1*15:01 and HLA DQB1*06:02. Neither risk alleles were associated with clinical outcome. Combination of risk alleles of HLA genes and PLA2R1 were not associated with clinical outcome.
Table 3. Replication and Combined analysis of significant HLA-DRB1 alleles. *OR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%. bP-value: Corrected P-value for the number of alleles tested.

| HLA-DRB1 alleles | IMN (2n = 258) No % | Control (2n = 772) No % | IMN (2n = 364) OR (95% CI) | Control (2n = 1610) No % | P-value | Replication analysis | Combined analysis |
|------------------|----------------------|-------------------------|----------------------------|---------------------------|---------|---------------------|---------------------|
| DRB1*0101        | 11 4.3               | 59 7.6                  | 0.54 (0.28–1.04)           | 13 3.6                     | 1.06    | 0.06                | 0.48 (0.27–0.86)    |
|                 |                      |                         |                            |                           |         |                     | 0.01                |
| DRB1*0401        | 9 3.5                | 11 1.4                  | 2.50 (1.02–6.10)           | 12 3.3                     | 0.03    | 0.77                | 2.58 (1.26–5.29)     |
|                 |                      |                         |                            |                           |         |                     | 0.22                |
| DRB1*0405        | 24 9.3               | 79 10.2                 | 0.90 (0.56–1.45)           | 31 8.5                     | 0.67    | N.S.                | 0.65 (0.44–0.97)     |
|                 |                      |                         |                            |                           |         |                     | 0.98                |
| DRB1*1101        | 12 4.7               | 15 1.9                  | 2.46 (1.14–5.33)           | 4.9                        | 0.02    | 0.53                | 2.15 (1.21–3.82)     |
|                 |                      |                         |                            |                           |         |                     | 0.21                |
| DRB1*1302        | 9 3.5                | 63 8.2                  | 0.41 (0.20–0.83)           | 3.3                        | 0.10    | 0.65                | 0.39 (0.22–0.72)     |
|                 |                      |                         |                            |                           |         |                     | 0.05                |
| DRB1*1454        | 16 6.2               | 23 3.0                  | 2.15 (1.12–4.14)           | 6.0                        | 0.02    | 0.55                | 2.05 (1.22–3.43)     |
|                 |                      |                         |                            |                           |         |                     | 0.16                |
| DRB1*1501        | 52 20.2              | 54 7.6                  | 3.36 (2.22–5.06)           | 20.1                       | 0.71    | 11.4E-09            | 3.09 (2.25–4.24)     |
|                 |                      |                         |                            |                           |         |                     | 3.94E-13            |

Table 4. Replication and Combined analysis of significant HLA-DQB1 alleles. *OR: Odds ratio; 95% CI: lower and upper limits of confidence interval at 95%. bP-value: Corrected P-value for the number of alleles tested.

| HLA-DQB1 alleles | IMN (n = 258) No % | Control (n = 772) No % | IMN (n = 364) OR (95% CI) | Control (n = 1610) No % | P-value | Replication analysis | Combined analysis |
|------------------|---------------------|------------------------|---------------------------|--------------------------|---------|---------------------|---------------------|
| DQB1*0401        | 23 8.9              | 76 9.8                 | 0.90 (0.55–1.46)          | 30 8.2                    | 0.66    | N.S.                | 0.64 (0.43–0.96)     |
|                 |                      |                        |                            |                           |         |                     | 0.40                |
| DQB1*0501        | 11 4.3              | 60 7.8                 | 0.53 (0.27–1.02)          | 14 3.8                    | 0.05    | N.S.                | 0.48 (0.27–0.85)     |
|                 |                      |                        |                            |                           |         |                     | 0.14                |
| DQB1*0502        | 11 4.3              | 14 1.8                 | 2.41 (1.08–5.38)          | 16 4.4                    | 0.03    | 0.38                | 2.34 (1.27–4.33)     |
|                 |                      |                        |                            |                           |         |                     | 0.07                |
| DQB1*0503        | 16 6.2              | 32 4.1                 | 1.53 (0.82–2.83)          | 23 6.3                    | 0.17    | N.S.                | 1.68 (1.03–2.76)     |
|                 |                      |                        |                            |                           |         |                     | 0.04                |
| DQB1*0602        | 51 19.8             | 50 6.5                 | 3.56 (2.34–5.41)          | 70 19.2                   | 0.01    | 0.20                | 3.10 (2.24–4.27)     |
|                 |                      |                        |                            |                           |         |                     | 8.90E-13            |
| DQB1*0604        | 9 3.5               | 54 7.0                 | 0.48 (0.23–0.99)          | 12 3.3                    | 0.01    | 0.20                | 0.44 (0.24–0.80)     |
|                 |                      |                        |                            |                           |         |                     | 5.64E-03            |

Discussion

The genetic association of PLA2R1 and HLA-DQA1 risk alleles with IMN in the Caucasian populations was reported by a GWAS that included three independent GWAS in three populations. Recent studies following this paper selected several SNPs as representative from PLA2R1 and the HLA regions. The diversity of HLA is well-known in human genetics and tightly bound to disease appearance and severity. Admittedly, it has been reported that IMN in Japanese has a better course and outcome. The purpose of this study was to clarify: (i) the primary risk SNPs of PLA2R1 in Japanese population, (ii) HLA typing in full detail, (iii) the interaction between the risk alleles of PLA2R1 and HLA genes, and (iv) clinical outcome according to risk alleles.

We 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 and additional 5 SNPs. In Japanese population, rs35771982 of PLA2R1 is reported to be most strongly associated with IMN. In agreement with Coenen et al. and Liu et al., we found that G allele of non-synonymous SNP rs35771982 (G/C) showed significantly increased risk of developing IMN. While Coenen et al. also reported that C allele of 5′UTR rs3749119 raised the risk of IMN, our data showed rs35771982 is in strong LD with 5′ UTR SNP rs3749119. Although Kim et al. reported that C allele of rs35771982 elevated the risk of IMN, our genotyping data is robust considering that we obtained the significant association of rs1511223 located in 3′ UTR with IMN which similar to a report by Saed et al.

Interestingly, our study identified the new significant associations of two intronic SNPs, rs2715928 and rs16844715. Both are coincidentally located in the first intron of PLA2R1. Our findings suggest that risk haplotypes from the combination of common variants and SNP-SNP interaction within PLA2R1 region may not explain the low occurrence of IMN in general population. In other words, HLA risk alleles possibly explain IMN development together with PLA2R1 risk alleles.

With respect to HLA association with IMN, recent studies have reported the strong association of HLA-DQA1 SNP rs2187668 with IMN in Caucasian and Chinese populations. HLA is highly polymorphic region spanning approximately 7.6 megabase pairs (Mb) of genomic sequence on the short arm of chromosome 6. 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 MN.

Our study identified the new significant association of HLA-DRB1*15:01 and DQB1*06:02 with IMN in Japanese population. These alleles are well known to form a common haplotype, HLA-DRB1*15:01-DQB1*06:02, in Japanese population. No such association with IMN has been reported in other populations. Therefore, this
IMN were reported to be IgG4 antibodies. IgG4 co-localized with PLA2R in the immune complex deposition of IMN in worldwide until today. Not frequent in the Japanese population. Only limited studies have been reported regarding the associations with Japanese IMN patients. HLA-C*07:04 was reported to increase relative risk for carbamazepine-induced cutaneous adverse reactions in HLA-C*07:04 with Japanese IMN patients. Significant after multiple correction. We also showed a potential risk of HLA-C*07:04 with Japanese IMN patients. Our results showed more than additive effects on the risk of IMN among the individuals with risk alleles of PLA2R1, HLA-DRB1*15:01 and DQB1*06:02. In patients with homozygous rs2715928 risk genotype AA, those alleles are associated with IgG4-related IMN has not been identified yet, and how or whether IgG4 may relate to IMN is still uncertain.

Table 5. Frequency distribution of DRB1-DQB1 haplotypes in Japanese IMN patients and controls.

| DRB1-DQB1 haplotypes       | IMN   | Control | OR (95% CI) | P-value |
|----------------------------|-------|---------|-------------|---------|
| DRB1*01:01-DQB1*05:01     | 13    | 115     | 0.48 (0.27–0.86) | 0.01    |
| DRB1*04:01-DQB1*03:01     | 12    | 66      | 2.71 (1.31–5.59) | 0.16    |
| DRB1*04:03-DQB1*03:02     | 6     | 46      | 0.57 (0.24–1.34) | 0.07    |
| DRB1*04:05-DQB1*04:01     | 30    | 165     | 0.65 (0.44–0.98) | 0.04    |
| DRB1*04:06-DQB1*03:02     | 14    | 71      | 1.01 (0.56–1.83) | 0.96    |
| DRB1*04:10-DQB1*04:02     | 2     | 11      | 0.42 (0.1–1.79)  | 0.12    |
| DRB1*08:02-DQB1*03:02     | 7     | 33      | 0.94 (0.41–2.13) | 0.16    |
| DRB1*08:02-DQB1*04:02     | 5     | 28      | 0.79 (0.3–2.05)  | 0.17    |
| DRB1*08:03-DQB1*06:01     | 18    | 124     | 0.62 (0.37–1.03) | 0.06    |
| DRB1*09:01 DQB1*03:03     | 35    | 192     | 0.66 (0.46–0.97) | 0.03    |
| DRB1*10:01 DQB1*06:05     | 1     | 5       | 0.55 (0.07–4.42) | 0.32    |
| DRB1*11:01 DQB1*03:01     | 18    | 99      | 2.34 (1.31–4.17) | 0.03    |
| DRB1*12:01 DQB1*03:03     | 7     | 38      | 0.83 (0.37–1.88) | 0.15    |
| DRB1*12:01 DQB1*03:03     | 4     | 2.2     | 1.77 (0.55–5.69) | 0.15    |
| DRB1*12:02 DQB1*03:01     | 3     | 1.6     | 0.49 (0.15–1.61) | 0.10    |
| DRB1*13:01 DQB1*06:03     | 3     | 1.6     | 1.66 (0.44–6.29) | 0.20    |
| DRB1*13:02 DQB1*06:04     | 12    | 6.6     | 0.44 (0.24–0.8)  | 6.10E-03 |
| DRB1*13:02 DQB1*06:09     | 0     | 0.0     | 0.19 (0.01–3.24) | 0.11    |
| DRB1*14:01 DQB1*03:01     | 4     | 2.2     | 0.65 (0.23–1.87) | 0.15    |
| DRB1*14:05 DQB1*05:03     | 11    | 6.0     | 1.70 (0.84–3.43) | 0.14    |
| DRB1*14:06 DQB1*03:01     | 9     | 4.9     | 1.8 (0.74–3.47)  | 0.08    |
| DRB1*14:54 DQB1*05:02     | 11    | 6.0     | 3.1 (1.43–6.73)  | 2.67E-03 |
| DRB1*14:54 DQB1*05:03     | 11    | 6.0     | 1.53 (0.77–3.07) | 0.22    |
| DRB1*15:01 DQB1*03:01     | 4     | 2.2     | 0.89 (1.63–48.87) | 0.01    |
| DRB1*15:01 DQB1*06:02     | 69    | 37.9    | 3.07 (2.22–4.24) | 1.89E-12 |
| DRB1*15:01 DQB1*06:02     | 42    | 23.1    | 1.09 (0.76–1.56) | 0.64    |
| DRB1*16:02 DQB1*05:02     | 5     | 2.7     | 2.47 (0.82–7.42) | 0.07    |

is the first report of this HLA haplotype in IMN. Because associations of HLA-DRB1*15:01-DQB1*06:02 with various immune disorders have been reported in different populations, it is conceivable that the determined haplotype should be functionally meaningful.

In a French population, increased frequency of HLA-DR3 and decreased frequency of HLA-DMA*01:02 with IMN were observed. 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. However, both DRB1*03:01 and DQB1*02:01 alleles are not frequent in the Japanese population. Only limited studies have been reported regarding the HLA associations of IMN in worldwide until today.

HLA-DRB1 could be associated with anti-PLA2R. Anti-PLA2R antibodies detected in 26 of 37 patients with IMN were reported to be IgG4 antibodies. 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. HLA association with IgG4-related IMN has not been identified yet, and how or whether IgG4 may relate to IMN is still uncertain.

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, we observed a 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 a weak association of HLA-C*01:02 with IMN before correction, although the association ceased to be significant after multiple correction. We also showed a 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. These HLA class I alleles are expected to be assessed in larger number of samples.

Our results showed more than additive effects on the risk of IMN among the individuals with risk alleles of PLA2R1, HLA-DRB1*15:01 and DQB1*06:02. In patients with homozygous rs2715928 risk genotype AA, those who have risk alleles of both HLA-DRB1*15:01 and DQB1*06:02 developed approximately 3.03 times as frequently as those who have one risk allele with either HLA-DRB1*15:01 or DQB1*06:02. This finding may suggest that HLA-DRB1*15:01 and DQB1*06:02 regulate immunological response including PLA2R via CD4-positive T cells.
The associations among renal survival, overall survival, steroid responsiveness, and HLA genes were analyzed in this study. This is because the HLA region may explain that both mild severity of IMN in Japanese and the impact of PLA2R on clinical course of IMN. However, even the strongest association of HLA-DRB1*15:01 and DQB1*06:02 with IMN was not related to renal survival, overall survival and steroid responsiveness. The fact provided that HLA-DRB1*15:01 and -DQB1*06:02 were only associated with IMN development and that they did not influence the better prognosis particularly observed in Japanese IMN patients. This means that HLA-DRB1*15:01 and -DQB1*06:02 are possibly the risk alleles in the other populations as well.

There are some limitations in the present study. Firstly, titers of PLA2R1 antibody was not measured because the DNA samples were obtained more than ten years ago and most of the patients were either deceased or unable for follow-up. However, median of observation period was as long as 11 years, which is sufficient to evaluate overall and renal survival though the number is small. In addition, although anti-PLA2R antibodies are found in Japanese IMN patients, the prevalence is lower than that of other countries. Our report partially explains why PLA2R1 serum level is not so frequently increased in Japanese IMN. The second limitation is the relatively small sample number, because IMN is not a common disease. Japan Renal Biopsy Registry in 2009 and 2010 announced that annual occurrence of total MN is approximately 200. The sample number of the present study is comparable to that of nationwide, and our results are statistically robust.

In summary, our study has identified PLA2R1 risk variants, the HLA risk alleles and haplotypes for association with IMN in Japanese population. Our study is the first report to perform high-density association mapping including the HLA region and reveal the increased risk of interaction effect between PLA2R1 risk variants and HLA risk haplotype in IMN. Individuals with PLA2R1 and HLA-DRB1*15:01–DQB1*06:02 risk alleles have higher risk of developing IMN while they were not associated with overall and renal survival. We also found novel two intronic SNPs (rs2715928 and rs16844715) and confirmed previous findings of PLA2R1 association with IMN.

### Methods

**Human subjects and sample collection.** This study included a total of 994 subjects comprised of healthy controls and cases with (biopsy-proven) IMN. The study was approved by the Ethical Committees at The Faculty of Medicine at The University of Tokyo, BioBank Japan and the Pharma SNP Consortium (Tokyo, Japan). Written informed consent was obtained from each participant before sample collection. The first set of study included 53 biopsy-proven IMN patients from The Tokyo University Hospital and 419 healthy controls residing around Tokyo area. Diagnosis of the IMN

| HLA          | PLA2R1 | IMN (N = 177) | Controls (N = 792) | OR (95%CI) | P-value |
|--------------|--------|---------------|-------------------|-----------|---------|
| DRB1*15:01-DQB1*06:02 | rs1511223: A/A |               |                   |           |         |
| +            | +      | 45 25.4 44 5.5 | 9 (5.32–15.24)    | 1.25E-19  |         |
| +            | −      | 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 23.2 360 45.5 | 1.0 |         |         |
| DRB1*15:01-DQB1*06:02 | rs35771982: G/G |               |                   |           |         |
| +            | +      | 41 23.2 27 3.4 | 15.91 (8.94–28.3) | 2.76E-29  |         |
| +            | −      | 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 | 1.0 |         |         |
| DRB1*15:01-DQB1*06:02 | rs2271928: A/A |               |                   |           |         |
| +            | +      | 33 18.6 15 1.9 | 17.53 (9.03–34.03) | 4.26E-26  |         |
| +            | −      | 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 | 1.0 |         |         |
| DRB1*15:01-DQB1*06:02 | rs16844715: C/C |               |                   |           |         |
| +            | +      | 34 19.2 18 2.3 | 15.91 (8.49–29.79) | 2.30E-26  |         |
| +            | −      | 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 | 1.0 |         |         |
| DRB1*15:01-DQB1*06:02 | rs3749119: C/C |               |                   |           |         |
| +            | +      | 41 23.2 27 3.4 | 15.86 (8.9–28.26) | 6.42E-29  |         |
| +            | −      | 21 11.9 82 10.4 | 2.87 (1.51–4.74) | 5.21E-04  |         |
| −            | +      | 72 40.7 234 29.5 | 3.21 (2.13–4.84) | 7.29E-09  |         |
| −            | −      | 45 24.3 449 56.7 | 1.0 |         |         |

Table 6. Interaction analysis between HLA-DRB1*15:01-DQB1*06:02 haplotype and PLA2R1 risk variants.
cases was established by kidney biopsy together with other routine clinical procedures and the patients with malignancy, chronic infectious disease including hepatitis B and C viruses, and drug induced secondary MN were excluded from the study. The second set of study that included 130 IMN cases from BioBank Japan and 392 healthy controls was recruited for replication purposes from The Tokyo University Hospital and the Pharma SNP Consortium (Tokyo, Japan). Both the discovery and replication studies included the Japanese individuals.

Single nucleotide polymorphism (SNP) selection of the PLA2R1 gene. SNP genotype information of PLA2R1 was downloaded from Phase III of the HapMap JPT population database (http://hapmap.ncbi.nlm.nih.gov/). HapMap data was analyzed using HaploView software (ver 4.1) and tag SNPs were selected by Tagger algorithm implemented in HaploView. SNPs were chosen by applying the following selection criteria: i) minor allele frequency (MAF) threshold of >0.10 in the HapMap JPT population, ii) r² threshold of greater than or equal to 0.8. A total of twelve tag SNPs meeting the criteria were selected together with additional three reported SNPs from previous literatures, to validate their association with IMN in the Japanese population.

Genotyping of PLA2R1 SNPs. 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). Genotyping of the total 15 SNPs were performed 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) by cycling parameters of 95 °C for 3 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. TaqMan SNP genotyping of significant variants was also performed in the replication study including 522 IMN cases and healthy controls.

HLA typing. Genotyping for six HLA genes (HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1) was performed in 53 Japanese IMN patients by the polymerase chain reaction (PCR)-Luminex typing method using the WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan). Briefly, target DNA was amplified by PCR (polymerase chain reaction) with biotinylated primers. 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 was performed automatically using the WAKFlow typing software. For healthy control samples, we utilized HLA data published previously. HLA typing of significant HLA regions, HLA-DRB1 and -DQB1 genes, was performed in the second set of samples for the replication study. HLA-DQA1 alleles were excluded from this study because of its low frequency in Japanese population.

All experiments were performed in accordance with approved guidelines and regulations.

Statistical analysis. Samples with failed genotyping were removed from data analysis. To compare the allele and genotype frequencies between case and control groups, Chi-square test or Fisher’s exact test was applied as appropriate. In interaction analysis of HLA and PLA2R1 risk variants, Chi-square test or Fisher’s exact test was also used. Departure from Hardy-Weinberg equilibrium was tested in all the SNPs by the chi-squared goodness-of-fit test. Association analyses of all the SNPs in PLA2R1 gene were performed by using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/). HaploView software and the sliding window analysis implemented in PLINK were used to infer the linkage disequilibrium structure of PLA2R1 SNPs and to perform a haplotype analysis of the PLA2R1 gene. In the HLA association analysis, HLA allelic associations were analyzed by Chi-square or Fisher’s exact test. HLA haplotype association analysis was performed using Arlequin algorithm. When any of the obtained value was zero, the odds ratio was calculated using Woolf’s correction. Bonferroni corrections were performed by standard method where P values were corrected for the number of alleles tested in each gene. More stringent multiple corrections using total number of 117 SNPs/alleles in PLA2R1, HLA-DRB1 and HLA-DQB1 were added when the SNPs/alleles were statistically significant in both sample sets.

References
1. Debiec, H. et al. Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies. N Engl J Med 346, 2053–2060 (2002).
2. Beck, L. H. Jr. et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 361, 11–21 (2009).
3. Beck, L. H. Jr. & Salant, D. J. Membranous nephropathy: recent travels and new roads ahead. Kidney Int 77, 765–770 (2010).
4. Stanescu, H. C. et al. Risk HLA-DQA1 and PLA(2)R1 alleles in idiopathic membranous nephropathy. N Engl J Med 364, 616–626 (2011).
5. Kim, S. et al. Single nucleotide polymorphisms in the phospholipase A2 receptor gene are associated with genetic susceptibility to idiopathic membranous nephropathy. Nephron Clin Pract 117, c253–c258 (2011).
6. Liu, Y. H. et al. Association of phospholipase A2 receptor 1 polymorphisms with idiopathic membranous nephropathy in Chinese patients in Taiwan. J Biomed Sci 17, 81 (2010).
7. Lv, J. et al. Interaction between PLA2R1 and HLA-DQA1 variants associates with anti-PLA2R antibodies and membranous nephropathy: J Am Soc Nephrol 24, 1323–1329 (2013).
8. Yokoyama, H. et al. Renal disease in the elderly and the very elderly Japanese: analysis of the Japan Renal Biopsy Registry (J-RRB). Clin Exp Nephrol 16, 903–920 (2012).
9. Bullich, G. et al. HLA-DQA1 and PLA2R1 polymorphisms and risk of idiopathic membranous nephropathy. Clin J Am Soc Nephrol 9, 335–343 (2014).
Author Contributions
K.T. and E.N. designed the study, supervised, and finalized the work. K.T., E.N. and K.H. reviewed the manuscript. H.S., K.D., K.W., M.N. and T.W. collected the samples and K.H. performed clinical data analysis, and K.T. and E.N. designed the study, supervised, and finalized the work. K.T., E.N. and K.H. reviewed the manuscript. H.S., K.D., K.W., M.N. and T.W. collected the samples and K.H. performed clinical data analysis, and K.T. and E.N. designed the study, supervised, and finalized the work.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Thiri, M. et al. High-density Association Mapping and Interaction Analysis of PLA2R1 and HLA Regions with Idiopathic Membranous Nephropathy in Japanese. Sci. Rep. 6, 38189; doi: 10.1038/srep38189 (2016).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016