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ORIGINAL ARTICLE

The novel and independent association between single-point SNP of *NPHP4* gene and renal function in non-diabetic Japanese population: the Takahata study

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Nephronophthisis (*NPHP*) *4* gene coding nephrocystin-4 is involved in the development of renal tubules and its congenital mutations cause juvenile end-stage renal disease, NPHP. To investigate the association between single-point single-nucleotide polymorphism (SNP) of *NPHP4* gene and renal function, we conducted a cross-sectional study in Japanese population. The subjects of this study were non-diabetic general population consisting of 2604 individuals >40 years in Takahata town, Japan. We genotyped 11 SNPs within *NPHP4* gene that displayed frequent minor allele frequencies (>0.1) in Japanese general population. Among 11 SNPs in *NPHP4* gene, only rs1287637 that induces amino acid substitution (A (Gln)/T (Leu)), located in the acceptor site of exon 21, showed a significant association with estimated glomerular filtration rate (eGFR; T/T: 81.3 ± 15.6 (*n*=1886), A/T: 82.0 ± 15.5 (*n*=652) and A/A: 87.4 ± 21.4 ml min^{-1} per 1.73m^2 (*n*=66); mean ± s.d., *P*=0.006). This SNP was not in linkage disequilibrium with the surrounding SNPs. The multivariate analysis adjusted with possible confounders showed that the A/T+T/T genotype of rs1287637 was independently associated with reduced renal function (eGFR <90 ml min^{-1} per 1.73m^2; odds ratio (OR) 1.75, 95% confidence interval (CI) 1.05–2.94, *P*=0.033). These results indicate the novel and independent association between single-point SNP rs1287637 in *NPHP4* gene and renal function in non-diabetic Japanese population.
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Keywords: general population; NPHP4; SNPs

INTRODUCTION

Familial and genetic studies have revealed that renal function is heritable and the genetic factors seem to have a role in the susceptibility and the progression of various renal diseases.[1,2] Among them, there are several types of inherited renal diseases that result in poor renal outcome. Nephronophthisis (NPHP), a group of autosomal recessive cystic kidney disorders, is the most common genetic cause of end-stage renal disease in the first three decades of life.[3] The renal histology of NPHP shows interstitial cell infiltration and fibrosis, tubular basement disruption and development of microcysts at the corticomedullary junction.[4]

NPHP is a genetically heterogeneous disorder and positional cloning has identified nine genes (*NPHP1–9*) causing NPHP.[5] Each of *NPHP1–9* genes encodes nephrocystin-1, inversin, nephrocystin-3–6, Gli-similar protein, RPGRIP1L protein and NEK8, respectively. The clinical and genetic aspects of *NPHP1,* the most frequently mutated *NPHP* gene, has been well documented in NPHP patients.[6–8] However, the impact of mutation of *NPHP* genes on renal function in general population is largely unknown. We previously investigated the association between renal function and genotypes of single-nucleotide polymorphisms (SNPs; rs33958626, rs35638834 and rs35367711) in *NPHP1* gene and found no significant association between them in general Japanese population.

In current study, we focused on *NPHP4*, the second major cause of NPHP, and examined whether its genotype has any effect on renal function in non-diabetic population.

MATERIALS AND METHODS

Study population

This study is a part of the ongoing molecular epidemiological study utilizing the regional characteristics of 21st century Centers of Excellence (COE) and global COE Program in Japan, as previously described in detail.[9] The aim of the

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present study is to determine the association between genetic variants and renal function in the general population in Japan. This study is a design-incorporated baseline survey that consisted of a self-administrated questionnaire on lifestyle, blood pressure measurement, anthropometrical measurement, and collections of blood and urine specimens from participants at annual health checkup. Genomic DNA was extracted from peripheral blood samples to determine the SNPs, as previously described.[10] The survey population in this study is the general population >40 years old in Takahata, Japan. In 2004 and 2005, a total of 3115 subjects (mean age 63; men 1380, women 1735) took part in the program and agreed to join the study. This study was approved by the Institutional Ethical Committee. All participants gave written informed consent.

Among 3115 subjects, 162 and 234 subjects were excluded from the present analysis owing to their incomplete data and having diabetes mellitus, respectively. Furthermore, to avoid the effect of potential renal diseases, we excluded the 115 subjects with increased urine albumin and β2-microglobulin-creatinine excretion. Thus, 2604 subjects were entered into final analyses (mean age 62; men 1156, women 1448).

Measurement

Clinical information regarding medical history, current medication, smoking habits and alcohol intake was obtained from a self-reported questionnaire. Blood pressures were determined by using a mercury manometer in subjects who had rested in a sitting position for at least 5 min before the measurement. Hypertension was defined as systolic blood pressure $\geqslant$140 mm Hg and/or diastolic blood pressure $\geqslant$90 mm Hg, and/or the use of anti-hypertensive medication. Body mass index was calculated from weight and height measures as weight (kg) divided by the square of height (m^2). Diabetes was ascertained either by self-reported physical diagnosis or by a measure of fasting blood sugar $\geqslant$126 mg dl^{-1} or hemoglobin A1c (HbA1c) value $\geqslant$6.5%. Hypercholesterolemia was ascertained by a measure of serum total cholesterol level $\geqslant$220 mg dl^{-1}, and/or the use of anti-hyperlipidemic medication. Urine albumin concentration was determined using immunoturbidimetry. Urine albumin-creatinine ratio was calculated from a single-spot urine specimen collected in the morning. Urine β2-microglobulin was assessed using the latex agglutination method. Urine β2-microglobulin levels corrected with urine creatinine (urine β2-microglobulin-creatinine ratio (UBCR)) were used for the analyses. It is widely accepted that proteinuria, almost equal to the urine albumin-creatinine ratio levels of >300 mg g^{-1}, is a risk for end-stage renal disease.[11] Another study showed that increased urine β2-microglobulin excretion (UBCR >1000 μg g^{-1}) was a risk for a higher 10-year mortality due to cardiovascular and renal disease.[12] Based on these reports, to avoid the effect of potential renal diseases, we excluded the subjects with urine albumin-creatinine ratio >300 mg g^{-1} or UBCR >1000 μg g^{-1} in this study. Serum creatinine was measured by enzymatic method and estimated glomerular filtration rate (eGFR) was obtained using the equation modified for Japanese.[13]

SNP selection and genotyping

NPHP4 gene is located on chromosome 1p36.22. We utilized Haploview software version 3.32 (http://www.broadinstitute.org/haploview/haploview) and dbSNP database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/) and International HapMap Project (http://www.hapmap.org/index.html) to extract all SNPs of the *NPHP4* gene with a minor allele frequency >0.1 in the Japanese general population. In all, 11 SNPs (rs11121648 in intron 29, rs489933 in intron 26, rs1287626 in intron 23, rs472651 in intron 23, rs905467 in intron 22, rs1287637 in exon 21, rs11121956 in intron 18, rs12058375 in intron 16, rs962662 in intron 11, rs1566724 in intron 9 and rs806104 in intron 2) were selected and have been genotyped. All SNPs gave accurate typing (call rate >99%) and were used in this study.

Genotyping of these 11 SNPs were performed as previously described.[10] Briefly, it was determined by Invader assay (Third Wave Technologies, Madison, WI, USA)[14,15] and Taqman allelic discrimination assay.[16] Reagents were purchased from Applied Biosystems (Foster City, CA, USA). TaqMan probes were designed and synthesized by Applied Biosystems and it distinguished the SNPs at the end of a PCR. One allelic probe was labeled with fluorescent FAM dye and the order with the fluorescent VIC dye. PCR was performed by TaqMan Universal Master Mix without UNG (Applied Biosystems), with PCR primers at a concentration of 900 nM and TaqMan MGB probes at a concentration of 200 nM. Reactions were performed in 384-well formats in a total reaction volume of 3 μl using 3 ng of genomic DNA. The plates were then placed in a GeneAmp PCR System 9700 (Applied Biosystems) and heated at 95 °C for 10 min, followed by 40 cycles at 92 °C for 15 s and 60 °C for 1 min, with a final soak at 25 °C. The plates were read by the Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity in each well of the plate was read.[16] Fluorescence data files from each plate were analyzed by the SDS 2 allele calling software (Applied Biosystems). Several data (signal intensity) were eliminated to preserve the reliability of the assay system (missing data are guaranteed to be <1%).

Statistical analyses

We used Student's *t*-test or an analysis of variance to evaluate the differences in means, and χ^2-tests to evaluate the differences in proportions. Some of the clinical and biochemical traits in each genotypic group did not distribute normally; we applied a non-parametric Mann–Whitney test or Kruskal–Wallis test. To examine the independent association between genotype and renal function, age, gender-adjusted and multivariate logistic regression analyses were performed. In multivariate model, possible confounders including age, gender, drinking, smoking, obesity, hypertension and hypercholesterolemia were adjusted. To confirm Hardy–Weinberg equilibrium among genotypes, χ^2-tests were used ($P \geqslant 0.05$). Linkage disequilibrium for the combination of variations was tested by D' and r^2 using Haploview. Data are expressed as mean ± s.d. except as otherwise indicated. A significant difference was defined as $P<0.05$. All statistical analyses were performed using SPSS version 15.0.1 J (SPSS, Chicago, IL, USA).

RESULTS

Characteristics of subjects

Baseline characteristics of 2604 subjects who entered into a final analysis were as follows; mean age 62 years, 1156 men (44.4%), 1373 subjects (52.7%) with hypertension, 746 subjects (28.6%) with obesity and 867 subjects (33.3%) with hypercholesterolemia.

Association of single-point SNPs of NPHP4 with eGFR

The chromosomal locations of 11 SNPs of *NPHP4* gene selected as in Methods section are shown in Figure 1 and their characteristics are summarized in Table 1. To investigate whether *NPHP4* gene SNPs are associated with renal function, we performed quantitative trait locus analyses using analysis of variance. It showed that only the genotypes of SNP6 rs1287637 was significantly associated with eGFR; the eGFR of T/T, T/A and A/A genotypes were 81.3 ± 15.6, 82.0 ± 15.5 and 87.4 ± 21.4 ml min^{-1} per 1.73 m^2, respectively (P=0.006; Table 2).

To examine the linkage disequilibrium of *NPHP4* gene, we performed linkage disequilibrium analysis using these 11 SNPs typing data and observed no obvious consistency of genotypes between SNP6 rs1287637 and the other 10 SNPs (Figure 2).

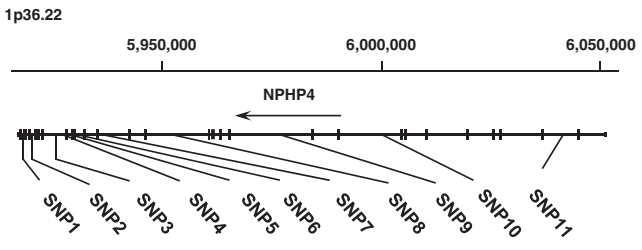

Figure 1 The chromosomal locations of 11 selected single-nucleotide polymorphisms (SNPs) in the nephronophthisis (*NPHP4*) gene coding exons are represented by thick blocks connected by lines representing introns.

Table 1 Polymorphisms in *NPHP4* genes examined in the study

					Allele		*Allele frequency*		*Genotype*			
SNP ID	*Gene symbol*	*NCBI SNP reference*	*SNP type*	*Public location*	*1*	*2*	*1*	*2*	*11*	*12*	*22*	*Heterozygosity*
SNP1	*NPHP4*	rs11121648	Intron 29	5923788	T	G	0.75	0.25	1281	1090	223	0.42
SNP2	*NPHP4*	rs489933	Intron 26	5925801	C	T	0.75	0.25	1308	1075	208	0.41
SNP3	*NPHP4*	rs1287626	Intron 23	5930977	G	A	0.75	0.25	1320	1062	209	0.41
SNP4	*NPHP4*	rs472651	Intron 23	5932517	C	T	0.83	0.17	1734	777	86	0.30
SNP5	*NPHP4*	rs905467	Intron 22	5934500	T	C	0.64	0.36	744	1290	561	0.50
SNP6	*NPHP4*	rs1287637	Exon 21	5935162	T (Leu)	A (Gln)	0.86	0.14	1886	652	66	0.25
SNP7	*NPHP4*	rs11121956	Intron 18	5941435	T	C	0.77	0.23	1379	1034	181	0.40
SNP8	*NPHP4*	rs12058375	Intron 16	5956909	C	A	0.79	0.21	1499	934	156	0.36
SNP9	*NPHP4*	rs962662	Intron 11	5978510	A	G	0.76	0.24	1362	1018	190	0.40
SNP10	*NPHP4*	rs1566724	Intron 9	6002803	A	G	0.77	0.23	1378	1029	190	0.40
SNP11	*NPHP4*	rs806104	Intron 2	6042934	A	G	0.77	0.23	1368	1018	192	0.39

Abbreviations: NCBI, National Center for Biotechnology Information; NPHP, nephronophthisis; SNP, single-nucleotide polymorphism.

Table 2 The association analysis of each SNP with eGFR

			Post-hoc *(LSD)* P-*value*			*eGFR (ml min^{-1} per 1.73 m^2) mean ± s.d.*		
SNP ID	*NCBI SNP reference*	*ANOVA* P-*value*	*11 versus 12*	*11 versus 22*	*12 versus 22*	*11*	*12*	*22*
SNP1	rs11121648	0.357	0.173	0.429	0.986	82.0 ± 15.9	81.1 ± 15.5	81.1 ± 15.8
SNP2	rs489933	0.356	0.166	0.479	0.955	81.1 ± 15.9	81.1 ± 15.7	82.0 ± 15.8
SNP3	rs1287626	0.376	0.164	0.625	0.782	82.0 ± 15.8	81.1 ± 15.7	81.4 ± 15.9
SNP4	rs472651	0.732	0.449	0.896	0.679	81.7 ± 15.7	81.2 ± 15.7	81.9 ± 17.3
SNP5	rs905467	0.291	0.352	0.118	0.377	82.2 ± 15.9	81.5 ± 15.8	80.8 ± 15.4
SNP6	rs1287637	0.006	0.332	0.002	0.007	81.3 ± 15.6	82.0 ± 15.5	87.4 ± 21.4
SNP7	rs11121956	0.148	0.112	0.143	0.532	82.1 ± 15.8	81.1 ± 15.5	80.3 ± 16.3
SNP8	rs12058375	0.308	0.764	0.156	0.127	81.6 ± 16.0	81.8 ± 15.4	79.7 ± 15.0
SNP9	rs962662	0.199	0.104	0.265	0.811	82.1 ± 15.7	81.0 ± 15.5	80.7 ± 17.0
SNP10	rs1566724	0.193	0.174	0.139	0.459	82.0 ± 15.5	81.2 ± 15.7	80.2 ± 16.1
SNP11	rs806104	0.290	0.191	0.250	0.659	82.0 ± 15.6	81.2 ± 15.6	80.6 ± 16.9

Abbreviations: ANOVA, analysis of variance; eGFR, estimated glomerular filtration rate; LSD, Fisher's least significant difference; NCBI, National Center for Biotechnology Information; SNP, single-nucleotide polymorphism.

The comparison of clinical parameters between the genotypes of SNP6 rs1287637

Next, we compared the clinical parameters between the genotypes of SNP6 rs1287637. There was no significant difference between them in clinical characters such as age, gender, the levels of blood pressure, body mass index and HbA1c. Neither urine albumin nor β2-microglobulin excretions, the markers of glomerular and tubular damages, showed any statistical difference (Table 3).

We further investigated the prevalence of cyst formation in 343 subjects using the results of renal ultrasonography. The simple cysts were observed in only one subject (11.1%) of A/A group (n=9) and 43 subjects (12.9%) of A/T+T/T group (n=334). None of them showed typical polycystic changes observed in NPHP. These results indicate that the difference in eGFR between these genotypes showed no significant association with the comorbidities or cystic formation.

The independent association between the genotypes of SNP6 rs1287637 and reduced renal function

To investigate the independent association between the genotypes of SNP6 rs1287637 and renal function, we performed age, gender-adjusted and multivariate logistic regression analyses. In age, gender-adjusted analysis, the rs1287637 A/A+A/T genotype showed a borderline significance with reduced renal function (eGFR $<$90 ml min^{-1} per 1.73 m^2; OR 1.66, 95% CI 0.99–2.78, P=0.054). In multivariate logistic regression analysis, the rs1287637 A/A+A/T genotype was independently related to reduced renal function (OR 1.75, 95% CI 1.05–2.94, P=0.033; Table 4).

DISCUSSION

In this study, we have revealed the novel and independent association between the genotypes of SNP rs1287637 in *NPHP4* gene and renal function in non-diabetic Japanese population $>$40 years old. This result indicates that genetic variation of *NPHP4* might be involved in the renal function not only in children, but also in the general population.

The renal function gradually declines along with aging. In this studied population, the eGFR values in the majority of subjects were within the predicted range for their age. Furthermore, we excluded the subjects with potential renal diseases in this study. Although the epidemiological setting does not allow us to specify the cause of renal decline individually, these findings suggest that the main reason for reduced renal function is not specific renal diseases, but aging in this population. In the process of renal aging, various factors are involved including hypertension, diabetes, dyslipidaemia, nephrotoxic agents, hormonal changes, lifestyle-related factors such as diet, smoking and drinking, and genetic property.[17] In this study, the subjects

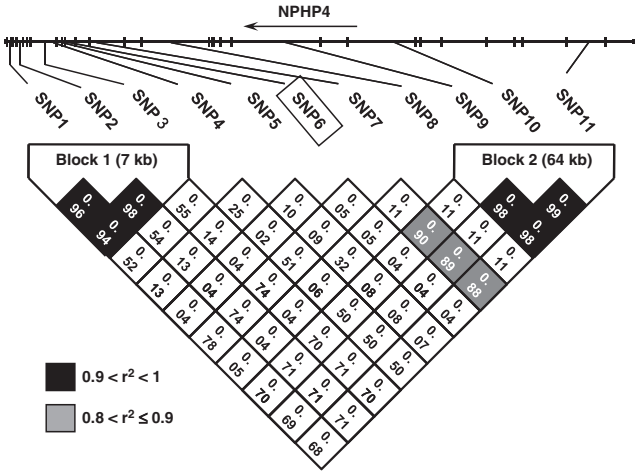

Figure 2 The linkage disequilibrium analysis of 11 single-nucleotide polymorphisms (SNPs) of nephronophthisis (*NPHP4*) gene.

with diabetes or potential renal diseases were excluded and the aforementioned possible factors were adjusted, however, we still found the independent association between the NPHP4 genotypes and renal function. Although it is not examined how the eGFR difference between rs1287637 genotypes is affected by aging, there is a possibility that the eGFR difference between these genotypes is attributed, at least in part, to the genetic factor itself.

The association of the defects in *NPHP* gene with premature renal failure has been vigorously investigated in various types of NPHP,[3] however, the interests of the researchers were mainly limited to the effect of gene mutation on renal deterioration in children. Usually, severe renal deterioration due to *NPHP4* genetic abnormalities occurs in young generation.[18,19] Interestingly, this study uncovered that the renal function was associated with *NPHP4* variants also in later generations, middle-aged and elderly. The location of SNP6 rs1287637 is further downstream of genetic variations previously reported in NPHP.[19] Taken together, our finding suggests that the genetic variation of rs1287637 might be the novel factor to modulate renal function in non-diabetic Japanese adults.

The nephrocystin-4 encoded by *NPHP4* is located in centrosomes in the cilia of renal tubular cells together with other types of nephrocystin and has an important role in regulating urine flow and intracellular calcium concentration.[3] It is speculated that these proteins regulate the dilatation of renal tubules, and the malfunction of these proteins induces the cystic formation.[20] In this study, the comparison of characters between genotypes of rs1287637 showed that this SNP was associated with renal function, with no significant difference in clinical parameters and cyst formation. Furthermore, the multivariate regression analysis showed that the association of the genetic variation of this SNP with renal function was significant after adjustment with known risk factors. These results suggest that the genetic variants of rs1287637 might affect the renal function not via the development of comorbidities or obvious structural changes. To clarify the precise mechanism of this phenomenon, the experimental study might be required.

Genome-wide analysis suggests the existence of multiple candidate genes related to renal function in general population.[1] Therefore, even though the difference in eGFR caused by single SNP was not so large, it is possible that the accumulated effects of these genetic variations including *NPHP4* cause a great influence on renal function in general population.

Table 3 The comparison of characters between the genotypes of rs1287637 in *NPHP4* gene

Genotypes	*A/T+T/T*	*A/A*
Number	2538	66
Male (%)	44.3	47.0
Age (years)	62.4 ± 10.3	62.3 ± 10.3
Systolic BP (mm Hg)	133.7 ± 15.8	131.7 ± ± 17.8
Diastolic BP (mm Hg)	79.3 ± 10.1	79.0 ± 10.5
Body mass index (kg m^{-2})	23.4 ± 3.1	23.8 ± 2.8
HbA1c (%)	5.12 ± 0.39	5.06 ± 0.33
Obesity (%)	28.9	27.1
Smoker (%)	32.4	34.7
Drinker (%)	41.3	32.9
Hypertension (%)	53.9	45.7
Hypercholesterolemia (%)	32.5	34.3
Serum creatinine (mg/dl)	0.67 ± 0.15	0.64 ± 0.15
eGFR (ml min^{-1} per 1.73m^2)	81.4 ± 15.6*	87.4 ± 21.4
UACR (mg g^{-1})	9.1 (4.8–19.5)	12.3 (5.7–24.7)
UBCR (μg g^{-1})	109.8 (70.7–178.3)	115.3 (75.4–223.5)

Abbreviations: BP, blood pressur; eGFR, estimated glomerular filtration rate; NPHP, nephronophthisis; UACR, urine creatinine-albumin ratio; UBCR, urine β2-microglobulin-creatinine ratio.
UACR and UBCR are expressed as median with interquartile range. *P<0.05.

Table 4 OR of rs1287637 (AT+TT) for reduced renal function (eGFR <90 ml min^{-1} per 1.73 m^2)

	Age, gender-adjusted		*Multivariate-adjusted*[a]	
Genotypes	*OR (95% CI)*	P-*value*	*OR (95% CI)*	P-*value*
rs1287637 (AT+TT; versus AA)	1.66 (0.99–2.78)	0.054	1.75 (1.05–2.94)	0.033

Abbreviations: CI, confidence interval; eGFR, estimated glomerular filtration rate; OR, odds ratio.
[a]Adjusted for age, gender, drinking, smoking, obesity, hypertension and hypercholesterolemia.

This study has several limitations. First, there is no information on how the genetic variation of rs1287637 causes the structural and functional changes in nephrocystin-4 protein. This point is beyond the scope of this study and should be clarified in further examination. Second, in this population, the mutation in other *NHPH* genes except *NPHP1* was not investigated. It is reported that NPHP sometimes accompanies simultaneous mutations in different *NPHP* genes.[21] Although the prevalence of renal abnormalities due to the mutation in other *NPHP* genes is low, there is a possibility that the difference in eGFR might be caused in part by the interaction between mutations in other *NPHP* genes.

In conclusion, our study showed the novel and independent association between single-point SNP rs1287637 in *NPHP4* gene and renal function. This SNP might be one of genetic factors that affect the renal function in non-diabetic Japanese adults.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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