Original paper

Modifier loci in non-mutant, female Wistar Kyoto rats influence cellular pathogenesis of nephronophthisis in Lewis polycystic kidney rats

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Genetic modifier loci influence the inheritance of diseases and lead to variability in phenotype progression. We report the influence of modifier loci in female Wistar Kyoto (WKY) rats on cellular pathogenesis of nephronophthisis inherited from Lewis polycystic kidney (LPK) rats. The loci modified cellular expression and progression of nephronophthisis in the backcross 1 (BC1) progeny. Mating experiments to produce BC1 progeny were carried out between three male LPK and seven female WKY rats. Fifteen female rats from the F1 generation were mated with the male LPK rats to produce the BC1 progeny. The rats with cystic kidney disease were identified and histology of the kidneys was carried out. Mapping studies and linkage analysis were carried out to identify the modifier loci. The BC1 progeny were less affected than the LPK strain with respect to disease severity and progression of the kidneys to end stage renal disease. It was found that the mean values of all the disease phenotypes of the mutant BC1 progeny were significantly different from those of the LPK rats, and these segregated with the genotypes of the markers located on chromosomes 5q34–q36 and 7q11–q34, giving maximum LOD scores greater than 3 ($p < 0.001$).

Key words: pathogenesis, nephronophthisis, modifier loci, LOD score, Wistar Kyoto rats.

Introduction

Genetic modifier loci are sections of the chromosomes, which influence the inheritance and progression of genetic diseases. These chromosomal sections contain genetic elements that interact with other chromosomal loci, especially the disease loci, and cause variability in disease presentation and progression. Nephronophthisis (NPHP) is one of these genetic diseases whose cellular progression is influenced by genetic loci of the background strains. Pathologically, NPHP simply means damaged nephrons; it is a form of autosomal recessive polycystic kidney disease (ARPKD) [1, 2], which is a chronic interstitial nephropathy whose cellular pathogenesis involves multiple organ systems and causes cystic kidneys, which eventually deteriorate to end stage renal disease [3].

Using different murine models, the pathogenesis of NPHP has been linked to many causative genetic mutations, some of which have been mapped to genes that are involved in centrosome and cilia functions [4, 5]. However, the pathogenesis of the classical ARPKD is caused by mutations only in the *Pkhd1* gene, which was mapped to human chromosome 6p21.1–p12 [6], and in murine models, the orthologs are intermittently located in different chromosomes [7, 8, 9]. Like NPHP, ARPKD is also characterized by mul-

tiple fluid-filled cystic kidneys, anemia, hypertension and eventually end stage renal disease [10].

Although the pathogenesis of NPHP and ARPKD results from mutations in different genetic loci [6, 11, 12, 13], both are monogenic renal cystic disorders, which share common cellular and macroscopic features of cyst development [2, 14, 15]. The disorders also have common clinical presentation, which include infantile incidence, renal failure, anemia, polyuria without hematuria, proteinuria and hypertension [10, 16]. It has, however, become clearer that the variability of disease phenotypes, especially the progression and extrarenal manifestation, is a feature common to both NPHP and ARPKD. This variability is attributed to individual genetic heterogeneity, but importantly to the genetic background of the parental strains [17]. Regardless of the different genetic mutations that lead to cellular pathogenesis of NPHP, it is now accepted that NPHP is a less common form of ARPKD [18, 19].

Recently, we carried out a rigorous study to elucidate the molecular pathogenesis and inheritance of NPHP in Lewis polycystic kidney (LPK) rats, and the quantitative trait locus (QTL) responsible for the disease phenotypes was mapped to chromosome 10q21–q26 [10]. The QTL contains genes that are important in signal transduction, cell growth, cell proliferation and cell differentiation [20, 21], and they were also associated with unregulated cell proliferation and cancer [22, 23, 24]. Fine mapping of the entire rat chromosome 10 using a dense set of markers identified a region within the regulator of chromosome condensation 1 (RCC1) in the never in mitosis A (NIMA)-related kinase 8 (*Nek8*) gene as the cause of molecular and cellular pathogenesis of the disease [25]. Previous studies established that mutations in the *Nek8* genes caused pathogenesis and development of NPHP in human and other animal species [26, 27, 28]. This resulted to the consideration of NPHP as a less common form of ARPKD [29, 30].

The current work is a further extension of the study in which a genome-wide linkage scan analysis identified a mutation in the *Nek8* gene as the cause of NPHP in LPK rats [10, 25, 31]. It is now reported that this mutation is influenced by genetic modifier loci in the non-mutant, female WKY rats. In this context, we document a clear influence of genetic background on cellular pathogenesis, severity and progression of the phenotypes in the backcross 1 (BC1) progeny. The BC1 progeny arose as a result of an intercross between distantly related mutant, male LPK and non-mutant, female Wistar Kyoto (WKY) rat strains.

Linkage analysis between phenotypes and genotypes of the BC1 progeny provided significant evidence for the presence of modifier genes on chromosomes 5q34–q36 and 7q11–q34. The association between phenotypes and genotypes of alleles amplified by markers that segregate with these genetic loci strongly suggests that cellular pathogenesis of NPHP, its severity and progression are modified by loci located in both chromosomes.

Material and methods

Background

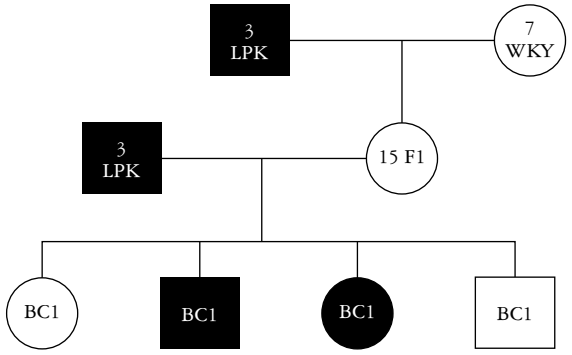
A colony of Lewis (LEW/SsNArc$^{-/-}$) rats spontaneously developed enlarged cystic kidneys at the Animal Resources Center of Western Australia. Mating experiments between male and female progeny with enlarged cystic kidneys produced 100% pups with bilaterally enlarged, fluid-filled cystic kidneys [10]. This colony is now called the Lewis polycystic kidney (LPK/SsNArc$^{+/+}$) rats.

Mating experiments

Three mutant, male Lewis polycystic kidney (LPK/SsNArc$^{+/+}$) rats were mated with seven non-mutant, female Wistar Kyoto (WKY/NArc$^{-/-}$) rats to produce first filial (F1) generations. Fifteen female rats from the heterozygote F1 progeny were mated with 3 mutant LPK parent male rats and produced 139 pups in the BC1 (Fig. 1). All the animals were kept in a 12-hour light/dark cycle and they had free access to the same food and water. The experiments were performed in accordance with the guidelines of the Animal Ethic Committees of Murdoch University and the Animal Resources Center of Western Australia [18].

Phenotypic traits analysis

The BC1 progeny were earmarked and recorded and each was palpated once every week from im-



■ CKD male; ● CKD female; □ non-CKD male; ○ non-CKD female

Fig. 1. Mating experiment between male LPK and female WKY to produce F1 and BC1 progeny. Mating experiments between mutants, homozygote male LPK/SsNArc$^{+/+}$ and non-mutants homozygote female WKY/NArc$^{-/-}$ rats to raise heterozygote F1 offspring were carried out. The F1 females were mated with the LPK/SsNArc$^{+/+}$ and produced BC1 progeny. None of the F1 progeny showed any disease phenotype. CKD – cystic kidney disease

mediately after birth until euthanasia to determine which rat had developed enlarged cystic kidneys. Enlarged kidneys were confirmed on euthanasia and the presence of cysts was established on histological examination of the kidneys. Systolic blood pressure was measured when the rats were 12 weeks old, by the tail-cuff method, using NIBP Controller (ADI Instruments, Castle Hill, NSW, Australia). Three measurements were taken and the average measurement was used in statistical analysis. However, telemetry will be used in future studies to measure systolic blood pressure [10].

Euthanasia

Euthanasia of the BC1 rats was carried out using a carbon dioxide and oxygen gas mixture, in the proportions of 80 : 20 respectively. After euthanasia, the rats were weighed and the mass recorded in grams. Blood was removed by cardiac puncture into lithium-heparin tubes. The rats were opened on the ventral side along the linear alba and kidneys and sections of liver and pancreas were removed. Each kidney was weighed and the mass was recorded and the kidneys and sections of the liver and pancreas were fixed in 4% formaldehyde solution [10].

Packed cell volume

The packed cell volume (PCV) was determined in 2 ml of blood samples at the Department of Pathology, Murdoch University, by microhematocrit technique using Haeraeus Biofuge hemo centrifuge, according to the manufacturer's instructions (RANDOX Laboratories Ltd, London, UK), as previously described [10].

Blood chemistry

Plasma total solid protein (TSP) was estimated on ethylenediamine tetra-acetic acid (EDTA) using a Refractometer according to the manufacturer's instructions (RANDOX Laboratories Ltd). To 20 μl of samples, blank and standard solution, 1000 μl of pyrogallol red was added. This was mixed and incubated at 37°C for 5 minutes. The absorbance of the samples (A_{sample}) and the standard ($A_{standard}$) was measured against the reagent blank at 600 nm wavelength using a spectrophotometer. Protein concentration was calculated according to the instructions of the manufacturer (RANDOX Laboratories Ltd):

Protein concentration (g/l) = $A_{sample} \times$ [standard]/$A_{standard}$, where [standard] is the concentration of the standard solution in g/l, A_{sample} is the absorbance of the sample, and $A_{standard}$ is the absorbance of the standard solution.

Plasma creatinine (PC) was determined as follows: To 20 μl of samples, sodium hydroxide and picric acid were added, as per the instructions provided by the manufacturer (RANDOX Instruments). Creatinine, in the presence of sodium hydroxide solution, reacts with picric acid to form a colored complex. The rate of formation of the complex was measured using the colorimetric method.

Plasma urea was measured on the Randox Daytona, the reaction catalyzed by urease enzyme (RANDOX Laboratories Ltd). Ammonia produced during the reaction combined with α-oxoglutarate and hydrogenated nicotinamide adenine dinucleotide (NADH), in the presence of glutamate-dehydrogenase, to yield glutamate and NAD^+. The NAD^+ produced was measured in μmol/l using the ultraviolet method, as previously described [10].

Histological examination of tissues

Histological examination of the sections of kidney, liver and pancreas were carried out at the Department of Histology, Murdoch University. The tissues were paraffin embedded and sectioned (4 μm thickness) using a microtome. The sections were fixed on glass slides, stained with hematoxylin and eosin and viewed with a light microscope, and the images were digitalized using a camera (Olympus, Perth, WA, Australia), as previously described [10].

Genetic analysis

Simple sequence repeat markers

The simple sequence repeat (SSR) markers used for the mapping studies were chosen from the rat genome database: http://rgd.mcw.edu/. One SSR marker was taken from the extreme ends of each chromosome and two or more markers between. A total of 150 SSR markers distributed across the 20 rat autosomes were screened in the study, but only 96 were found informative, and therefore used in the mapping studies, as described in the previous study [10].

DNA extraction and PCR analysis

The extraction of deoxyribonucleic acid (DNA) from liver tissue was carried out according to the instructions provided in the Standard Tissue Kit Protocol (QIAamp DNA Mini Kit from Qiagen, Melbourne, Australia). The DNA was quantified using a NanoDrop, ND-1000 (BIOLAB, Wilmington, USA).

The polymerase chain reaction (PCR) was performed in a total volume of 10 μl containing 10% (w/v) Cresol Red solution; PCR buffer [6.7 mM Tris-HCl, pH 8.8, 1.66 mM $(NH_4)_2SO_4$, 0.045% Triton X-100, 0.02 mg/ml gelatin]; 0.25 mM of total dNTPs; 10 pM each of forward and reverse primers; 1U Taq polymerase; 1.5 mM $MgCl_2$, and 20 ng/μl genomic DNA template in the reaction mixture. The amplification of the DNA was performed using touchdown conditions, with initial denaturation tem-

perature of 94°C for 3 minutes, followed by 8 cycles at 94°C for 30 seconds, 63°C for 30 seconds and 72°C for 30 seconds, and the temperature decreased to 55°C, one cycle/1°C. A further 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds were carried out. A final extension at 72°C for 5 minutes was allowed.

The PCR products were separated using 8% polyacrylamide (acrylamide/bis-acrylamide solutions, 40% w/v) gels and electrophoresis was carried out for 20 hours using 1X Tris-base-Boric acid-EDTA (TBE) buffer. The potential difference for electrophoresis was set at 4 volts/cm and the gels were stained with ethidium bromide solution from Sigma-Aldrich, Pty. Ltd. Sydney, Australia (E1510; 10 mg/ml). The image of the gel was visualized using ultraviolet light on a trans-illuminator.

Scoring of genotypes and linkage analyses

The alleles amplified by each SSR in all the BC1 progeny were independently scored by three people either as homozygote parent A or heterozygote parent H (Fig. 2). Where there was no agreement in the score because the alleles were not informative, the fragment was scored as a dash. Linkage analysis between the genotype and the phenotypes to identify the quantitative trait locus (QTL) that controlled the phenotypic trait variation in the LPK rats was carried out using Map Manager QTX20b [32]. A minimum $\log_{10}$ likelihood ratio (LOD) score of 3 was used to determine the presence of a QTL [33] and the additive effect for each QTL was calculated [32, 34].

Results and statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences, SPSS16 (Chicago, Ill., USA). The analysis of variance and multiple comparison tests with post hoc analysis of the combined male and female data sets were carried out and significance was considered at $p < 0.05$, unless otherwise stated. The segregation ratio showed the inheritance of a recessive mutation in a single gene (Table I).

Cysts were only found in the kidneys of rats with the disease; see Fig. 2 (left) and Fig. 3 (above). On histological examination, the normal kidneys did not have any cysts (Fig. 4), but the enlarged kidneys had cysts, some coalescing to form very large cysts (Fig. 5). The blood chemistry (Table II) showed that the cysts in the kidneys led to the deterioration of kidney function. As a result, the kidneys were unable to regulate the concentrations of protein, urea and creatinine. The variation of the mean values of the phenotypic traits between the mutant and the non-mutant prog-

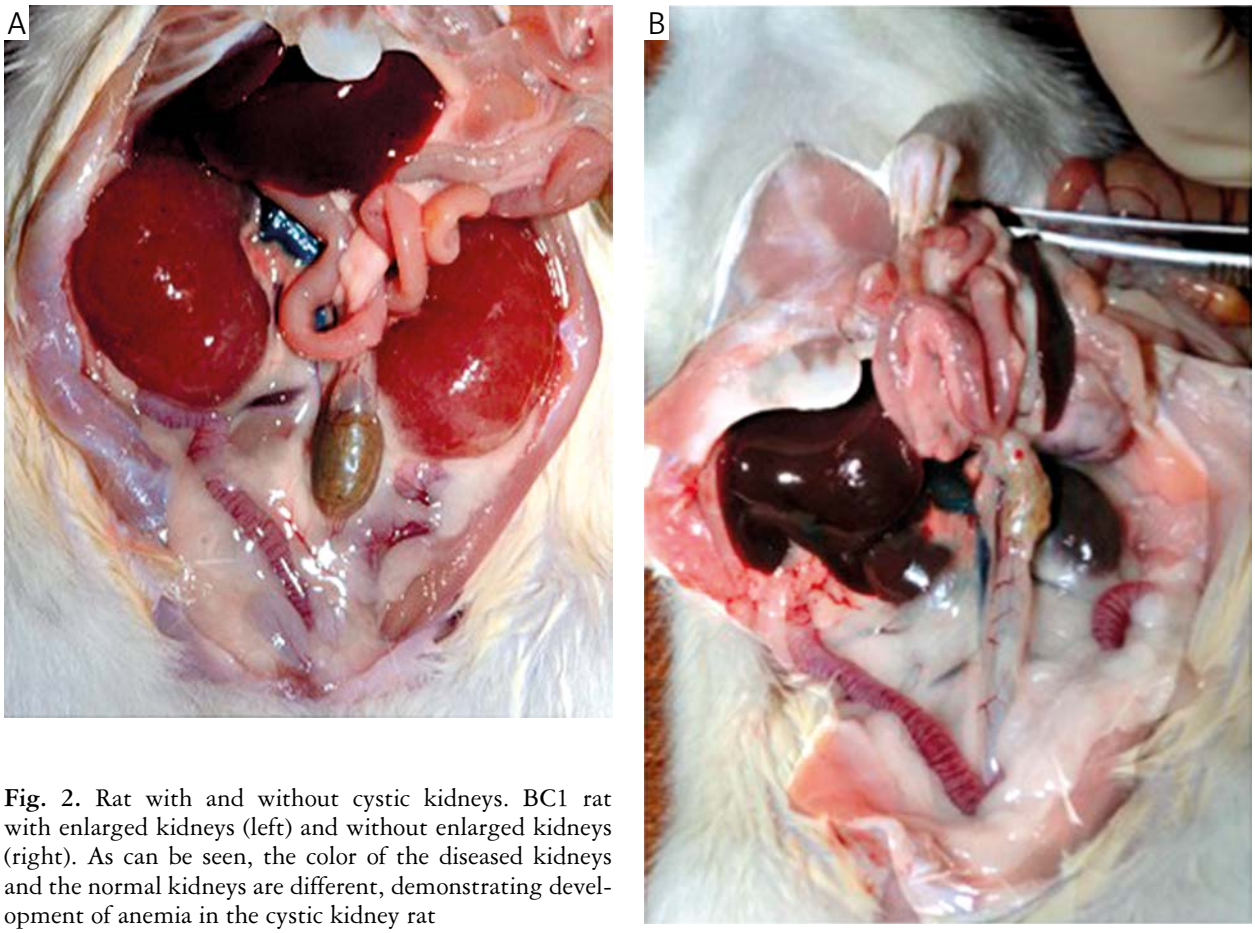


Fig. 2. Rat with and without cystic kidneys. BC1 rat with enlarged kidneys (left) and without enlarged kidneys (right). As can be seen, the color of the diseased kidneys and the normal kidneys are different, demonstrating development of anemia in the cystic kidney rat

Table I. The segregation ratio in the back cross 1 progeny

Generation	Progeny	Expected	Observed	Statistical ratio	χ^2 Square	P-value
BC1	139	69.5	67	1 : 1	0.2	> 0.05

BC1 – Backcross 1 generation; Expected number of rats with disease; Observed number of rats with disease; χ^2 – chi square values.

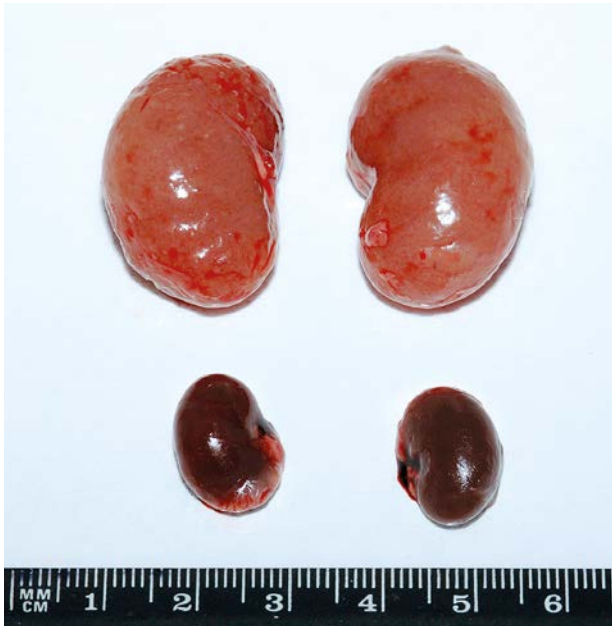


Fig. 3. Kidneys of rats with NPHP are compared to those without NPHP. Comparing the enlarged kidneys (above) with normal kidneys (below) to scale in cm. The enlarged kidneys are three to four times larger than the normal kidneys

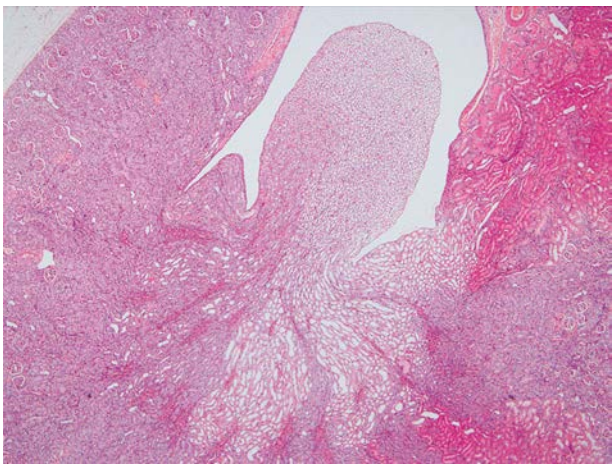


Fig. 4. Histology of non-cystic kidneys of the BC1 rat. The normal kidneys did not develop any cysts. Magnification 4×

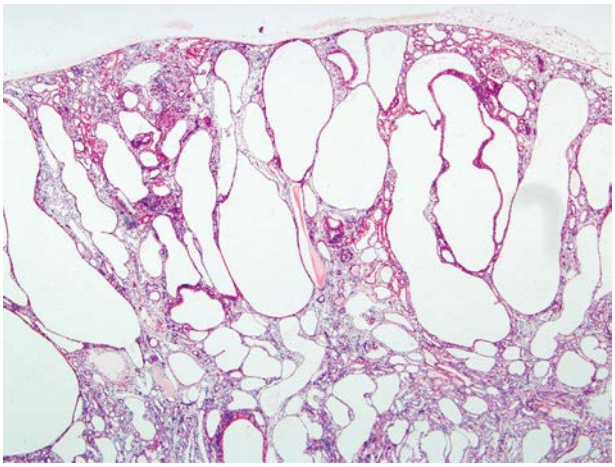


Fig. 5. Histology of cystic kidney from the BC1 rat. The diseased kidneys developed cysts. Some cysts coalesce to form larger cysts. Magnification 4×

eny suggests the presence of modifier loci somewhere along the chromosomes in the WKY rat strain.

Figure 6 shows the alleles in the BC1 rats. Table III shows the LOD scores on chromosome 10, and Table IV shows an epistatic interaction between modifier loci on chromosomes 5q34–q36, 7q11–q34 and the *Nek8* gene on chromosome 10q25. These modifier loci significantly influenced the cellular pathogenesis of NPHP, its severity and progression to end stage renal disease (Fig. 7 and Fig. 8). The effect of the genetic mutation (Fig. 9) located on the *Nek8* gene led to interconnected events, but influenced by modifier genes on chromosomes 5q34–q36 and 7q11–q34.

Discussion

We have established that cellular pathogenesis and expression of NPHP in the BC1 rats is modified by genetic loci on chromosomes 5q34–q36 and 7q11–q34 of the non-mutant, female WKY rats. Genetic markers D5Rat111 and D5Rat132, and D7Rat36 and D7Rat11 flank these chromosomal loci respectively. This finding was established by the use of a dense set of polymorphic simple sequence repeat markers that mapped the genetic loci. Previously, we identified mutation in the *Nek8* gene, located on the RCC1 on chromosome 10q25, as the cause of pathogenesis of NPHP [10, 18]. Later, we observed that the BC1 progeny had much more variable phenotypes than the LPK parental strain. The disease severity and its progression were less severe in the BC1 progeny than the LPK parental strain. This finding suggested that at least one genetic modifier loci was introduced by the WKY background strain. By analyzing the association between phenotypic and genotypic trait variations in the BC1 populations, presumptive modifying loci on chromosomes 5q34–q36 and 7q11–q34 were identified. We now report that these genetic loci in the non-mutant, female WKY rats are important

Table II. Phenotypic trait variation in the mutant and the non-mutant rats

Phenotypes	Rats	N	Mean ± SEM	95% Confidence Interval for mean		Minimum	Maximum
				Lower bound	Upper bound		
Body weight (grams)	Lewis	11	262.18 ±20.72	216.01	308.35	190.00	349.00
	LPK	9	209.78 ±14.29*	176.83	242.72	139.00	288.00
	BC1	76	234.49 ±5.87	222.80	246.17	113.00	365.00
	BC1	63	225.43 ±5.70*	214.03	236.83	150.00	340.00
Percentage kidney/body weight	Lewis	7	0.92 ±0.03	0.84	0.10	0.75	1.01
	LPK	9	7.36 ±1.0***	5.06	9.66	0.00	10.78
	BC1 (non-mutant)	76	0.85 ±0.02	0.80	0.89	0.62	2.19
	BC1 (mutant)	63	4.53 ±0.20***	4.13	4.93	1.55	9.54
Packed cell volume	Lewis	6	0.51 ±0.02	0.47	0.55	0.44	0.56
	LPK	7	0.32 ±0.03**	0.25	0.39	0.18	0.42
	BC1 (non-mutant)	55	0.50 ±0.00	0.49	0.51	0.36	0.56
	BC1 (mutant)	47	0.44 ±0.01**	0.43	0.45	0.34	0.54
Urine specific gravity	Lewis	8	1.03 ±0.01	1.01	1.04	1.00	1.06
	LPK	9	1.01 ±0.00*	1.01	1.01	1.01	1.02
	BC1 (non-mutant)	56	1.04 ±0.00	1.03	1.04	1.01	1.06
	BC1 (mutant)	58	1.03 ±0.00*	1.02	1.03	1.01	1.05
Urine protein (g/l)	Lewis	9	0.49 ±0.11	0.23	0.74	0.15	1.00
	LPK	6	0.87 ±0.13**	0.53	1.20	0.42	1.21
	BC1 (non-mutant)	65	0.61 ±0.07	0.48	0.75	0.04	2.24
	BC1 (mutant)	58	0.40 ±0.03**	0.33	0.47	0.06	1.51
Urine creatinine (μmol/l)	Lewis	8	4420.59 ±1036.39	1969.90	6871.27	1027.00	8975.50
	LPK	6	1524.00 ±147.36***	1145.19	1902.81	1113.00	2047.00
	BC1 (non-mutant)	65	6861.85 ±401.38	6059.99	7663.70	800.00	14780.00
	BC1 (mutant)	58	3566.90 ±229.04***	3108.25	4025.55	1240.00	7960.00

Table II. Cont.

Urine protein: creatinine ratio	Lewis	8	1.12 ±0.18	0.69	1.55	0.24	1.73
	LPK	6	5.35 ±1.04***	2.67	8.04	2.00	8.00
	BC1 (non-mutant)	65	0.79 ±0.07	0.64	0.94	0.09	2.48
	BC1 (mutant)	58	1.32 ±0.16***	0.99	1.64	0.12	6.20
Plasma protein (g/l)	Lewis	7	67.40 ±1.40	64.00	70.80	63.1	74.83
	LPK	8	57.02 ±1.33**	53.88	60.16	50.85	62.90
	BC1 (non-mutant)	55	73.05 ±0.62	71.80	74.30	59.00	88.00
	BC1 (mutant)	47	66.83 ±0.70**	65.42	68.24	59.00	80.00
Plasma urea (mmol/l)	Lewis	9	6.71 ±0.51	5.53	7.89	4.19	9.42
	LPK	8	33.59 ±8.03***	14.59	52.59	14.10	66.69
	BC1 (non-mutant)	74	5.80 ±0.14	5.51	6.08	0.40	8.00
	BC1 (mutant)	62	10.82 ±0.51***	9.81	11.84	0.10	26.40
Plasma creatinine (μmol/l)	Lewis	10	59.01 ±1.92	54.67	63.35	49.08	71.00
	LPK	8	108.74 ±22.43***	55.69	161.79	59.00	197.98
	BC1 (non-mutant)	74	59.26 ±0.89	57.48	61.04	41.00	78.00
	BC1 (mutant)	62	67.31 ±1.67**	63.97	70.64	19.00	121.00
Systolic blood pressure (mm Hg)	Lewis	7	106.26 ±5.28	93.33	119.19	87.33	125.00
	LPK	9	178.86 ±10.01***	155.77	201.95	144.67	232.00
	BC1 (non-mutant)	76	116.74 ±1.42	113.91	119.56	86.00	146.00
	BC1 (mutant)	63	166.90 ±2.18***	162.55	171.26	134.00	215.00

*The mean values of the phenotypes and their variation among the rats: Lewis, Lewis polycystic kidney rats and the backcross 1 progeny. The values are presented as mean ± SEM at 95% confidence interval. *$p \leq 0.01$; **$p \leq 0.001$; ***$p \leq 0.0001$ and N is the number of rats in each group.*

Lewis – non-mutant parental strain from which LPK arose; LPK – Lewis polycystic kidney rat; BC1 – backcross 1 progeny, some of which developed cystic kidneys and some did not develop cystic kidneys.

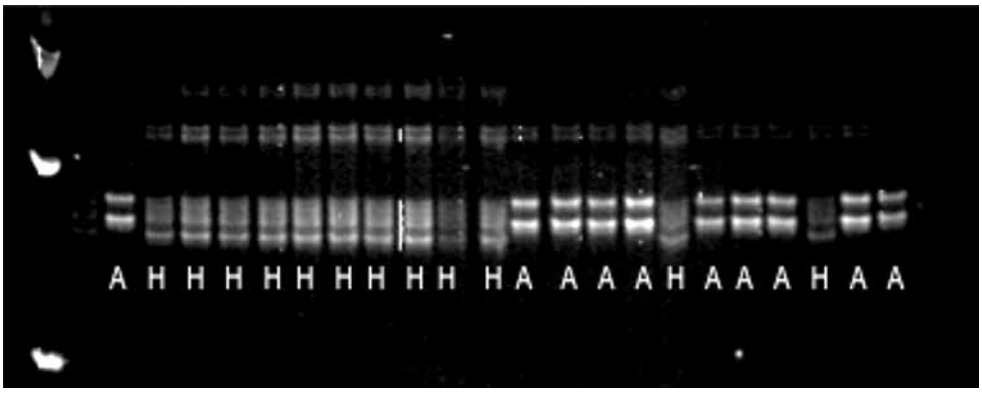


Fig. 6. The genotype of the backcross 1 rats. The stepladder is 50 bp and genotypes show alleles for homozygote parent A or heterozygote parent H

in the modification of cellular pathogenesis of NPHP in the LPK rats.

General inspection of the locus on chromosome 5q34–q36 identified many candidate genes, including the regulator of chromosome condensation 2 (RCC2), as possible modifier genes. The genes in this genetic locus are important in cellular regulation, but RCC2 in particular is a known regulatory element, which was previously reported to be required in the signaling pathways [35]. The RCC2 gene regulates directional cell migration and alignment of chromosomes on the spindle during specific stages of the cell cycle [36]. By localizing the modifier elements to this genetic locus, it means that the RCC2 gene and some of the regulatory elements found within the locus are important in regulating the cellular pathogenesis of NPHP, and therefore influence the severity and progression of the kidneys to end stage renal disease in the BC1 progeny. What have not been identified are the specific gene responsible for the modification and the pathway through which this modification occurs.

We also identified that the pathogenesis of NPHP in the mutant BC1 progeny was modified by loci on chromosome 7q11–q34 of the WKY rats. This section of the chromosome contains a number of candidate genes, but the most important is the Cyp11b1 (11β-hydroxylase) gene. A previous study reported that mutation in this gene resulted in the development of hypertension in congenic Dahl rat strains [37]. By localizing the modifier locus to this genetic region, it strongly suggests that this genetic locus is essential in the modification of systolic blood pressure in the mutant BC1 progeny.

The LOD scores for the association between genotypes and phenotypes within both chromosomal intervals were greater than 3. LOD scores greater than 3 provide significant evidence for linkage [7, 38] and show that the loci associated with the phenotypic traits are responsible for cellular modification and progression of the disease phenotypes. Therefore, these modifier loci caused significant variations in the mean values of the phenotypic traits between the LPK and the BC1 rats with NPHP.

During mapping of the QTLs responsible for the trait variation, assigning the allele fragments to the BC1 progeny was not biased. The accuracy in data preparation for the analysis was considered more important than the ease of computation. In addition, we did not underestimate that variation in quantitative traits is also influenced by environmental factors. In the study, however, we controlled the environmental factors during the growth of the pups because these factors are important features for increasing the precision of mapping the QTLs.

Since the inheritance of NPHP follows Mendelian ratios for traits controlled by single recessive genes, we expected that when an informative marker was linked to the locus that modified the disease phenotype or when the segregation of the mean values of the phenotypic traits was dependant on the segregation of the informative marker, then the alleles would show at this locus an increasing frequency of homozygosity for the alleles inherited from LPK rats over the frequency of 50% [39]. However, this percentage frequency also depends on the disease penetrance and its expressivity and also on the distance of the marker from the modifier loci [40, 41, 42, 43].

In the phenotypic study, we established that only 48% of the BC1 progeny carried the LPK phenotype and this percentage varied for every marker used in genotyping. The variation in the percentage frequency of the marker genotypes is a result of the varying number of informative alleles for the markers used. However, the linkage of all phenotypic traits to the same QTLs strongly demonstrates the relationship that exists among loci during pathogenesis of diseases [34, 44].

This study has provided evidence that modifier loci on chromosomes 5q34–q36 and 7q11–q34 of the non-mutant, female WKY rats significantly influenced cellular progression of NPHP in the mutant BC1 progeny. Therefore, we propose that a new QTL mapping framework, where an interval test separates

Table III. Linkage analysis between phenotypes and genotypes in the BC1 progeny

Trait	Chromosome	Locus	Stat	%	P	CI	Add Value	Marker Interval	LOD
SBP	10	D10Rat43	9.7	37	0.00189	57	15.22	D10Rat218-D10Rat43	7.9
	10	D10Rat26	10.0	10	0.00158	56	20.30	D10Rat43-D10Rat26	5.1
%K/BW	10	D10Rat43	6.0	34	0.01435	90	0.88	D10Rat218-D10Rat43	7.9
	10	D10Rat26	15.4	15	0.00009	37	1.84	D10Rat43-D10Rat26	5.1
P/C Ratio	10	D10Rat43	4.8	34	0.02771	112	0.42	D10Rat218-D10Rat43	17.6
	10	D10Rat26	4.6	4	0.03216	118	0.39	D10Rat43-D10Rat26	13.2
PCV	10	D10Rat26	8.5	41	0.00361	66	–0.03	D10Rat26-D10Rat43	5.1
TSP	10	D10Rat26	8.0	11	0.00480	70	–4.02	D10Rat26-D10Rat43	5.1

Statistic – The likelihood ratio statistic (LRS) for the association of the trait with the locus, % – The amount of the total trait variance, which would be explained by the QTL at this locus, as a percentage, P-value – The probability of an association this strong happening by chance, CI – An estimation of the size of a 95% confidence interval for a QTL, Additive – The additive regression coefficient for the association, LOD Score – Logarithm of odds score.

Table IV. Epistatic interaction between genetic loci on chromosomes 5q34–q36, 7q11–q34 and *Nek8* gene on chromosome 10q25. The chi square statistic was considered significant at $p = 10^{-5}$

Trait	Chromosome	Locus1	Chromosome	Locus2	LRS	IX	Main1	Main2
SBP	5	D5Rat111	10	D10Rat26	26.5	14.9	4.1	11.3
PCV	7	D7Rat36	10	D10Rat26	35.8	11.7	4.9	15.5
TSP	7	D7Rat35	10	D10Rat26	23.4	7.10	4.4	15.5
% K/BW	7	D7Rat36	10	D10Rat26	35.0	7.70	7.1	15.4

LRS – Total likelihood ratio statistics for interaction between locus1 and locus2, IX – Interaction LRS, Main1 – LRS for locus 1 main effect, Main2 – LRS for locus 2 main effect.

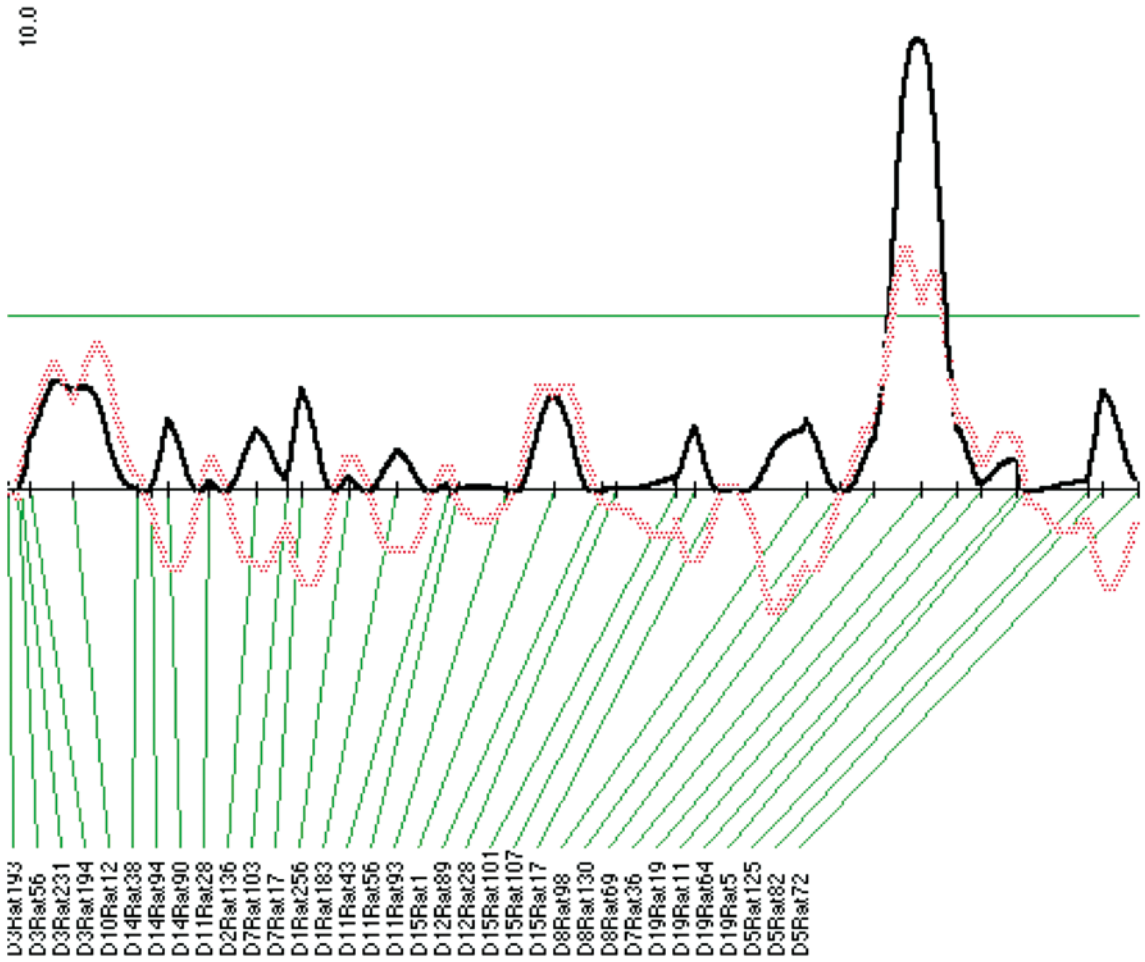


Fig. 7. Linkage between systolic blood pressure phenotypes and BC1 genotype. Linkage analysis associated systolic blood pressure phenotypic trait to chromosome 10 (D10Rat12) and chromosome 7 (D7Rat36). Other phenotypes are also associated with same locus on chromosome 7q11–q34

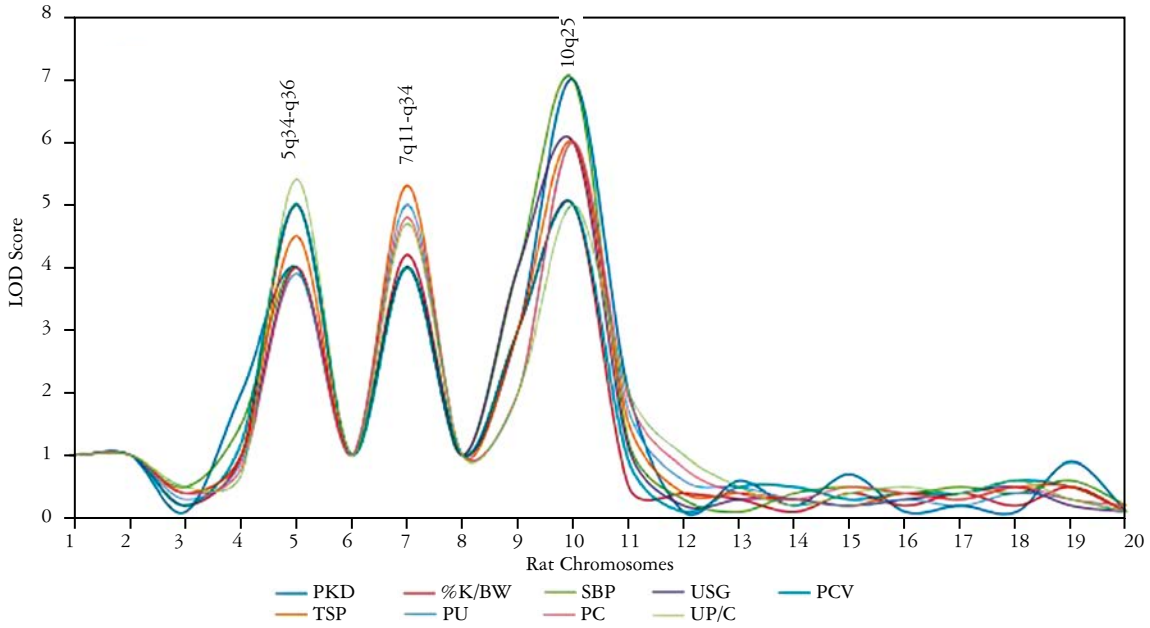


Fig. 8. The LOD scores resulting from association between phenotypes and loci on the rat chromosomes. The LOD scores show that loci on chromosomes 5q34–q36 and 7q11–34 influence the inheritance of the disease phenotypes caused by mutation in the *Nek8* gene. This is significant evidence for the presence of modifier genes within both chromosomes

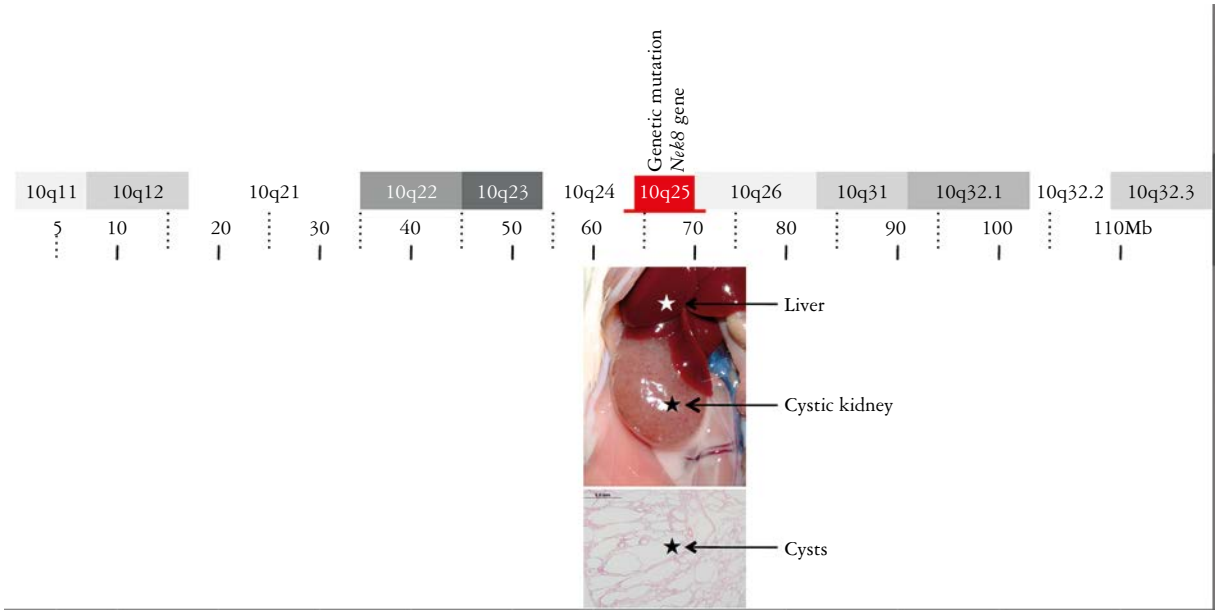


Fig. 9. Association between genetic locus and phenotypes controlled by the locus. This is the length of chromosome 10 in Mb and the locus where the Nek8 gene is located. Mutation in this locus resulted in the development of NPHP in the LPK rats. However, this locus is modified by genetic loci on chromosomes 5q34–q36 and 7q11–q34 of the non-mutant female WKY rats

and isolates the effects of individual QTLs, be carried out. This framework will improve the precision of mapping and will isolate the QTLs that specifically modify the disease phenotypes. This mapping framework will be particularly necessary because NPHP in our model is highly heritable and the genetic variation of the background strains can be easily controlled.

Therefore, mapping studies and linkage analysis have provided invaluable evidence that cellular pathogenesis of NPHP in this rat model is modified by genetic loci on the non-mutant, female WKY rats located on chromosomes 5q34–q36 and 7q11–q34. These modifier loci caused the phenotypic trait variation between the LPK parental strain and the mutant BC1 rats. The LOD scores associated with the genetic loci are greater than 3 and therefore provide substantial evidence for the presence of modifier genes within both loci. The study has therefore presented an opportunity to identify the modifier genes and the pathways through which modification of NPHP phenotypes in the mutant BC1 rats occurs. Presently, work is in progress to establish this.

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References

1. Arts HH, Knoers NV. Current insights into renal ciliopathies: what can genetics teach us? Pediatr Nephrol 2013; 28: 863-874.
2. Benzing T, Schermer B. Clinical spectrum and pathogenesis of nephronophthisis. Curr Opin Nephrol Hypertens 2012; 21: 272-278.
3. Chung EM, Conran RM, Schroeder JW, et al. From the radiologic pathology archives: pediatric polycystic kidney disease and other ciliopathies: radiologic-pathologic correlation. Radiographics 2014; 34: 155-178.
4. Chaki M, Hoefele J, Allen SJ, et al. Genotype-phenotype correlation in 440 patients with NPHP-related ciliopathies. Kidney Int 2011; 80: 1239-1245.
5. Yokoyama T. Renal cystic diseases as a ciliopathy. Nihon Rinsho 2011; 69: 1495-1503.
6. Guay-Woodford LM, Muecher G, Hopkins SD, et al. The severe perinatal form of autosomal recessive polycystic kidney disease maps to chromosome 6p21.1-p12: implications for genetic counseling. Am J Hum Genet 1995; 56: 1101-1107.
7. Zerres K, Mücher G, Bachner L, et al. Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. Nat Genet 1994; 7: 429-432.
8. Nagasawa Y, Matthiesen S, Onuchic LF, et al. Identification and characterization of Pkhd1, the mouse orthologue of the human ARPKD gene. J Am Soc Nephrol 2002; 13: 2246-2258.
9. Masyuk TV, Huang BQ, Masyuk AI, et al. Biliary dysgenesis in the PCK rat, an orthologous model of autosomal recessive polycystic kidney disease. Am J Pathol 2004; 165: 1719-1730.
10. Yengkopiong JP. Polycystic kidney disease gene in the Lewis polycystic kidney rat is mapped to chromosome 10q21-q26. Adv Genomics Genet 2012; 2: 55-66.
11. Halbritter J, Porath JD, Diaz KA, et al. Identification of 99 novel mutations in a worldwide cohort of 1,056 patients with

a nephronophthisis-related ciliopathy. Hum Genet 2013; 132: 865-884.
12. Hoff S, Halbritter J, Epting D, et al. ANKS6 is a central component of a nephronophthisis module linking NEK8 to INVS and NPHP3. Nat Genet 2013; 45: 951-956.
13. Rajanna DK, Reddy A, Srinivas NS, Aneja A. Autosomal recessive polycystic kidney disease: antenatal diagnosis and histopathological correlation. J Clin Imaging Sci 2013; 3: 13.
14. Hurd TW, Hildebrandt F. Mechanisms of nephronophthisis and related ciliopathies. Nephron Exp Nephrol 2011; 118: e9-14.
15. Wolf MT, Hildebrandt F. Nephronophthisis. Pediatr Nephrol 2011; 26: 181-194.
16. Bisceglia M, Galliani CA, Senger C, et al. Renal cystic diseases: a review. Adv Anat Pathol 2006; 13: 26-56.
17. Iakoubova OA, Dushkin H, Beier DR. Localization of a murine recessive polycystic kidney disease mutation and modifying loci that affect disease severity. Genomics 1995; 26: 107-114.
18. Yengkopiong JP, Lako JD. Genetic background of nonmutant Piebald-Virol-Glaxo rats does not influence nephronophthisis phenotypes. Int J Nephrol Renovasc Dis 2013; 6: 39-46.
19. Soloukides AP, Moutzouris DA, Papagregoriou GN, et al. Renal graft outcome in autosomal dominant medullary cystic kidney disease type 1. J Nephrol 2013; 26: 793-798.
20. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 1990; 348: 125-232.
21. Hoshino M, Yoshimori T, Nakamura S. Small GTPase proteins Rin and Rit Bind to PAR6 GTP-dependently and regulate cell transformation. J Biol Chem 2005; 280: 22868-22874.
22. Margolis B, Skolnik EY. Skolnik, Activation of Ras by receptor tyrosine kinases. J Am Soc Nephrol 1994; 5: 1288-1299.
23. Garbay C, Liu WQ, Vidal M, Roques BP. Inhibitors of Ras signal transduction as antitumor agents. Biochem Pharmacol 2000; 60: 1165-1169.
24. Cowley BD Jr, Chadwick LJ, Grantham JJ, Calvet JP. Elevated proto-oncogene expression in polycystic kidneys of the C57BL/6J (cpk) mouse. J Am Soc Nephrol 1991; 1: 1048-1053.
25. McCooke JK, Appels R, Barrero RA, et al. A novel mutation causing nephronophthisis in the Lewis polycystic kidney rat localises to a conserved RCC1 domain in Nek8. BMC Genomics 2012; 13: 393.
26. Bollée G, Fakhouri F, Karras A, et al. Nephronophthisis related to homozygous NPHP1 gene deletion as a cause of chronic renal failure in adults. Nephrol Dial Transplant 2006; 21: 2660-2663.
27. Tory K, Rousset-Rouvière C, Gubler MC, et al. Mutations of NPHP2 and NPHP3 in infantile nephronophthisis. Kidney Int 2009; 75: 839-847.
28. Zalli D, Bayliss R, Fry AM. The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis. Hum Mol Genet 2012; 21: 1155-1171.
29. Zhang JW, Wang C, Wang CY, Qiu ZQ. Gene analysis and literature review of autosomal recessive polycystic kidney disease. Zhonghua Er Ke Za Zhi 2013; 51: 684-687.
30. Zhou XH, Hui ZY, Li Y. Clinical and pathological features of a neonate with autosomal recessive polycystic kidney disease caused by a nonsense PKHD1 mutation. World J Pediatr 2013; 9: 76-79.
31. Yengkopiong JP, Lako JD. Genetic background of nonmutant Piebald-Virol-Glaxo rats does not influence nephronophthisis phenotypes. Int J Nephrol Renovasc Dis 2013; 6: 39-46.
32. Manly KF, Cudmore RH Jr, Meer JM. Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome 2001; 12: 930-932.
33. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 1995; 11: 241-247.
34. Abiola O, Angel JM, Avner P, et al. The nature and identification of quantitative trait loci: a community's view. Nat Rev Genet 2003; 4: 911-916.
35. Grigera PR, Ma L, Borgman CA, et al. Mass spectrometric analysis identifies a cortactin-RCC2/TD60 interaction in mitotic cells. J Proteomics 2012; 75: 2153-2159.
36. Humphries JD, Byron A, Bass MD, et al. Proteomic analysis of integrin-associated complexes identifies RCC2 as a dual regulator of Rac1 and Arf6. Sci Signal 2009; 2: ra51.
37. Cicila GT, Garrett MR, Lee SJ, et al. High-resolution mapping of the blood pressure QTL on chromosome 7 using Dahl rat congenic strains. Genomics 2001; 72: 51-60.
38. Woo DD, Nguyen DK, Khatibi N, Olsen P. Genetic identification of two major modifier loci of polycystic kidney disease progression in pcy mice. J Clin Invest 1997; 100: 1934-1940.
39. Eversley CD, Clark T, Xie Y, et al. Genetic mapping and developmental timing of transmission ratio distortion in a mouse interspecific backcross. BMC Genetics 2010; 11: 1-6.
40. Risch N. Linkage strategies for genetically complex traits. III. The effect of marker polymorphism on analysis of affected relative pairs. Am J Hum Genet 1990; 46: 242-253.
41. Risch N. Linkage strategies for genetically complex traits. II. The power of affected relative pairs. Am J Hum Genet 1990; 46: 229-241.
42. Risch N. Linkage strategies for genetically complex traits. I. Multilocus models. Am J Hum Genet 1990; 46: 222-228.
43. Risch N. Corrections to "Linkage strategies for genetically complex traits. III. The effect of marker polymorphism on analysis of affected relative pairs". Am J Hum Genet 1992; 51: 673-675.
44. Doerge RW. Mapping and analysis of quantitative trait loci in experimental populations. Nat Rev Genet 2002; 3: 43-52.

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