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MOLECULAR STUDY OF PKD_1 & PKD_2 GENES BY LINKAGE ANALYSIS AND DETERMINING THE GENOTYPE/PHENOTYPE CORRELATIONS IN SEVERAL IRANIAN FAMILIES WITH AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

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ABSTRACT

Background: Autosomal dominant polycystic kidney disease (ADPKD) is an inherited disorder with genetic heterogeneity. Up to three loci are involved in this disease, PKD1 on chromosome 16p13.3, PKD2 on 4q21, and a third locus of unknown location.

Methods: Here we report the first molecular genetic study of ADPKD and the existence of locus heterogeneity for ADPKD in the Iranian population by performing linkage analysis on 15 affected families.

Results: Eleven families showed linkage to PKD1 and two families showed linkage to PKD2. In two families, PKD1 markers are common in all affected members but PKD2 markers were not informative.

Conclusion: The results of this study demonstrate significant locus heterogeneity in autosomal dominant PKD in Iran. Analysis of clinical data confirms a milder ADPKD phenotype for PKD2 families. Our results showed relatively high heterozygosity rates and PIC values for some markers, while the most informative markers were KG8 and 16AC2.5 for PKD1 gene and AFM224x6 for PKD2 gene.

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Keywords: ADPKD, microsatellite marker, genetic diagnosis, linkage analysis.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is an hereditary systemic nephropathy (PKD [MIM 173900]), characterized by the development of cysts in the ductus organs (mainly the kidneys and the liver), with gastrointestinal and cardiovascular abnormalities.[42] The kidney cysts gradually enlarge and their number grows, destroying in this way the functional parenchyma (Fig. 1). As a consequence, the patients develop chronic renal failure (CRF), which progresses to end-stage chronic renal failure (ESCRF), requiring substitutional therapy (hemodialysis or kidney transplantation). ADPKD patients may benefit if the diagnosis can be established before symptoms develop.[58]

ADPKD is one of the most common genetic disorders.

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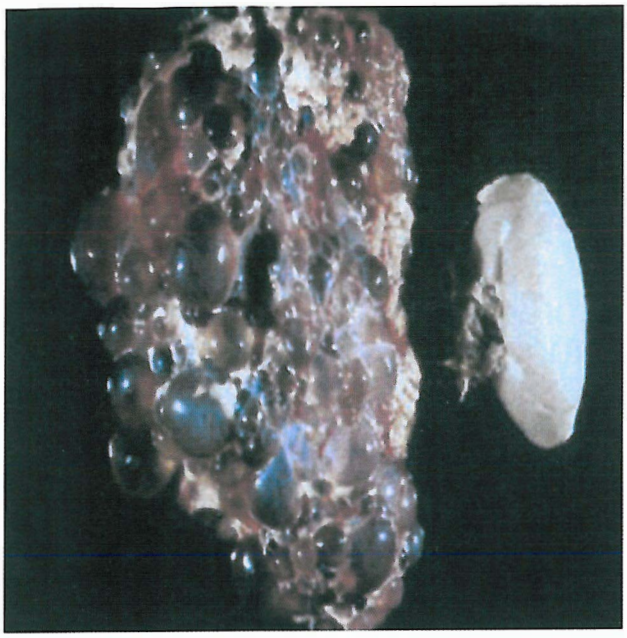

Fig. 1. Comparison between enlarge polycystic kidney (left) and normal kidney (right).

The gene frequency in the general population has been estimated to be about 1 in 400-1,000 in the Western countries.[42] ADPKD is genetically heterogeneous and there are at least three possible loci involved in this disease.[4] Mutations of the polycystic kidney disease 1 gene (PKD1 [MIM 601313]) on chromosome 16p13.3 are the most common and account for about 85% of all cases of ADPKD.[5] The PKD1 gene has been cloned, and the structure of its encoded complex protein, called polycystin, determined.[7] The polycystic kidney disease 2 gene (PKD2 [MIM 173910]) is located on chromosome 4q21-23.8 It is defective in most of the remaining ADPKD families.[5,8] A small number of families are affected by one or more undetermined loci (PKD3 [MIM 600666]), which do not map to the above loci.

Direct screening for PKD1 mutations in ADPKD is difficult due to the large gene size and extensive homology of PKD1 to other parts of the human genome.[9] The PKD2 gene is more amenable because of its smaller size and absence of extensive homology to other parts of chromosome 4. However, no "hot-spot" mutation has been identified in the PKD1 or PKD2 genes.[10] Genetic diagnosis through screening for defects in the PKD1 and PKD2 is technically possible but the cost and time involved are prohibitive and severely limit its clinical application. Alternative ways that identify the transmission of ADPKD are needed. In this communication, we report our finding of using DNA microsatellite marker analysis to determine the transmission of ADPKD in Iranian families.

MATERIAL AND METHODS

Normal population

Genomic DNA of 150 unrelated healthy individuals from Iran were obtained. Based on those samples, we determined the allele frequencies, heterozygosity rate, and PIC values of the six microsatellite markers in our local population.

ADPKD families and clinical evaluation

Ethical approval for this study was given by the various ethics committees of Iran. Informed consent was obtained from all individuals involved. Several families with the history of hereditary renal cysts were recruited and informed consent was obtained before our investigation. The following criteria were used for diagnosis of ADPKD: (i) the presence of at least two renal cysts (unilateral or bilateral) in an at-risk individual aged (30 years; (ii) the presence of at least two renal cysts in each kidney in an at-risk individual aged 30-59 years; (iii) the presence of at least four renal cysts in each kidney in an at-risk individual aged >60 years. All living relatives in our four families were invited for this study. They had blood pressure (BP) measurements and WHO criteria for hypertension were used; patients were regarded as hypertensive if the systolic blood pressure was 160 mmHg or higher or the diastolic blood pressure (Korotkoff phase V) was 95 mmHg or higher. Abdominal ultrasonography and full biochemical studies was arranged, including serum urea, creatinine, uric acid, urine microscopy, and a renal ultrasound. Urine studies for fractional extraction of sodium (FENa), fractional excretion of uric acid (FEur), and protein/creatinine ratio were also performed. Twenty-four hour urine collections for total protein excretion were performed in those with protein-urine on urine analysis studies. Creatinine clearance was calculated according to sex, age, lean body mass (LBM), and serum creatinine based on the equation (140-age)×LBM/72×serum creatinine for males, or (140-age) × LBM/72×serum creatinine×85% for females and was corrected for a body surface area of 1.73 m^2. Following their clinical assessments by nephrologists, the blood samples of each patient and first-degree relatives were collected for DNA extraction. A total of 4 affected kindred were included in this study following informed consent. A detailed family history was obtained from each subject. For some of the cases included here, since they died many years ago, there are no accurate clinical data and their children provided the information that they suffered from renal failure.

Controls

Controls were spouses of those affected in each fam-

ily as well as unaffected siblings of individuals with *PKD1* or *PKD2* (normal renal imaging and linkage results showed that their genetic risk was less than 1%). Affected individuals were matched with their partners whenever possible, irrespective of whether they were alive or dead. Whereas siblings had to have evidence that they were not affected to be eligible, spouses did not. Because in the absence of evidence of assortive mating for this disorder, affected individuals who could not be matched by partners were paired with individuals from the unaffected siblings selected on the basis of region, sex, and date of birth. For controls who had died, the age at death was recorded, and for the one control who had ESRD (cause unknown), the age at onset of this disease. Non-affected siblings who had died were included as controls only if there was proof that they did not have ADPKD.

PKD1 and *PKD2* linkage studies and haplotype construction

Large ADPKD families, selected only because of their suitability for study by genetic linkage, were tested for evidence of linkage to the *PKD1* and *PKD2* loci by means of polymorphisms flanking both loci. Two-point linkage analysis was performed and LOD scores calculated by the FASTLINK suite of programs.[59] Data available from

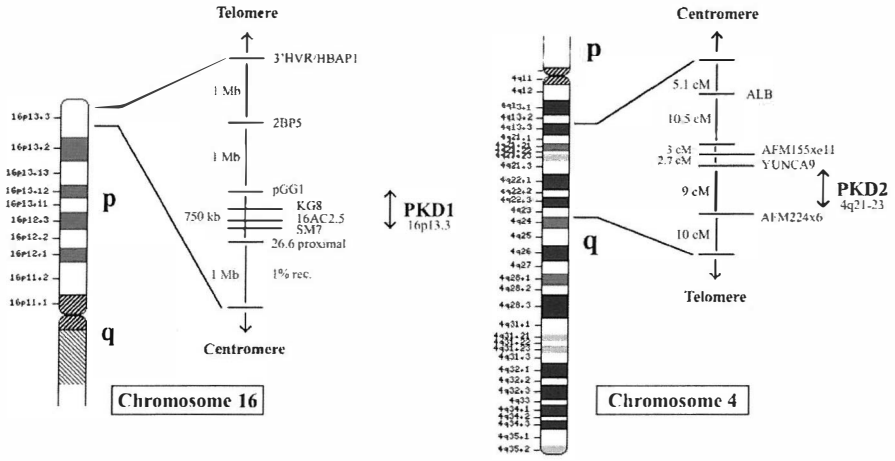

Fig. 2. Genomic locations of the microsatellite markers relative to *PKD1* on chromosome 16 and *PKD2* on chromosome 4.[16, 38]

Table I. Sequences and PCR characteristics of *PKD1* & *PKD2* specific primers.

	Name of Markers	Length (mer)	Tm (°C)	% GC	Primer Sequence	Size (bp)	Gel running time	Annealing T (°C) (hr. min)
PKD1	KG8	21	63	66	F: CTCCCAGGGTGGAGGAAGGTG	116-130	3.55	69
		21	65	71	R: GCAGGCACAGCCAGCTCCGAG			
	16AC2.5	25	60	48	F: GCAGCCTCAGTTGTGTTTCCTAATC	154-170	4.15	63
	(D16S291)	25	61	52	R: AGTGCTGGGATTACAGGCATGAACC			
	SM7	21	54	42	F: ACATATGTAGTCTTCTGCAGG	81-107	3.20	57
	(D16S283)	21	54	42	R: ACAAGAGTGAATCTCTGACAG			
PKD2	AFM155xe11	18	50	44	F: ATTCAGTTTCAGCCCCAT	146-158	4.15	55
	(D4S1534)	18	57	61	R: ACCAGCCCAAGGTAGAGG			
	YUNCA9	21	48	28	F: AGATGAGTATGTTATTATACC	154-169	4.45	55
	(D4S231)	18	53	50	R: TGCTAGAGTTCCCTAGTG			
	AFM224x6	21	56	47	F: TTGAGTAGTTCCTGAAGCAGC	103-125	3.50	55
	(D4S423)	21	56	47	R: CAAAGTCCTCCATCTTGAGTG			

Table II. No. of alleles, heterozygosity rates and PIC values of 6 microsatellite markers for linkage analysis of ADPKD in the Iranian population rate.

	Marker	Locus Symbol	Maker Type	Allele Size	Allele No.	Heterozygosity (h)	PIC
PKD1	KG8	N/A	(AC)n	116-130	6	0.80	0.772
	16AC2.5	D16S291	(AC)n	154-170	5	0.73	0.686
	SM7	D16S283	(AC)n	81-107	4	0.68	0.624PKD2
PKD2	AFM155xe11	D4S1534	(AC)n	146-158	4	0.60	0.519
	YUNCA9	D4S231	(AC)n	154-169	4	0.64	0.570
	AFM224x6	D4S423	(AC)n	103-125	5	0.76	0.712

the linked markers for *PKD1* and *PKD2* used in each family were combined by means of a Bayesian weighting formula to estimate the likelihood that a family shows linkage to one or other locus. Marker-allele frequencies were obtained from married-in individuals and from reconstruction of founder genotypes. The locations of the markers relative to *PKD1* and *PKD2* are shown in Fig. 2, respectively. The haplotypes of these microsatellite markers in members of the ADPKD families were determined.

Microsatellite Variant Repeat PCR (MVR-PCR)

Three microsatellite markers KG8,[61] 16AC2.5[61] and SM7[31,57] for *PKD1* gene, YUNCA9,[48] AFM155xe11[21,29] and AFM224x6[21,62] for *PKD2* gene, were amplified by polymerase chain reaction (PCR) using specific primers (Table I and Fig. 3). The following is a brief description of the procedure we used. Genomic DNA (30 ng) was PCR amplified in a final volume of 12.5 µL containing, 1×Cetus buffer II (PE Applied Biosystems, Warrington, UK), 1.5 mmol/lit $MgCl_2$, 200 mol/lit of each dATP, dGTP, dCTP and dTTP, 3pmol of each primer and one unit of Amplitaq Gold (Applied Biosystems). Amplification conditions were an initial denaturing step (6 min for 95°C), followed by 29 cycles of 94°C for 60 seconds, the primer specific annealing temperature (T_a) for 60 seconds and 72°C for 60 seconds. A final extension step for 60 seconds at T_a and 10 minutes at 72°C was performed. The Ta was 69°C for KG8, 63°C for 16AC2.5, 57°C for SM7 and 55°C for *PKD2* markers (D4S1534, D4S231 and D4S423). Polyacrylamide gel electrophoresis was used to resolve the markers according to the molecular size. 5 mL of the denatured PCR product were loaded onto a 12% denaturing polyacrylamide gel. The gel was run in Tris-Borate-EDTA buffer at 35 watts until the xylene cyanol dye reached approximately 5 cm from the bottom of the gel. It was colored by silver nitrate. The gel was then developed and kept for permanent record.

RESULTS

Population studies

We will concentrate mainly on the polymorphic microsatellite repeats closely linked to PKD1 and PKD2 genes. Before the analysis of linkage in our population,

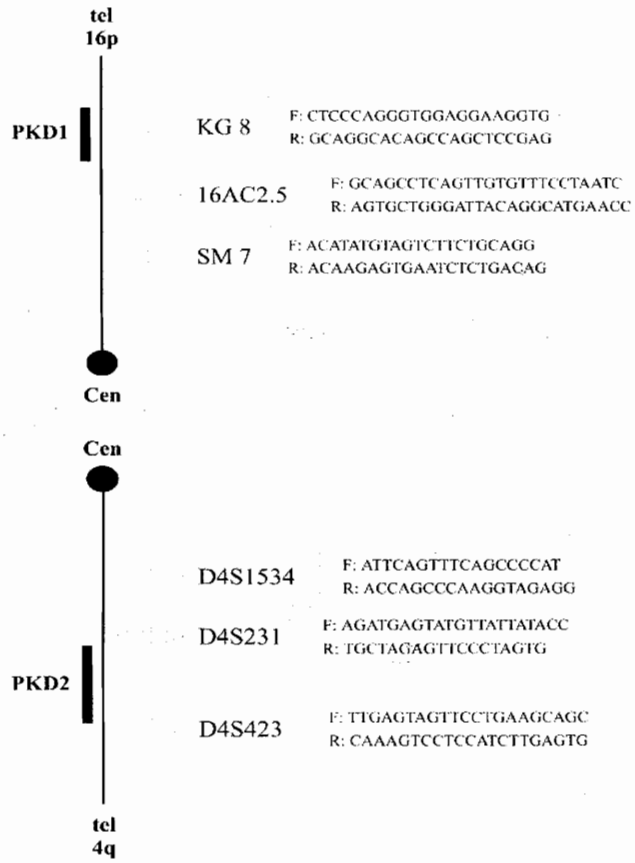

Fig. 3. Location of PKD1 & PKD2 specific primers.[38]

Table III. Clinical findings and specific characteristics of Iranian ADPKD patients.

Manifestation	Frequency
External	
Gastrointestinal	
o Hepatic cysts	Approximately 53%
o Pancreatic cysts	Approximately 11%
o Colonic diverticula	80% of patients with ESRD
Cardiovascular	
o Cardiac valvular abnormalities	27%
o Intracranial aneurysms	8%
Genital	
o Ovarian cysts	Unknown
o Testicular cysts	Unknown
o Seminal vesicle cysts	Unknown
Renal	
Anatomic	
o Renal cysts	100%
o Renal adenomas	27%
o Cyst calcification	97%
Hormonal alternations	
o Increased renin production	Approximately all affected hypertensive adults
o Preserved erythropoietin production	All patients with ESRD
Complications	
o Hypertension	87% (most patients with ESRD)
o Hematuria and / or hemorrhage	57%
o Acute and chronic pain	75%
o Urinary tract infection	Common
o Nephromegaly	100%
o Renal failure	47% of patients by age >50

ESRD= end-stage renal disease

we characterized the number of alleles, their sizes, polymorphic information content (PIC) and heterozygosity content of the microsatellite markers in 150 unrelated individuals in Iranian population (Table II). All the microsatellites except SM7 and AFM155xe11 had high heterozygositiy rates and were suitable for linkage analysis of ADPKD in Iran.

Family studies and haplotype constructions

Haplotype construction was performed at both of the PKD1 and PKD2 locus. Three microsatellite markers were identified at the *PKD1* locus including 16AC2.5-CA (D16S291), SM7-CA (D16S283) and KG8-CA that were closely linked to *PKD1* locus and three pairs of selected polymorphic markers including YUNCA9 (D4S231), AFM155xe11 (D4S1534) and AFM224x6 (D4S423) which were closely linked to the *PKD2* locus. Haplotype analysis revealed that each family had *PKD1* or *PKD2*-linked disease with a unique disease-associated haplotype. Our molecular study of 15 Iranian affected families was suggested that 11 of the 15 families linked to *PKD1* gene whereas 2 families was linked to PKD2 gene. In 2 families, *PKD1* markers were common in all affected members but *PKD2* markers were not informative. In this report, we describe 3 of 15 Iranian families with clear haplotype (two families linked to *PKD1* and one family linked to *PKD2* gene) (Figs.

Table IV. Comparison of ADPKD-1 and ADPKD-2 in Iranian patients.

	At-risk subjects <30 years old with renal cysts (%)	All subjects <40 years old with hypertension (%)	Subjects with cysts <40 years old with renal insufficiency (%)	Mean age of end-stage renal disease (yr)
ADPKD-1	48	27	11	56
ADPKD-2	11	8	0	73

4-6).

Family 1203:

In this family there has been a history of kidney disease with hypertension of fairly early onset. Multipoint analysis with polymorphic markers around *PKD1* and *PKD2* genes gave evidence for linkage within the consensus region. Haplotype of *PKD1* markers {KG8, SM7, 16AC2.5}: 3,4,4 was segregated by the ADPKD members, II:3, III:3 and III:7 for *PKD1* markers (Fig. 4). This haplotype was neither carried by I:2 and II:5.

A common haplotype of PKD2 markers {D4S1534, D4S231, D4S423}: 2,3,2 carried by the ADPKD affected member (II:3 and III:3) as well as the unaffected II:5 and

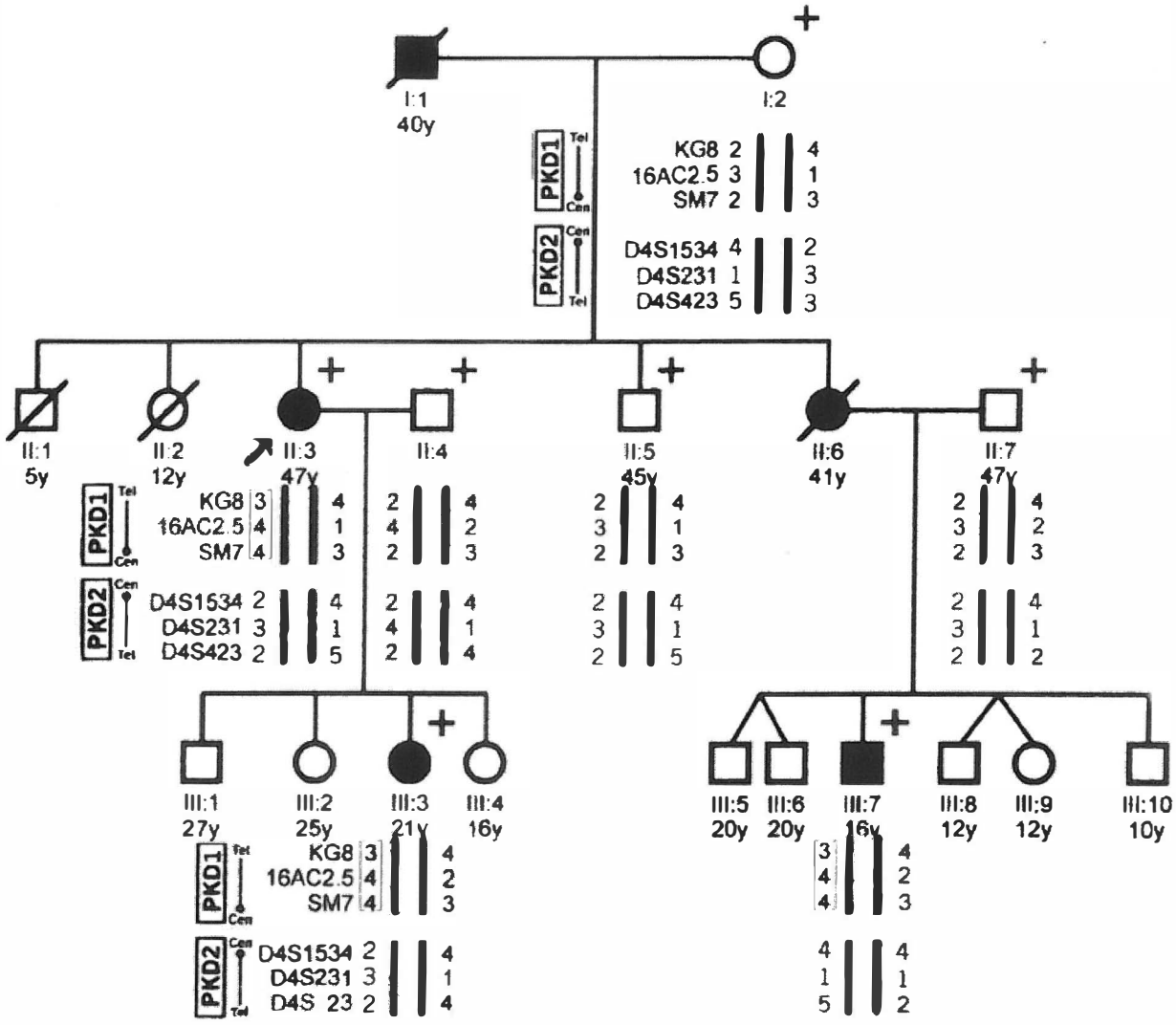

Fig. 4. Phasing and haplotype construction for PKD1 and PKD2 linked markers in family 1203.

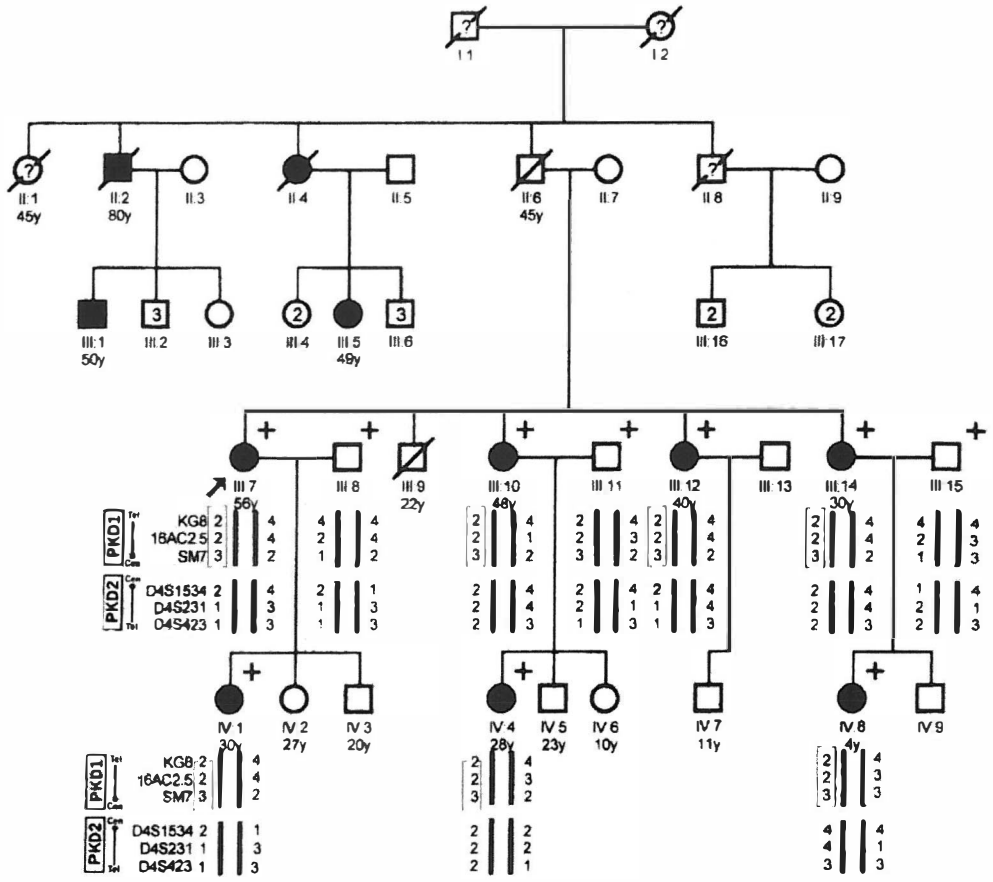

Fig. 5. Phasing and haplotype construction for *PKD1* and *PKD2* linked markers in family 1208.

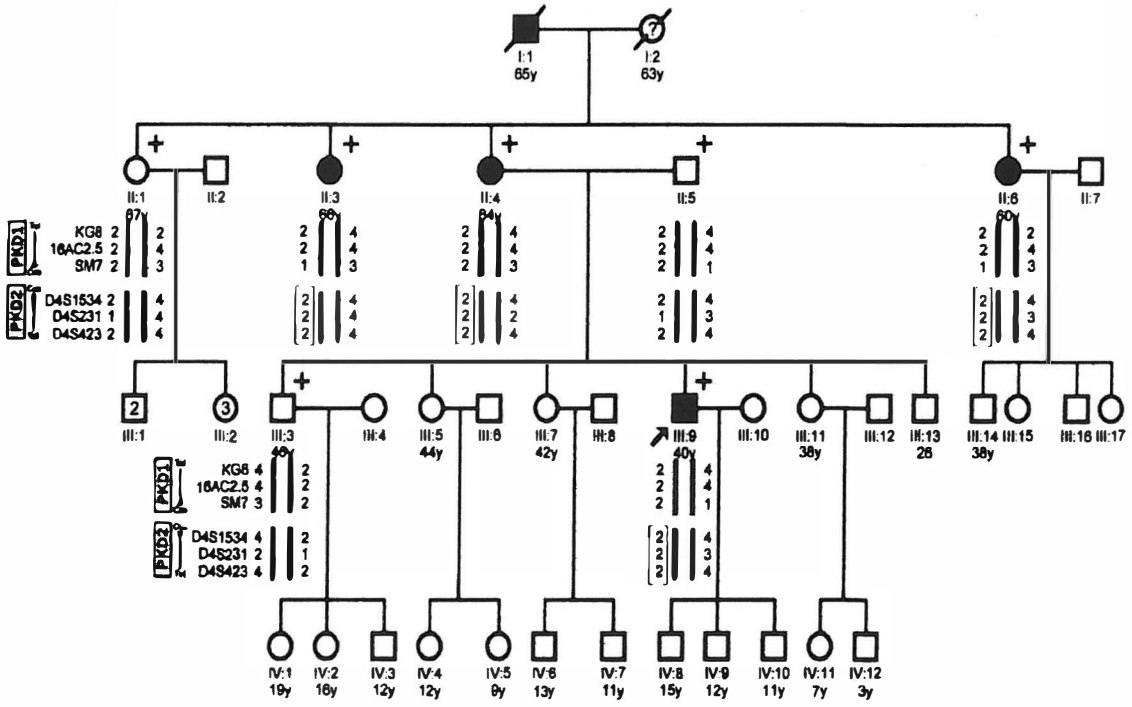

Fig. 6. Phasing and haplotype construction for *PKD1* and *PKD2* linked markers in family 1214.

it is not carried by affected III: 7. Therefore, it is concluded that ADPKD is linked to PKD1 but not PKD2 in this family. Clinical evaluations confirmed this finding.

Family 1208:

In this family haplotype for *PKD1* markers: 2,2,3 was common in all affected members (III:7, III:10, III:12, III:14, IV:1, IV:4 and IV:8) but for *PKD2* markers since affected members have not a common haplotype in this family, the disease phenotype is most probably co-inherited with haplotype 2,2,3 and ADPKD is linked to *PKD1* locus (Fig. 5).

Family 1214:

On the other hand, a different linkage pattern was observed in family 1214. This family showed statistically significant evidence of non-linkage to *PKD1*. The analysis of chromosome 4 markers showed positive linkage to *PKD2* locus. In this family four ADPKD and three unaffected members were studied. There was no common *PKD1*-linked haplotype shared by all the ADPKD affected members (Fig. 6). There was common haplotype of *PKD2* markers: 2,2,2 Carried by the ADPKD affected member II:3, II:4, II:6 and III:9. This haplotype was neither carried by unaffected persons (II:1 and III:3). This finding indicating likely linkage to the PKD2 gene.

Clinical findings and genotype/phenotype correlations

A total of 135 subjects from 15 Iranian ADPKD families were studied for the purpose of this report. The total number of patients originating from linked to *PKD1* was 68 and that of patients from PKD2 families was 25. Totally, 93 investigated subjects were affected (ADPKD1 or ADPKD2), 31 blood relatives were unaffected (at risk subjects), and 11 were spouses. In the *PKD1* families, all members 30 years of age with affected relatives and a *PKD1* associated haplotype, had detectable cysts (Table III). No member entered ESRD below the age of 40. Seventeen subjects developed chronic renal failure at mean age of 48 years. However, members without the associated *PKD1* haplotype did not enter ESRD before age of 60 year. Haplotype analysis revealed the presence of a unique disease-associated haplotype in each family. Interfamily differences were detected when comparing overall survival. Family 1206 had the shortest survival, with a median age of 47.5 years, and family 1213 had the longest, with a median age of 77.4 years. For renal survival, the difference between families was highly significant (P= 0.0001).

PKD2 patients, compared with those who have *PKD1*, present with symptoms later in life, live longer, have a lower risk of progressing to renal failure, and have fewer complications (Table IV). In this study, *PKD2* was associated with a reduction of 17 years in median survival to death or onset of ESRD compared with the survival of those without ADPKD. To overcome this case-mix, as well as regional differences, we defined the endpoint for overall survival as death or onset of ESRD. The current true survival figures taking into account survival on dialysis will almost certainly be more optimistic, particularly for those with *PKD1* who have a higher risk of developing ESRD. Although the prevalence of hypertension in affected ADPKD1 individuals increased with age (X^2= 26.4, p<0.00001), there was no significant difference between affected males and females (X^2 = 0.14, p= 0.71). Against of this finding, males with ADPKD2 had significantly worse overall survival (p= 0.0085), as well as renal survival (p= 0.03) than females.

DISCUSSION

The diagnosis for presymptomatic ADPKD is mostly done by ultrasonography.[24] The age dependent manifestation of ADPKD renders imaging techniques less effective diagnostic tools than genetic studies. PKD1 is a big gene that generates a 14 kb transcript, and there are 3 regions highly homologous with the α-globin genes at its 5' end.[35] Mutations falling within the homologous regions are difficult to identify.[54]

With the discovery of microsatellite markers closely associated with the *PKD1* and *PKD2* gene loci, PCR amplification of these markers is increasingly being applied on presymptomatic and antenatal diagnosis.[6,16] In the present study, we used six markers for PKD1 and PKD2 genes. Our survey of the allele frequencies of the microsatellites in the local population (Table II) provided data for a rational choice of markers in future ADPKD genetic linkage studies. KG8 and 16AC2.5 had the highest heterozygosity rates and PIC values and were the most informative markers for the *PKD1* locus. SM7 had intermediate PIC values. *PKD2* markers were not very informative individually, but were highly informative when used in combination. AFM155xe11 had a low PIC value. The results of this study demonstrate significant locus heterogeneity in autosomal dominant PKD in Iran. The proportion of families linked in *PKD1* is estimated to be 78%.

In our study, haplotypes were constructed in a number of ADPKD families using respective markers. Assignment of the disease gene loci was performed following phasing and haplotype construction, genotype/phenotype correlations were deduced from the constructed haplotypes. In studying the genotype/ phenotype correlations we have focused on our patient's phenotype

and found that high blood pressure is more prevalent in ADPKD patients than in non-affected relatives.

Our study confirms the fact that *PKD2* is a milder form of the disease. Although the age of ESRD seems not to be affected by prompt and effective treatment in order to avoid vascular complications. A clearer appreciation of the relative frequency of complications among people with *PKD1* and *PKD2* has the potential to provide practical management guidance for those affected with ADPKD. Because of the technical demands and expense of both mutation detection and linkage analysis in ADPKD, identification of the genotype is not currently possible in the majority of cases. Nevertheless, the findings of a higher prevalence of hypertension and the poorer cumulative overall survival probability in *PKD1* than in *PKD2*, support views that hypertension in ADPKD has a deleterious effect, particularly on renal survival, although the more rapid progression of *PKD1*-associated disease may be due to pathogenetic mechanisms unrelated to hypertension. Although our data have confirmed that PKD2 is clinically milder than *PKD1*, the adverse effect on survival shows that it cannot be regarded as a benign disorder.

In the course of our study we encountered some limitations of genetic linkage studies and some of the investigated families have no linkage to either of the known gene loci; the absence of clear linkage in these families does not necessarily imply the presence of another disease locus and the existence of a third locus for ADPKD remains uncertain at this study.[53] A number of potential confounders, including genotyping errors, no paternity, misdiagnosis, and bilineal disease, could lead to false exclusion of linkage and will need to be vigorously excluded in these families. But having more siblings within the families generally gives more information for haplotype construction and data interpretation.[59] With increased emigration and a tendency to have smaller families, it could be difficult to establish the haplotype of markers associated with the disease in some families. This problem is compounded in the study of ADPKD due to its genetic heterogeneity.

ACKNOWLEDGEMENT

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ELECTRONIC-DATABASES

Electronic-Websites for data in this article are as follows: Online Mendelian Inheritance of Man (OMIM), http:// www.ncbi.nlm.nih.gov/omim/(for PKD [MIM 173900], *PKD1* [MIM 601313], *PKD2* [MIM 173910] and *PKD3* [MIM 600666]).

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