GENETIC TESTING
Volume 12, Number 2, 2008

Pp. 1–6
DOI: 10.1089/gte.2007.0110

GTE-07-0110-Voskarides
Type: research-article

COL4A3 Founder Mutations in Greek-Cypriot Families with Thin Basement Membrane Nephropathy and Focal Segmental Glomerulosclerosis Dating from Around 18th Century

AU1 ▶

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Mutations in the *COL4A3*/*COL4A4* genes of type IV collagen account for about 40% of cases of thin basement membrane nephropathy, a condition that is estimated to affect 1% or more of the general population. We recently described 10 Cypriot families with familial hematuria and thin basement membrane nephropathy in the presence of focal segmental glomerulosclerosis, with founder mutations on *COL4A3* gene. Seven of the families carried mutation G1334E on haplotype K, and another three carried mutation G871C on haplotype Ky. In this report we performed extension of the haplotypes with additional polymorphic markers, 12 for haplotype K and 22 for haplotype Ky, to estimate the linkage disequilibrium value between the mutation and flanking non-common markers. Haplotype Ky extended to 13.71 Mb, but we did not attempt further analysis owing to the small number of chromosomes. Haplotype K extended to 3.83 Mb, thereby suggesting that it was a much older event compared to mutation G871C. Mutation G1334E was calculated to be about 5–10 generations old with a possible origin between 1693 and 1818 AD, during the Ottoman ruling of the island. Both mutations are clustered in specific geographic regions with apparently formerly isolated populations, although mutation G1334E has been detected elsewhere on the island. The identification of founder mutations in large families with microscopic hematuria greatly facilitates presymptomatic diagnosis and provides useful information on the history of the population, while it may also assist in association studies in search for disease modifier genes.

Introduction

Thin basement membrane nephropathy (TBMN) is a frequent condition that may affect up to 1% or more of the general population. Approximately 40% of TBMN cases are caused by mutations in one of the two collagen type IV genes at locus 2q36-37, *COL4A3*, or *COL4A4* (Kashtan, 2002; Rana *et al.*, 2005; Tryggvason and Patrakka, 2006). TBMN is largely considered a benign disease with excellent prognosis, with microscopic hematuria as the cardinal and mostly isolated finding, only occasionally progressing to proteinuria and chronic renal failure. It has also been described in several other glomerulopathies such as minimal change disease, IgA nephropathy, and, rarely, focal segmental glomerulosclerosis (FSGS) (Jefferson *et al.*, 1997; van der Loop *et al.*, 2000; Ciccarese *et al.*, 2001; Pescucci *et al.*, 2004; Torra *et al.*, 2004).

Familial FSGS is often a severe primary disease associated with autosomal dominant heterozygous mutations in several genes, those being *ACTN4*, *TRPC6*, *CD2AP*, or *WT1* (Kaplan *et al.*, 2000; Kim *et al.*, 2003; Ruf *et al.*, 2004; Winn *et al.*, 2005), or with homozygous recessive inheritance mutations, mostly in childhood, such as podocin-related mutations in *NPHS2*, which is expressed at high levels in podocytes (Boute *et al.*, 2000). At the same time, however, FSGS can be a secondary development from several other primary conditions. Of particular interest is the published work by several investigators which suggests that TBMN and collagen 4 mutations can be a predisposing condition that may lead to FSGS and renal impairment (Nieuwhof *et al.*, 1997; Nogueira *et al.*, 2000; Norby and Cosio, 2005).

Recently, we described nine families among the Greek-Cypriot population of Cyprus that presented with the

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histological finding of FSGS, on single or multiple renal biopsies. Upon detailed investigation we considered the FSGS phenotype to be of secondary nature, as all families segregated familial microscopic hematuria in the presence of TBMN and heterozygous mutations in the *COL4A3*/*COL4A4* genes. Most striking was the finding that of 82 heterozygous patients for whom we had detailed clinical information, 31 patients (38%) had progressed to chronic renal failure, while 16 of them (19.5%) had progressed to ESRD (Voskarides *et al.*, 2007). These numbers do not represent the norm in the existing literature; rather, they describe an exceptionally high percentage of patients with TBMN who progress to FSGS and ESRD, and do not support the current belief that TBMN and benign familial hematuria is always a benign disease. At this point it is worth mentioning that this complex phenotype of FSGS-associated TBMN adds to the phenotypic heterogeneity, and one way to deal with the significant variable expressivity of carriers of *COL4A3*/*COL4A4* mutations is to collectively refer to them as *COL4A3*/*COL4A4* nephropathies, thereby treating them under a unified molecular terminology (Torra *et al.*, 2004).

Here we extended the haplotypes of the chromosomes with identified mutations in an effort to estimate the degree of linkage disequilibrium and if possible date the mutations. We based our interest on the striking fact that several families with the same causative mutation shared the limited haplotype we constructed while doing linkage analysis, thereby permitting the hypothesis that we dealt with a strong founder effect in a population with geographic clustering in a region near Nicosia, the capital of Cyprus.

Materials and Methods

Subjects, DNA isolation, and linkage analysis

All patients and relatives participated in this study as part of a research program aimed at deciphering the etiology of FSGS in several families, which proved to be on the background of collagen 4 mutations that caused TBMN. Peripheral blood for DNA isolation was collected upon informed written consent. DNA linkage was found for locus 2q36-37 with four markers initially (Fig. 1) and mutations identified in both *COL4A3* and *COL4A4* genes of type 4 collagen (Voskarides *et al.*, 2007). All samples of the present work belonged to 10 Greek-Cypriot families, 7 of which shared haplotype K with mutation G1334E and 3 shared haplotype Ky with mutation G871C. In one of the families both haplotypes segregated, whereby in a younger generation two siblings were compound heterozygotes and developed Alport syndrome in the presence of sensorineural deafness.

Linkage disequilibrium analysis and mutation dating

For linkage disequilibrium analysis and mutation dating, one patient was chosen per family (the oldest proband) who clearly had the *COL4A3* G1334E or G871C mutation, for extended haplotype construction. Hence, eight chromosomes were analyzed for G1334E mutation and three for G871C. DNA samples from offspring and spouses were analyzed to phase the haplotypes (except family CY5326, where only one sample was available) (Fig. 2).

To date the G1334E mutation we used the *D2S1363* (5′ of haplotype) and *D2S1392* (3′ of haplotype) STR markers (Fig. 2B). The genetic distance between the mutation and the markers was calculated by linear regression analysis of genetic versus physical map position by use of eight markers (*D2S279*, *D2S2390*, *D2S1363*, *D2S2354*, *D2S159*, *D2S401*, *D2S1239*, and *D2S2297*) in proximity to *COL4A3*, with known cM (Marshfield map; UniSTS—NCBI) and Mb (Ensembl) information. Marshfield map cM information does not exist for *D2S1392*, instead we considered that cM position of the very close marker *D2S427* (*D2S1392*: 231.914444 Mb, *D2S427*: 231.914508 Mb) is the same with *D2S1392*. Kosambi function was applied to convert genetic map distance (cM) into recombination fraction (θ). The linkage disequilibrium (LD or δ) index was calculated using the Bengtsson and Thomson (1981) formula: $\delta = (P_d - P_n)/(1 - P_n)$, where P_d and P_n are the frequencies for marker allele on mutation-bearing and normal chromosomes, respectively. Allele frequencies in unrelated normal chromosomes were found by genotyping 62 unrelated individuals of the general population. Estimation of the age of the mutation was based on the following formula: generations $= \log \delta / \log (1 - \theta)$ (Risch *et al.*, 1995; see also Colombo *et al.*, 2000, and Chan *et al.*, 2004).

Results and Discussion

Initial molecular investigation revealed that 7 of the 10 families carried *COL4A3* mutation G1334E on common haplotype K and another 3 carried *COL4A3* mutation G871C on haplotype Ky, thereby strongly suggesting founder effects (Fig. 1). This prompted us to seek detailed family histories in an effort to locate common ancestors. No obvious relationship could be identified based on personal communication. No written records were available.

Four of the families that shared haplotype K originated from a village near Nicosia, named Kaimakli, one was from a small village, Mosphiloti in Larnaca prefecture, and two more were from the village of Xylotymbou also in Larnaca prefecture further south-east (Fig. 3). The three families that shared haplotype Ky originated in three neighboring villages near the north coast, from where they were expelled in the summer of 1974 during the Turkish invasion of Cyprus. This strong geographic clustering in formerly isolated populations and the haplotype sharing is a very strong indication of founder effects. Based on the shared haplotype K in seven reportedly unrelated families, we calculated values for linkage disequilibrium in an attempt to date this mutation. We did not observe this disease haplotype K in 40 unrelated samples using markers *D2S1363*, *D2S159*, *D2S401*, and *D2S439*, which were initially utilized for linkage analysis. We observed the disease haplotype Ky only in 2 individuals out of 40 unrelated samples.

Haplotype K encompassed eight polymorphic markers in total, and was flanked by varying STR *D2S1363* at 5′-end and STR *D2S1392* at 3′-end (Fig. 2B). Haplotype Ky was more extensive encompassing 15 polymorphic markers and was flanked by varying STRs *D2S377*/*D2S338* (Fig. 2B). The physical length of the G871C haplotype Ky is 13.71 Mb and is much larger than G1334E haplotype K, which extends to 3.83 Mb. This is an indication that G871C mutation is "younger" than G1334E mutation. We did not attempt dating of mutation G871C because we found only three unrelated chromosomes to carry this mutation (small statistical power for LD estimation).

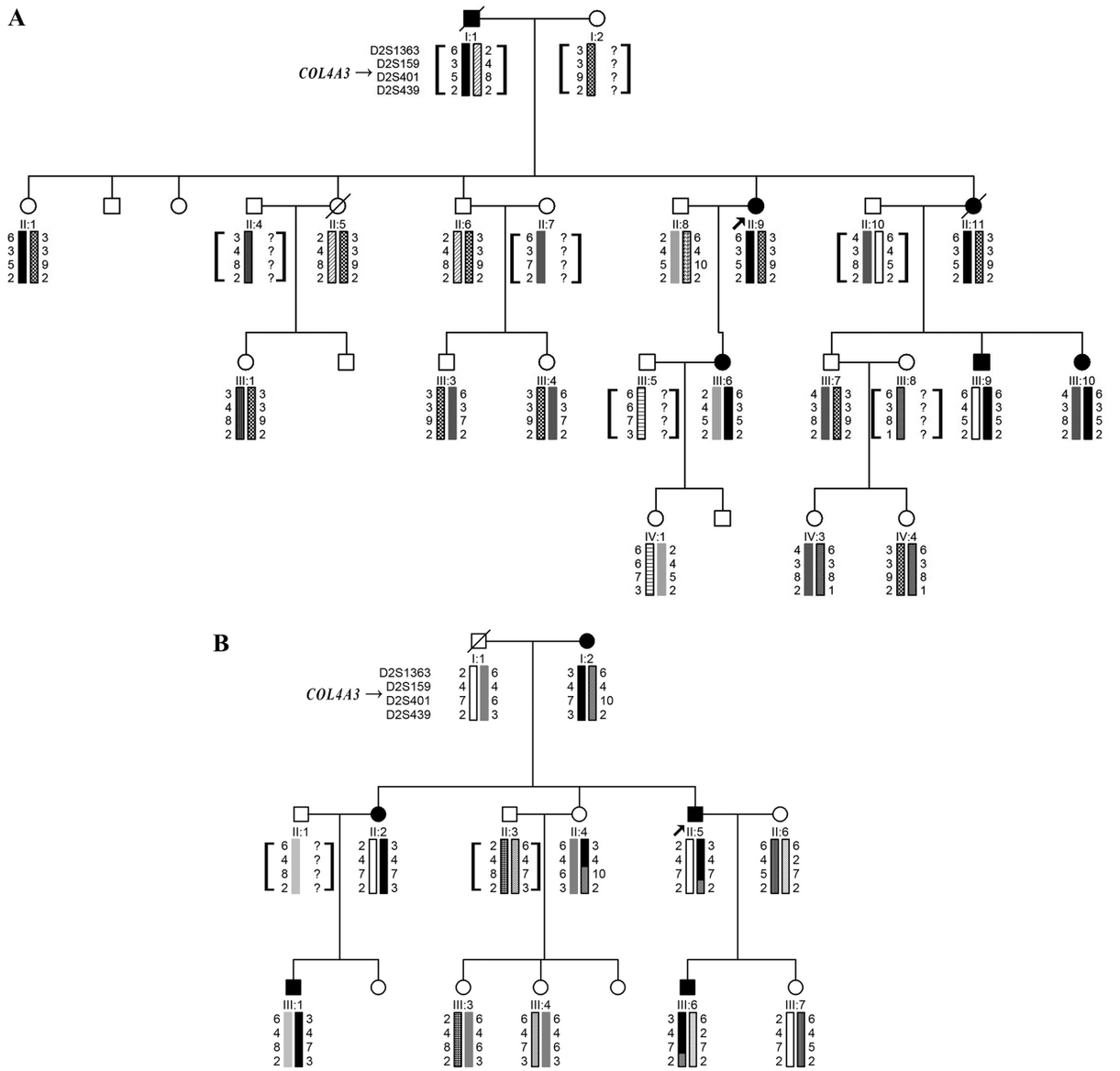
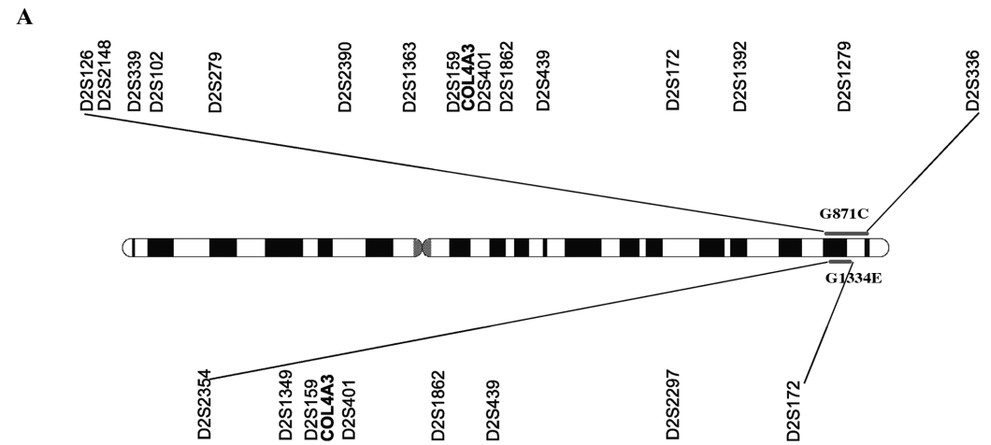


FIG. 1. (**A**) Pedigree of family CY5304, which segregates mutation G1334E on haplotype K. Filled black squares and circles are affected males and females, respectively. Diagonal lines denote deceased members; black bars denote the inherited affected haplotype. Haplotypes in brackets are deduced. (**B**) Pedigree of family CY5306, which segregates mutation G871C on haplotype Ky. Symbols are as in (**A**). Haplotypes of individuals II:4 and II:5 are the result of distal recombination events.

The cM position of the G1334E mutation was found to be 229.1 cM. In order to date the G1334E mutation we used the *D2S1363* (5′-end of haplotype) and *D2S1392* (3′-end of haplotype) with disease allele frequencies 0.345 and 0.250, respectively, among 62 healthy unrelated samples. Applying Kosambi function and LD equation by Bengtsson and Thomson (Bengtsson and Thomson, 1981; Risch *et al.*, 1995), we found the following values: *D2S1363*–G1334E: $\theta = 0.021$, $\delta = 0.809$; G1334E–*D2S1392*: $\theta = 0.076$, $\delta = 0.667$. Using these data, the mutation is dated to be 10 generations old based on marker *D2S1363* and 5 generations old based on marker *D2S1392*. Assuming an average generation length of 25 years and an average age of 63 years for the eight unrelated probands, we can place the possible time period of G1334E origin between 1693 and 1818 AD, during the Ottoman ruling of the island.

Cyprus is a rather small island with a Greek-Cypriot population of 646,400 according to the 2005 demographic data. The work presented here perhaps describes the most extensive documented series of founder mutations in the Cypriot population for an autosomal dominant disease. It is reasonable to assume that a founder of mutation G1334E was a resident of the village of Kaimakli, an urban area near the capital of Nicosia, 5–10 generations ago. Limited migration and traveling resulted in an increase in the mutation frequency in that region, explaining why all mutant chromosomes

A



B

	D2S / FAM	2390	1363	2354	1349	159	401	1862	439	2297	172	1392	1279
		225.64	226.73	227.05	227.51	227.53	227.96	228.32	228.88	230.08	230.88	231.91	233.65
COL4A3-G1334E (haplotype K)	CY4201	6	6	7	3	3	5	5	2	1	11	3	8
	CY5301	6	6	7	3	3	5	5	2	1	11	3	8
	CY5304	7	6	7	3	3	5	5	2	1	11	3	8
	CY5307a	6	6	7	3	3	5	5	2	1	11	2	8
	CY5307b	6	6	7	3	3	5	5	2	1	11	3	8
	CY5313	7	6	7	3	3	5	5	2	1	11	3	8
	CY5314	7	3	7	3	3	5	5	2	1	11	3	4
	CY5326	-	6	7	3	3	5	5	2	1	11	2	3

	D2S / FAM	164	2250	1242	377	126	2148	339	102	279	2390	1363	159	401	1862	439	172	1392	1279	336	338	2968	125
		217.6	219.4	220.9	221.5	221.7	221.8	222.4	222.7	223.8	225.6	226.7	227.5	227.9	228.3	228.8	230.8	231.9	233.6	235.4	236.9	237.7	240.8
COL4A3-G871C (haplotype Ky)	CY4201	11	3	6	4	3	2	4	6	3	7	3	4	7	5	2	10	3	4	3	2	3	5
	CY5306	8	4	4	1	3	2	4	6	3	7	3	4	7	5	3	10	3	4	3	4	2	6
	CY5323	2	4	5	1	3	2	4	6	3	7	3	4	7	5	3	10	3	4	3	1	2	1

FIG. 2. (**A**) Chromosome 2 ideogram focusing on the region around locus *COL4A3/COL4A4*. As illustrated, the length of the diseased conserved shared haplotype that bears mutation G871C is much more extensive compared to the one bearing mutation G1334E. (**B**) Disease haplotypes for each one of the families with the *COL4A3*–G1334E (haplotype K) or *COL4A3*–G871C (haplotype Ky) mutations. *D2S* microsatellite markers are shown in the first row, and their Mb physical position, according to Ensembl, is shown in the second row. The least common haplotypes are in bold. Marker *D2S439* allele 2 in family CY4201 differs in haplotype Ky, most probably owing to a spontaneous mutation. The *COL4A3/COL4A4* locus is located between markers *D2S159* and *D2S401*. Note that family CY4201 carries both haplotypes, K and Ky. Note also that in the text we refer to seven families that share haplotype K, while here we show eight haplotypes. This is because family CY5307b is actually a single patient from Kaimakli village, married into family CY5307a (also from Kaimakli), who incidentally happened to carry the same mutation G1334E on haplotype K. No specific known relationship could be identified, although during a long conversation with the authors the two spouses alluded to an undefined probable common ancestor, several generations back.

◀AU9

bearing mutation G1334E are identical by descent, as evidenced by the common haplotype K, the high linkage disequilibrium, and the geographic clustering. Even with limited migration rates, and the rather young age of the founding of this mutation, we found it on identical haplotype K in two other places further south (Fig. 3). The extensive traveling in present-time Cyprus in combination with more random mating practices is expected to promote gene flow and the spread of the mutation all over the island. The same scenario holds for the clustering of *COL4A3* mutation G871C in villages at the northern coast, in the Kerynia prefecture. However, this finding is presently more of a historic interest, as all Greek-Cypriots of that region were expelled and forced to move further south during the hostilities with Turkey in July 1974. Also, the fact that mutations G871C and G1334E have been reported only in Cypriot patients may imply that they originated here.

To our knowledge no other occurrence of *COL4A3/COL4A4* founder effect has been reported in the past in other populations. A well-documented founder of an Alport syndrome mutation has been reported by Arrondel *et al.* (2004), who described a large tandem duplication within the *COL4A5* gene in the French Polynesia. In addition to the founder effect presented here, similar phenomena and impressive geographic clustering have been investigated and documented before for several other mutations or diseases among the Hellenic population of Cyprus. Best examples are the high carrier frequency of 1:14 for *CFTR* gene mutation F508del, in the village of Athienou, and the frequency of 1:7 to 1:10 for Friedreich Ataxia gene *FRDA*, in the neighboring villages Arodes and Kathicas and in the surrounding population, in Pafos, at west of the island (Dean *et al.*, 1988; Yiallouros *et al.*, 2007). Other examples, this time for dominant diseases, are medullary cystic kidney disease type 1 with

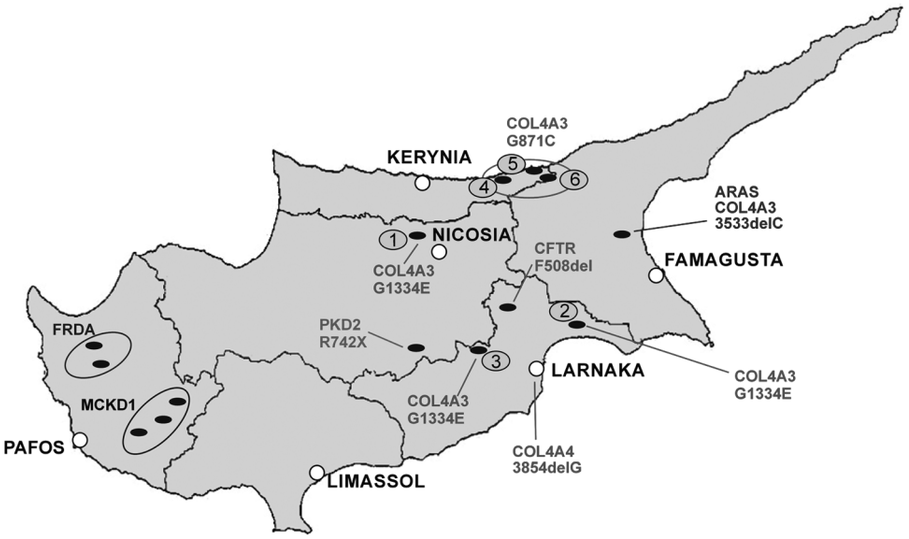


FIG. 3. Modified genetic map of Cyprus, as it has been prepared and published by Deltas (2004). Shown are clusters or regions with increased frequency of *COL4A3*/*COL4A4* mutations, as evidenced by the identification of families. At the area near Nicosia, five large families, apparently with a common founder and sharing haplotype K, carry mutation G1334E in *COL4A3*, which is also found in families at two other villages further south. One large family (not described in this report) with classical autosomal recessive Alport syndrome originates from a village near Famagusta (ARAS, *COL4A3* 3533delC) (Heidet *et al.*, 2001). Shown are also paradigms of instances of geographic clustering and implied founder effects for renal and other diseases. Several large *MCKD1* families in the area of Pafos are clustered in a triangle of three neighboring villages with formerly isolated populations, and segregate a common haplotype (Stavrou *et al.*, 2002). A *PKD2* gene mutation, R742×, segregates in a huge family (nearly 90 affected subjects) in the Lythrodontas village near Nicosia. *PKD2*, polycystic kidney disease type 2; *MCKD1*, medullary cystic kidney disease type 1, adult type; *CFTR*, cystic fibrosis transmembrane regulator; *FRDA*, Friedreich ataxia. Geographic key (circled numbers)—1: Kaimakli, four families; 2: Xylotympou, two families; 3: Mosphiloti, one family; 4: Hartzia, one family; 5: Kalograia, one family; 6: Ayios Nikolaos, one family.

a putative founder in the area of Pafos (Stavrou *et al.*, 2002) and polycystic kidney disease type 2 with a founder of a huge family of nearly 90 affected subjects carrying the *PKD2* gene mutation R742× in the Lythrodontas village near Nicosia (Mochizuki *et al.*, 1996) (Fig. 3). The widespread practice of marrying within one's close local geographic area that perpetuated these recessive and dominant mutations is expressed by the following well-known and old village Cypriot saying:

Παπούτσιν που τον τόπον σου τζιας εν κομμαδκιασμένον.
[You should better obtain shoes from your own place, even though they may be full of holes.]

As regard the dominant diseases referred to above, no homozygous case came to our attention, despite the presumed nonrandom mating within close small communities, perhaps because of nonviability (for extensive description of founder effects in Cyprus, see Deltas, 2004).

For large and multiexon genes such as *COL4A3* and *COL4A4*, molecular investigations like this study that result in the finding of only few mutations to account for the pathology in many families make diagnosis and genetic counseling much easier for people at risk. In cases where mutation screening is needed, either for therapeutic purposes or for identifying potential kidney donors, one can start the analysis by testing for the mutations that have been shown to be founders, before resolving to more general gene screening and sequencing. Also, from the epidemiologic perspective, the easy identification of carriers of frequent mutations, especially locally, may justify wider population screening and application of awareness campaigns for preventive purposes. This is also especially useful from the clinical point of view, since familial hematuria is a frequent presenting symptom that often requires extensive and expensive investigations that can include urine cytology, cystoscopy, IVP and CT studies, and renal biopsy. ◀AU4◀AU5

Molecular analysis and correct diagnosis will permit correct and fast clinical decisions that enable correct long-term management of mutation carriers, while it will relieve the anxiety and prevent unnecessary frequent visits to the nephrologist, of those who are diagnosed not to have inherited the causative mutation. Finally, linkage disequilibrium studies and dating of mutations provides useful information on the history of the population, while it may also assist in association studies in search for disease modifier genes.

Acknowledgments

We thank the patients and their families for participating in this study. This work was funded mainly through a grant from the Cyprus Research Promotion Foundation, ΕΝΙΣΧ/0505/02, and partly by the Cyprus Ministry of Health, the Cyprus Kidney Association (scholarship to K.V.), and the University of Cyprus Research Activities 3/312.

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AU6: In "Dean G, ... (1988)" Ref. entry, please mention the inclusive page range.
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AU8: Arrow and other kinds of bars that are present in Figure 1 are not explained in the figure legend. Please check.
AU9: Please check the phrase "length of the diseased conserved shared."