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PAX2 mutations in oligomeganephronia

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***PAX2* mutations in oligomeganephronia.**

Background. Oligomeganephronia (OMN) is a rare congenital and usually sporadic anomaly. It is characterized by bilateral renal hypoplasia, with a reduced number of enlarged nephrons. The mechanisms involved in this deficient nephrogenesis are unknown. The paired box transcription factor PAX2 plays a fundamental role in renal development. Heterozygous *Pax2* mutants in mice are characterized by renal hypoplasia and retinal defects, and in humans, *PAX2* mutations have been described in the renal-coloboma syndrome.

Methods. To assess whether OMN could be related to *PAX2*, we searched for *PAX2* mutations in nine patients presenting with sporadic and apparently isolated OMN.

Results. Heterozygous *PAX2* mutations were found in three patients. A limited optic nerve coloboma was secondarily detected in two cases and a very mild optic disk dysplasia in one patient. None of these patients had visual impairment.

Conclusions. Ocular anomaly and *PAX2* mutations should be sought in all patients with OMN.

Oligomeganephronia (OMN) is a rare congenital anomaly characterized by bilateral renal hypoplasia without dysplasia or urinary tract abnormalities. Kidneys are small and have a normal shape on radiological examination. Histologically, they show a striking reduction in the number of nephrons, which are markedly enlarged [1]. Polyuria and polydypsia appearing within the first two years of life are the main manifestations of the disorder. The glomerular filtration rate (GFR) is impaired from birth, increases progressively to a maximum during the first years of life, and remains stable in early childhood, ultimately decreasing in late childhood. The mechanisms responsible for the deficit in nephrogenesis are not identified. They could result from a deficient metanephric blastema [2] or from a defect in the growth and branching of the ureteric bud. Although OMN usually occurs as a sporadic and isolated malformation, several lines of evidence, namely the existence of syndromic renal hypoplasia including branchio-oto-renal [3], acro-renal [4], or renal-coloboma syndromes (RCSs) as well as the association with chromosomal abnormalities [5, 6], suggest that genetic factors could be involved. Moreover, the description of some cases of familial recurrence is consistent with a role of genetic events in OMN [7].

PAX genes encode paired box-containing transcription factors that are critical during embryonic development. In the human embryo, *PAX2* is expressed in the kidney as well as in the optic cup, the otic vesicle, and other parts of the central nervous system [8]. *Pax2* loss-of-function mutations in mice (krd, $Pax2^{1Neu}$, and targeted *Pax2* gene knockout) are associated with renal hypoplasia and retinal defect [9–11]. Interestingly, the kidneys have a reduced cortical thickness with fewer nephrons, a feature very similar to what is observed in OMN [9].

Renal-coloboma syndrome (MIM 120330) is an autosomal congenital anomaly characterized by the association of renal hypoplasia, vesicoureteral reflux (VUR), and optic nerve coloboma [12]. Based on the similarity between this condition and the phenotype of *Pax2* mutant mice, *PAX2* has been regarded as a good candidate gene for RCS. Indeed, in 1995, Sanyanusin et al reported heterozygous *PAX2* mutations in two RCS families [13, 14]. Thirty-two RCS patients with nine different *PAX2* mutations have hitherto been reported (Table 1) [13–20], but the absence of *PAX2* mutations in some RCS patients suggests genetic heterogeneity of the syndrome (data not shown) [18]. To investigate whether *PAX2* could be implicated in OMN, nine patients with apparently isolated OMN were evaluated for *PAX2* mutations.

METHODS

Patients

Nine children with OMN defined as renal hypoplasia with a normal shape on radiological examination were included in the study. Informed consent was signed by

Key words: coloboma, transcription factor, inheritance, renal hypoplasia, retinal defects in *Pax2*, optic disk dysplasia in OMN.

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Table 1. Renal and ocular phenotype in 32 patients with *PAX2* mutations

Patient	*PAX2* mutation (exon)	Age *years*	Renal hypoplasia	Renal insufficiency	VUR	Optic nerve coloboma[a]	Visual impairment	References
1	1104delC (5)	15	+	+	+	+	+	[14]
2		10	+	+	−	+	+	[14]
3		6	+	+	+	+	+	[14]
4		35	−	+	−	+	+	[14]
430	619insG (2)	5	−	+	−	+	+	[13]
431		12	−	+	−	+	+	[13]
579	674del22 (2)	11	+	+	−	+	+	[15]
656	619insG (2)	48	+	+	−	+	+	[15]
657		25	+	+	−	+	+	[15]
2646	619insG (2)	20	+	+	−	+	+	[15]
TRN	t(10; 13)	5	−	+	−	+	+	[17]
985	611delT (2)	6	−	+	+	+	ND	[18]
III-8	G769A (3)	70	−	+	+	+	−	[19]
IV-2		52	+	+	−	+	+	[19]
IV-3		46	+	+	−	+	−	[19]
IV-6		40	+	+	−	+	−	[19]
IV-7		35	+	+	−	+	−	[19]
V-2		16	+	+	−	+	−	[19]
F2	768ins6 (3)	17	+	+	+	+	+	[19]
I-1	C1289T (7)	70	−	+	−	+	+	[20]
II-5		37	+	+	−	+	+	[20]
II-7		40	−	−	−	+	−	[20]
II-9		39	−	+	−	+	+	[20]
III-9		14	−	−	−	+	−	[20]
III-11		18	−	−	−	+	−	[20]
III-13		21	+	+	−	+	+	[20]
III-16		4	−	−	+	+	−	[20]
III-17		6	+	+	−	+	+	[20]
X2003	619insG (2)	13	+	+	−	+	+	[20]
12961	619insG (2)	5	ND	ND	+	+	+	[16]
12962		33	ND	ND	ND	+	+	[16]
8961	619delG (2)	4	+	+	ND	+	+	[16]

Abbreviations are: VUR, vesicoureteral reflux; ND, not determined.
[a] Optic disk dysplasia rather than optic nerve coloboma is present in some patients

the parents. Clinical data are presented in Table 2. Patients with renal hypoplasia associated with high-grade VUR (III or more) or other renal or extrarenal malformations had been excluded from the study. Renal histologic analysis was available on the nephrectomy specimens of five patients who were transplanted. It confirmed the diagnosis of OMN by showing (*1*) a striking reduction in the number of glomerular generations on strictly orthogonal cortico-medullary sections [21], (*2*) the absence of dysplastic features, and (*3*) the marked enlargement of preserved nephrons with glomerular and tubular diameters two or three times normal (Fig. 1).

Detection of PAX2 mutation

Genomic DNA was extracted from peripheral blood lymphocytes by standard methods. Fragments spanning the 12 exons of *PAX2* were amplified from genomic DNA by use of intronic polymerase chain reaction (PCR) primers. Primer sequences and PCR conditions have been described previously [8]. Mutation screening was performed by single strand conformation polymorphism (SSCP) as previously described [8]. When an abnormal SSCP pattern was observed, genomic DNA was directly sequenced on both strands using the fluorometric method (DyeDeoxy or BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA). To detect partial or total deletion of the gene, the heterozygosity for *PAX2* was analyzed in each patient by amplification of a (CA)n dinucleotide repeat within intron 9 as previously described [22].

Pax2 expression in the kidney

To assess the consequences of the mutations on the renal expression of PAX2 protein, we performed PAX2 immunostaining on the kidneys of five OMN patients who progressed to end-stage renal disease (2 with and 3 without identified *PAX2* mutation). Control kidneys were from two fetuses of 22 and 28 gestational weeks obtained at autopsy after spontaneous abortion or termination of pregnancy for medical reasons and six children or adults (3 nontransplanted kidneys and 3 normal tissue specimens adjacent to renal carcinoma). PAX2 antibody (Zymed Laboratories Inc., San Francisco, CA, USA) is a rabbit antibody generated against a fusion protein derived from the C-terminal domain of the murine PAX2 protein. Immunostaining was performed as previously

Table 2. Clinical and genetic characteristics of patients with OMN

Patient	Birthweight *kg*	Kidney size[a]	Circumstances of diagnosis *age*	Maximal creatinine clearance[b]	Age at ESRF or actual creatinine clearance[b]	Renal histology	VUR	Eye anomalies	Other anomalies	*PAX2* mutation
1	3.13	0.70 (21)	Dehydration (neonatal)	68	20 y	no	no	Coloboma	no	658–663del
2	2.60	0.60 (48)	Polyuria (4 years)	15	5.5 y	yes	no	Papillar dysplasia	no	619insG
3	3.15	0.70 (1)	Vomiting (neonatal)	15	7 y	yes	no	Coloboma	no	619insG
4	2.90	0.55 (6)	Failure to thrive (neonatal)	22	13 y	no	no	no	no	no
5	4.08	0.70 (24)	Dehydration (neonatal)	10	6.5 y	no	no	no	no	no
6	3.50	0.60 (6)	Acidosis (neonatal)	16	14 y	yes	no	no	Clubfoot	no
7	2.70	0.55 (birth)	Abdominal US (neonatal)	56	30 mL/min at 18 y	no	no	no	Testicular ectopia, Mental retardation	no
8	2.94	0.25 (birth)	Oligoamnios (antenatal)	8	1.5 y	yes	no	no	no	no
9	2.87	0.55 (1)	Oligoamnios (antenatal)	6	3.5 y	yes	grade II	no	no	no

[a] Kidney size is expressed as a fraction of the mean normal size for the same age, age is indicated in month

[b] Maximal creatinine clearance was calculated by means of the formula K × T/C, where T is the size expressed in centimeters, C represents the plasma level of creatinine in micromoles per liter, and K stands for a constant the value of which varies according to the weight, the results are expressed in mL/min/1.73 m^2; y, year; ESRF, end-stage renal failure; VUR, vesicoureteral reflux.

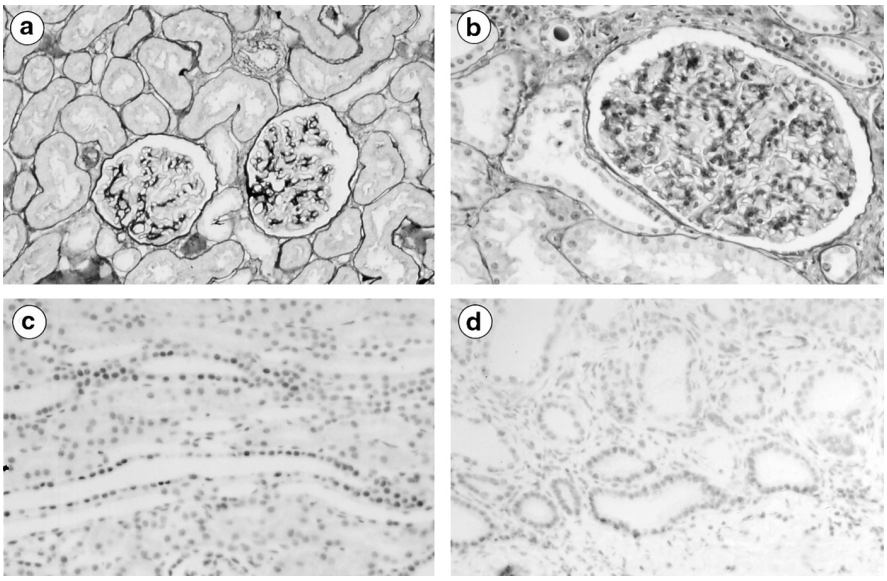

Fig. 1. Silver methenamine (*a*), periodic acid-Schiff stain (*b*), and PAX2 immunolabeling (*c* and *d*) of normal (a and c) and oligomega-nephronia (OMN; b and d) human kidneys. A comparison between a and b shows a marked increase in size of OMN nephrons. Nuclear labeling of collecting duct cells is present in normal adult kidney (c) and preserved in OMN kidney (d). (Original magnification ×135).

described [23]. Three-micrometer thick deparaffinized sections were pretreated by microwave heating in a urea solution (2 × 5 min in 0.8 mol/L urea, pH 6.4). PAX2 antibodies were diluted to 1/25 to 1/50. Immunoperoxidase staining was carried out using the Vectastain Elite ABC kit (Vector, Burlingame, CA, USA). Labeling was examined with a Leitz Orthoplan microscope. Control stainings were performed in the same conditions with the primary antibody omitted. No labeling was observed in control sections.

RESULTS

By screening the entire coding sequence of the *PAX2* gene in nine unrelated patients with apparently isolated OMN, we detected heterozygous *PAX2* mutations in three of them: a single nucleotide (guanosine) insertion at position 619 in two (patients 2 and 3), and a 6 nucleotide deletions located in exon 2 in one (patient 1; Fig. 2). The guanine insertion occurred in exon 2 after a stretch of seven guanines and resulted in a frameshift predicting the premature termination of the protein 27

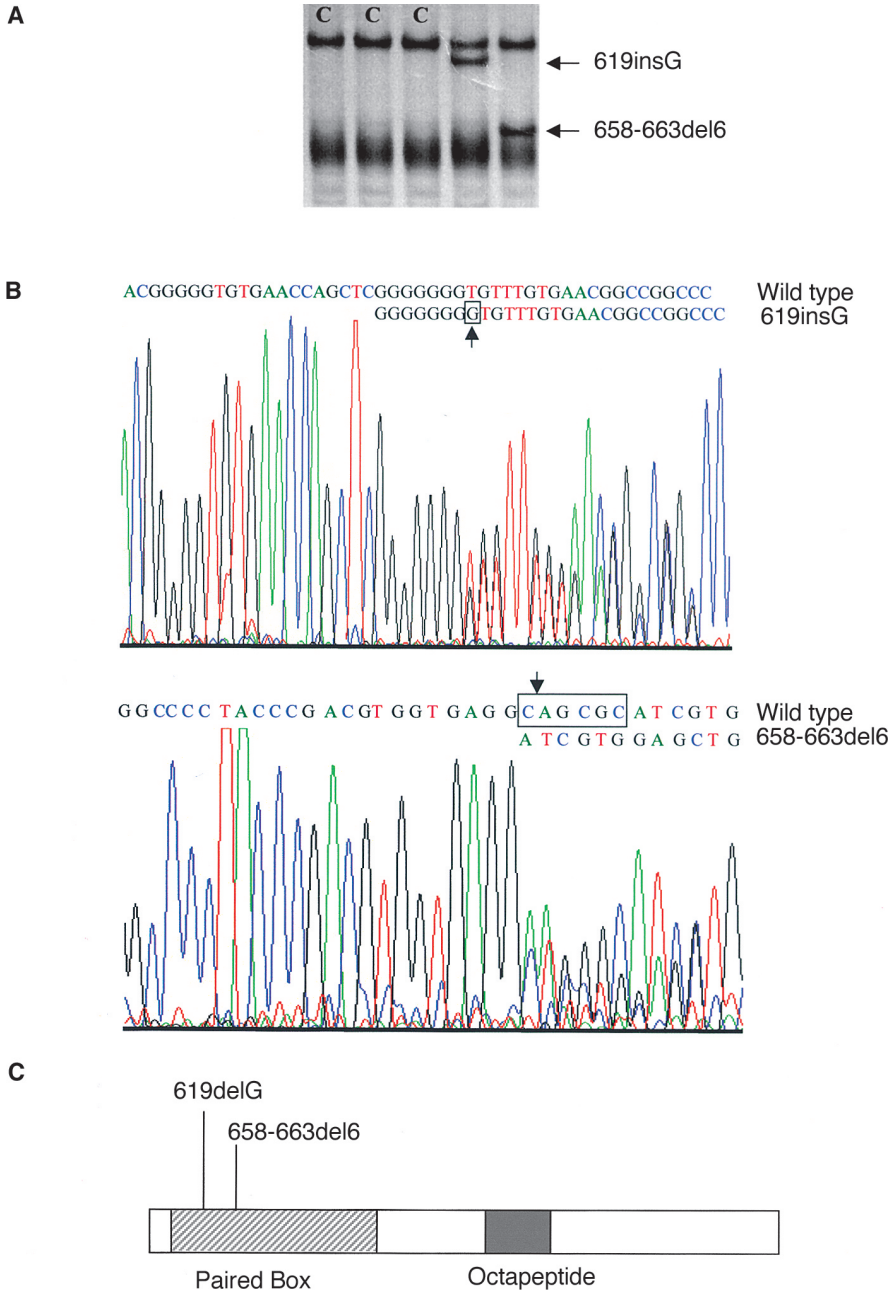

Fig. 2. *PAX2* mutations in OMN probands. (*A*) SSCP autoradiograms showing shift of the G619insG mutant allele (lane 4) and del6 bp658-663del allele (lane 5) compared with control (lane 1 through 3). (*B*) Control and mutant DNA sequence analysis. (*C*) Schematic representation of the *PAX2* gene coding regions. The conserved paired box domain (▨) and the octapeptide domain (■) are represented. The two mutations identified in the present study are depicted: the insertion of a G in position 619, which results in a frameshift and a truncated protein within the paired domain, and a 6 bp deletion, which results in a two amino acid deletion within the paired domain.

amino acids downstream. The truncated PAX2 mutant protein lacked the major part of the paired domain. The six-nucleotide deletion (658-663del) in exon 2 resulted in the ablation of two highly conserved amino acids in the paired domain. These two *PAX2* mutations were not found in 100 control chromosomes, indicating that they were not common polymorphic variants. All nine patients carried two alleles of the $(CA)_n$ repeat within intron 9 excluding chromosomic deletion at the *PAX2* locus. Mutation in patients 2 and 3 occurred de novo, as their parents were shown not to carry the mutation.

In patients 1 and 3, bilateral coloboma limited to the optic disk was revealed by fundus examination. In patient 2, the optic disk appeared dysplastic with apparent thickening of the papilla (Fig. 3).

Within the kidney, nuclear expression of the protein was observed in most preserved distal and collecting ducts of patient 3 carrying a 619insG*PAX2* mutation (Fig. 1). The distribution was comparable to that in normal control kidneys. Conversely, in patient 2 carrying the same mutation, most distal tubules and collecting ducts were PAX2 negative. Of the three patients with

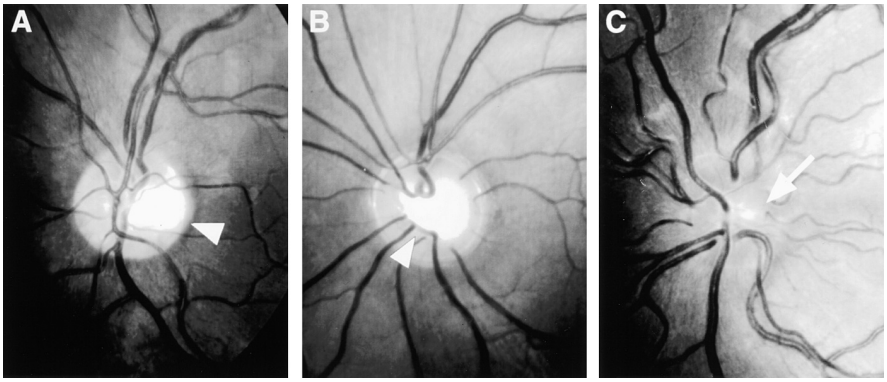

Fig. 3. Optic disk appearance (left eye) in the three patients with OMN and *PAX2* mutation. Patients 1 (*A*) and 3 (*B*) exhibit characteristic features of optic disk coloboma with a deep temporal excavation (arrowheads). In patient 2 (*C*), the optic disk is dysplastic with thickening (arrow) and abnormal vessels emergence.

OMN without *PAX2* mutation, two had normal expression of PAX2, whereas the expression was reduced in the other patient (data not shown).

DISCUSSION

The pathogenesis of OMN is still unknown. The characteristic reduction in the number of nephrons could result from a failure in the process of epithelial transformation of the metanephric blastema or from deficient branching of the ureteric bud, as suggested by the frequent finding of unipapillary or paucipapillary kidneys. Genetic and environmental factors have both been incriminated. In recent years, advances in the basic mechanisms of developmental biology have provided new insight into the mechanisms of nephrogenesis, and a start has been made toward elucidating the underlying molecular controls of this process. For example, it has been shown that OMN observed in some branchio-oto-renal patients is due to mutation in the EYA1 gene expressed in the condensing mesenchyme surrounding the "just divided" ureteric branchs [3].

Pax genes are a family of developmental genes encoding nuclear transcription factors, with a paired domain harboring the DNA binding activity. *Pax2* is one of the first genes expressed during nephrogenesis in the nephric duct, then in the ureteric branch derivatives, and in the condensed metanephric cells that will differentiate into nephrons [8, 24]. It plays a crucial role during urinary tract development, as established by mutant mice. Deletion of a single *Pax2* allele, in $Pax2^{1Neu/+}$ mice, impairs metanephric growth and results in a reduction of the number of nephrons, often associated with megaureter suggesting vesicoureteral reflux [10]. Interestingly, the mechanism leading to renal hypoplasia in $Pax2^{1Neu/+}$ mice seems to be enhanced apoptosis of the ureteric epithelium at E15 [20].

In humans, heterozygous *PAX2* mutations have been reported in patients with optic nerve coloboma associated with renal hypoplasia [13]. Histologic analysis is compatible with renal dysplasia in some cases and with OMN in others. In our study, the finding of PAX2 mutations in three patients with OMN clearly shows that this condition can be considered part of the RCS.

As previously shown, PAX2 is expressed in the distal tubule and collecting duct, and the expression persists in adulthood [23]. In one OMN patient, PAX2 expression was preserved, whereas it was not detected in the second one with the same 619Gins. Similar findings were obtained in OMN patients without PAX2 mutation, showing the difficulties in interpreting changes observed in end-stage kidneys and the need for animal models [20].

Optic nerve coloboma, a malformation resulting from incomplete closure of the embryonic optic cup fissure, may be large, resulting in a more or less serious visual deficit, or may be limited and asymptomatic [19]. Indeed, none of the children studied here had visual impairment, and repeat fundus examinations were initially considered normal. After careful re-evaluation, mild coloboma was found in two patients, and mild optic disk dysplasia was found in the third. The detection of these ocular changes, even mild and asymptomatic in the three patients, confirms that eye anomalies are a constant feature of *PAX2* mutations and that the RCS is the sole clinical expression of this gene defect. This is confirmed by previous findings that among a large cohort of patients with optic nerve coloboma, *PAX2* mutations were detected only in patients with associated renal malformation [18]. It thus seems important to search for renal hypoplasia or VUR in patients with coloboma, and conversely to perform careful eye examination, with specific attention to discrete signs of optic disk anomalies, in patients with apparently isolated renal hypoplasia.

In conclusion, our report confirms the critical role of PAX2 in human renal development and suggests that OMN is genetically heterogeneous. Furthermore, this study extends the phenotypic variability of the RCS associated with *PAX2* mutations in the human.

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