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# ***RET* and *GDNF* mutations are rare in fetuses with renal agenesis or other severe kidney development defects**

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Key words: renal agenesis; renal hypodysplasia; variants ; evolutionary conserved non-coding regions ; CNVs

Word count : 3655

## ABSTRACT

**Background** The RET/GDNF signalling pathway plays a crucial role during development of kidneys and enteric nervous system. In humans, *RET* activating mutations cause multiple endocrine neoplasia, whereas inactivating mutations are responsible for Hirschsprung disease. *RET* mutations have also been reported in fetuses with renal agenesis, based on analysis of a small series of samples.

**Objective and Methods** To better characterize the involvement of *RET* and *GDNF* in kidney development defects, we studied a series of 105 fetuses with bilateral defects including renal agenesis, severe hypodysplasia or multicystic dysplastic kidney. *RET* and *GDNF* coding sequences, evolutionary conserved non-coding regions (ECRs) in promoters, 3'UTRs and *RET* intron 1 were analysed. Copy number variations (CNVs) at these loci were also investigated.

**Results** We identified: (i) a low frequency ( $< 7\%$ ) of potential mutations in the *RET* coding sequence, with inheritance from the healthy father for four of them; (ii) no *GDNF* mutation; (iii) similar allele frequencies in patients and controls for most SNP variants, except for *RET* intron 1 variant rs2506012 that was significantly more frequent in affected fetuses than in controls (6% vs. 2%,  $P=0.01$ ); (iv) distribution of the few rare *RET* variants unidentified in controls into the various 5'-ECRs; (v) absence of CNVs.

**Conclusion** These results suggest that genomic alteration of *RET* or *GDNF* is not a major mechanism leading to renal agenesis and other severe kidney development defects. Analysis of a larger series of patients will be necessary to validate the association of the *RET* intron 1 variant rs2506012 with renal development defects.

## INTRODUCTION

Congenital abnormalities of the kidney and urinary tract (CAKUT) are frequently observed in children and represent a significant cause of morbidity, accounting for more than 40% of pediatric end-stage renal failure, and mortality.[1] Kidney development defects (KDD) include: (i) bilateral/unilateral renal agenesis (BRA/URA); (ii) renal hypodysplasia (RHD) characterized by a reduction in the number of nephrons leading to a small overall kidney size and frequent dysplasia with or without cysts; and (iii) multicystic dysplastic kidney (MCDK). These various alterations can be observed together in a same patient or in different members of the same family, suggesting that they belong to a same continuum of phenotypes. Although most cases are sporadic and isolated, syndromic and familial cases suggest that genetic factors are involved. In particular, dominant mutations with variable penetrance have been found in several syndromic forms of KDD. The most frequently mutated genes are *PAX2* in patients with renal-coloboma syndrome, *EYA1* and *SIX1* in patients with branchio-oto-renal (BOR) syndrome and *HNF1B* in patients with renal cysts and diabetes association.[2-6] Analysis of genotype-phenotype correlations showed that the severity of the renal disease associated with these mutations is extremely variable.[2,7] Other less frequent syndromes including KDD are associated with mutations in developmental genes such as *SALL1* (Townes-Brocks syndrome), *WNT4* (Rokitanski syndrome), *KALI* (Kallman syndrome), *FRAS1* and *FREMI* (Fraser syndrome), *GATA3* (RHD, hypoparathyroidism and sensorineural deafness) and *GLI3* (Pallister-Hall syndrome).[8-14] Moreover, comparative genomic hybridization and familial studies have highlighted several chromosomal regions that could carry other genes involved in KDD.[15,16].

Mammalian kidney development results from a reciprocal induction between the ureteric bud (UB) and the metanephric mesenchyme (MM). Signalling by GDNF secreted by the MM and the RET tyrosine kinase receptor and its co-receptor GFR $\alpha$ 1 expressed on the UB plays a critical role for normal growth and branching of the UB.[17,18] The *RET* gene encodes two major splicing isoforms, RET9 and RET51, that differ in their carboxy terminus. These two isoforms appear to have redundant roles for kidney development.[19,20] The RET/GDNF signalling also plays a critical role during the development of the enteric nervous system. Homozygous knockout of *Ret* or *Gdnf* in mice leads to loss of enteric ganglia as well as severe kidney aplasia or hypodysplasia caused by a failure of UB outgrowth.[21,22] In humans, heterozygous loss-of-function mutations in *RET* resulting in haploinsufficiency are the most frequent alterations reported in patients with segmental intestinal aganglionosis known as Hirschsprung disease (HSCR).[23,24] *RET* is also an oncogene involved, through

activating mutations, in predisposition to multiple endocrine neoplasia type 2A (MEN2A) characterized by medullary thyroid carcinoma (MTC), pheochromocytoma and/or parathyroid hyperplasia.[25] Several studies have reported the association of HSCR and MTC in patients with *RET* mutations affecting the cysteine-rich extracellular domain of the protein. Both activating and inactivating effects have been demonstrated for the C620R mutation (so called the Janus mutation) because, when expressed in kidney cells, it promotes cell proliferation and impairs migration and branching in three-dimensional culture in response to GDNF.[26] Interestingly, association of HSCR with CAKUT has also been described and a *RET* mutation has been reported in few cases,[27] suggesting a common genetic basis for these two pathologies. Renal agenesis was also reported in a family with MTC and the Janus *RET* C620 mutation.[28] Recently, both activating and inactivating *RET* mutations have been reported in a small series of fetuses with renal agenesis.[29]

To better assess the role of the RET/GDNF signalling in KDD, we analysed coding and regulatory sequences of *RET* and *GDNF* in a series of 105 fetuses with severe bilateral defects.

## **PATIENTS AND METHODS**

### **Patients**

We studied a series of 105 fetuses with bilateral KDD contributing to anamnios or severe oligohydramnios and that had motivated termination of pregnancy. This included 65 cases with BRA, 24 cases with URA and an abnormal contralateral kidney (RHD in 8 cases, MCDK in 16 cases), 4 cases with bilateral RHD, 10 cases with bilateral MCDK, one case with RHD on one kidney and MCDK on the other and one case with URA and ureteral duplication. Kidney defects were defined by the fetopathologists of the Société Française de Foetopathologie upon histological examination. Five fetuses were from consanguineous families, 11 cases (including 4 sib-pairs) were from 7 non-consanguineous families in which the mother underwent several terminations of pregnancy for anamnios, and five other cases had relatives with renal abnormalities. Twenty-one fetuses had extra-renal abnormalities, namely uterine agenesis (6 cases), bifid uterus (1 case), epididymal hypoplasia or cysts (2 cases), or more complex syndromic associations (growth retardation, craniofacial dysmorphism, hand and limb anomalies, cardiopathy). No *GLI3* mutation was identified in 3 fetuses with symptoms suggestive of Pallister-Hall syndrome. There was no indication of renal-coloboma or BOR syndromes in relatives of any of the studied fetuses. Moreover, we excluded the presence of *HNF1B* mutations / deletions in the fetuses with MCDK or RHD with cysts.

This study was fully approved by the Comité de Protection des Personnes pour la Recherche Biomédicale Ile de France 2 and informed consent was obtained from all parents. DNA was extracted from frozen liver, lung, spleen or kidney samples. For three fetuses, RNA was also extracted from frozen kidney samples.

### Sequencing of *RET* and *GDNF* coding and non-coding sequences

We screened the 20 *RET* exons encoding both protein isoforms (RET9: exons 1 to 19b, RET51: exons 1 to 19a, 20) and the three *GDNF* exons (non-coding exon 1 and coding exons 2 and 3). Evolutionary conserved non-coding regions (ECRs) located upstream of *RET* and *GDNF* and in *RET* intron 1 (<http://www.dcode.org>) were also analyzed. Their positions relative to exon 1 are given in Table 1. Finally, as variations in the 3'UTR could potentially disrupt genetic regulation by micro-RNAs, these regions were also analyzed. The 3'UTRs of RET9 and RET51 transcripts were identified from <http://www.ensembl.org/>. For analysis of the 3'UTR of *GDNF*, we focused on the first 1231 bp that were evolutionary conserved (Table 1). PCR and sequencing primers are available upon request.

**Table1** Evolutionary conserved sequences in 5' and 3' of *RET* and *GDNF*

Evolutionary Conserved Region	Position on chromosome	Position related to the first exon	Size (bp)	Homology <sup>1</sup>	More distant species with >70% homology
RET-ECR1E	chr10:42794983-42795367	5' (-97kb)	385	78% (O)	chicken
RET-ECR1B	chr10:42803235-42803698	5' (-89 kb)	464	78% (M)	frog
RET-ECR1	chr10:42880879-42881488	5' (-11.6kb)	610	77% (M)	chicken
RET-ECR4	chr10:42883631-42883905	5' (-8.9kb)	275	74% (M)	mouse
RET-ECR8	chr10:42883965-42884464	5' (-8.5kb)	500	77% (M)	mouse
RET-ECR2	chr10:42886856-42887495	5' (-5.7kb)	640	79% (M)	opossum
RET-ECR6	chr10:42895408-42895741	intron 1	334	76% (M)	mouse/rat
RET-ECR7	chr10:42902036-42902350	intron 1	315	72% (M)	opossum
GDNF-ECR1	chr5:37876398-37876866	5' (-5.5kb)	469	81% (O)	opossum
GDNF-ECR2	chr5:37877173-37877521	5' (-6.2kb)	349	75% (O)	opossum

GDNF-ECR3	chr5:37850279-37851510	3'-UTR	1231	82% (M)	opossum
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Positions on chromosomes are given according to the NCBI36/hg18 built ;

<sup>1</sup> % of homology of the selected region with the mouse (M) or the opossum (O) sequence

## Statistical analyses

Fisher exact tests were used to compare frequencies of the variants in patients and controls. As 80% of the fetuses were of European origin, frequencies in controls were mostly evaluated by analysis of 189 unrelated Caucasians. Algerian and Turkish controls (34 and 33 cases, respectively) were also tested for polymorphisms present in patients originating from these countries. Moreover, we considered frequencies generated from the HapMap-CEU (120 chromosomes), Pilot.1.CEU (72 chromosomes) and AGL\_ASAP (74 chromosomes) populations (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). For statistical study of the *RET* variant rs2506012, we analysed a second series of 171 patients, including 53 fetuses and 118 living children, with the same spectrum of bilateral KDD as the first series.

## Analysis of *RET* cDNA

The effect of the c.1353 G>A variant (T451T) on splicing was analysed by RT-PCR, using primers located in exons 6 and 8. Quantitative RT-PCR was performed using the ABsolute Sybr green ROX mix (Thermo Scientific) and GAPDH was used as a control.

## Analysis of copy number variations

For 41 samples, the quality and the available amount of DNA allowed us to perform genomic analysis on Illumina Infinium HumanOmni1 beadchips. Hybridizations were performed as recommended by the manufacturer. We used the GenomeStudio software for normalization and genotyping. For identification of copy number variations (CNVs), we used the GenomeStudio plug-in CNVpartition and the PennCNV algorithm with default parameters.[30]

## RESULTS

### Analysis of *RET*

Sequencing of the 20 exons of *RET* in 105 fetuses with severe bilateral KDD identified 7 previously unreported variations in the coding region, including one nonsense mutation, four missense and two neutral changes (Table 2). All variations were heterozygous and none of



them was identified in 180 controls. The nonsense mutation resulted in a stop codon located 58 and 16 amino acids before the C-terminus of RET51 and RET9 isoforms, respectively. PolyPhen-2 predicted variations D567N and V787I as probably damaging with scores of 0.984 and 0.979 respectively and L56M as possibly damaging with a score of 0.281. Although R57Q was predicted as benign by PolyPhen-2, it was qualified as possibly damaging with the previous version of PolyPhen (score=1.558) and it affects a very conserved aminoacid.

For fetuses with the R57Q, D567N and W1056X mutations and the P992P neutral variant, DNA from the parents was available. In all four cases, the variation was also present in the heterozygous state in the father, in whom presence of the two normal-sized kidneys was ascertained by renal echography. This demonstrates that none of these variants alone, even the nonsense mutation, is sufficient to explain the renal development defect.

**Table 2** Variants identified in the *RET* coding sequence

Nucleotide Change <sup>1</sup>	Variant	Consequence	Exon	Phenotype	Features of the variants
c.166 C>A	p.L56M	Missense	2	BRA and uterine agenesis	Possibly damaging <sup>2</sup>
c.170 G>A	p.R57Q	Missense	2	MCDK	Benign <sup>2</sup> Present in healthy father
c.1353 G>A	p.T451T	Neutral	7	URA/MCDK	Potential effect on splicing <sup>3</sup>
c.1699 G>A	p.D567N	Missense	9	BRA	Probably damaging <sup>2</sup> Present in healthy father
c.2359 G>A	p.V787I	Missense	13	URA/RHD	Probably damaging <sup>2</sup>
c.2976 G>A	p.P992P	Neutral	18	BRA	Present in healthy father
c.3167 G>A	p.W1056X	Nonsense	19	BRA	Present in healthy father

<sup>1</sup> c. positions are given according to the coding sequence of NM\_020975

<sup>2</sup> identified with PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>);

<sup>3</sup> identified with Alamut (<http://www.interactive-biosoftware.com/>) and

ESE finder (<http://rulai.cshl.edu/tools/ESE/>):

BRA : bilateral renal agenesis ; URA : unilateral renal agenesis ; RHD : renal hypodysplasia.

MCDK: multicystic dysplastic kidney

We questioned whether the neutral variations c.1353 G>A (T451T) located in exon 7 and c.2976 G>A (P992P) in exon 18 could have an effect on splicing, using the mutation interpretation software Alamut (<http://www.interactive-biosoftware.com/>) and the Exonic Splicing Enhancer analysis software ESE finder (<http://rulai.cshl.edu/tools/ESE/>). While we did not identify any effect of the c.2976 G>A variant, both methods identified creation of sites for splicing factors SF2, SC25 and SPP40 by the c.1353 G>A variant (scores = 4.5, 2.5 and 4.5, respectively), suggesting that this variant might alter splicing of exon 7.[31] To test this hypothesis, we amplified a cDNA fragment spanning *RET* exons 6 to 8 from kidney samples obtained for fetus H637 with the c.1353 G>A exon 7 variant, two other fetuses with KDD and no *RET* mutation, and three control fetuses without renal pathologies. We did not identify any abnormal-sized band associated with the variant (data not shown). To analyse if the variant could decrease the amount of *RET* transcript, we performed quantitative RT-PCR using the same exon 6 and exon 8 primers. In the three KDD kidney samples, the level of *RET* expression was 10 times lower than in the normal fetal kidney samples. However, *RET* expression in H637 kidney sample with the c.1353 G>A variant was not different from *RET* expression in the two other KDD kidney samples without *RET* mutation (data not shown). Altogether, these results did not allow us to validate any effect of the c.1353 G>A variant on *RET* expression. Whether the low level of *RET* expression in KDD kidneys is a cause or a consequence of the altered kidney development remains an open question.

Several variations corresponding to known SNPs were also identified in the coding sequence as well as in flanking intronic sequences. We compared their frequencies in the KDD fetuses to controls (Table 3A). No significant difference was observed. Evolutionary sequence conservation has proven a valuable approach to identify genomic regions important for gene expression regulation. Using the ECR Browser (<http://ecrbrowser.dcode.org/>), we identified 8 ECR regions that were at least 70% conserved until mouse (Table 1). Six ECRs were located upstream of the transcript region (ECR1, -1B, -1E, -2, -4 and -8,) and sequencing of these ECRs in the 105 samples led to the identification of 11 variants (Table 3A): five of them were referenced in the SNP database (<http://www.ncbi.nlm.nih.gov/snp/>) and their frequencies were similar in cases and controls, whereas the 6 other were unreported variations that were heterozygous in 1% to 6% of the fetuses (allele frequencies between 0.005 and 0.03). We only identified the most frequent one, ECR2/504, in controls (Table 3A). ECR6 and ECR7 were located in intron 1 and included 7 variants that were all referenced in the SNP database. One of these variants, ECR6/271, was significantly more frequent in the KDD fetuses than in controls (heterozygous in 14/96 fetuses vs 7/189 controls,  $P=0.002$ )

(Table 3A). To confirm this result, we analysed a new series of 171 cases with bilateral KDD including 53 fetuses and 118 children. Based on analysis of all of the samples (variant heterozygous in a total of 24/267 fetuses/children) or fetuses only (variant heterozygous in a total of 17/149 fetuses), the variant frequency remains significantly higher in cases than in controls ( $P=0.04$  or  $P=0.01$ , respectively). The presence of the variant in fetuses with BRA but also in fetuses and children with MCDK suggests that it could confer predisposition to the whole spectrum of KDD.

Sequencing of the 3'UTR of both RET9 and RET51 transcriptional isoforms revealed 8 referenced SNPs and 6 previously unreported rare variants (Table 3A). There was no significant difference in their frequencies in cases vs. controls.

**Table 3A** Polymorphic variants in the *RET* sequence

	Position	Variation in non coding sequence	Variation in coding sequence	Frequency of the minor allele in KDD fetuses (in controls)	SNP referenced in NCBI
5' evolutionary conserved ECRs <sup>1</sup>	ECR1E/83	C>A		0.01 (-)	rs73262104
	ECR1E/176	C>A		0.01 (-)	
	ECR1B/36	G>A		0.23 (0.23)	rs1547930
	ECR1B/136	C>T		0.01 (-)	
	ECR1B/262	C>T		0.01 (-)	rs12572318
	ECR1B/293	G>A		0.005 (0.007)	
	ECR8/48	C>T		0.29 (0.24)	rs7910199
	ECR2/63	T>C		0.005 (-)	
	ECR2/189	C>G		0.47 (0.51)	rs2505992
Intron 1 ECRs <sup>1</sup>	ECR2/230	delTCAC		0.005 (-)	
	ECR2/504	C>T		0.03 (0.02)	
	ECR6/44	G>A		0.09 (0.10)	rs1897002
	ECR6/198	C>T		0.36 (0.37)	rs1864411
	ECR6/213	C>T		0.33 (0.28)	rs10900298
	ECR6/224	G>T		0.20 (0.23)	rs1864410
	ECR6/271	C>G		<b>0.07 (0.02)</b> <sup>5</sup>	rs2506012
	ECR7/27	C>T		0.21 (0.19) <sup>4</sup>	rs2435357
	ECR7/244	C>A		0.21 (0.18) <sup>4</sup>	rs2506004
Exonic and flanking intronic sequences <sup>2</sup>	c.73+53	G>A		0.15 (0.10) <sup>3</sup>	rs12267460
	c.73+104	T>C		0.005 (nd)	
	c.73+171/172	GC>AA		0.30 (0.35)	rs34327391
	c.73+173	insGGGCGGC		0.15 (0.10) <sup>3</sup>	
	exon2		A45A	0.23 (0.29)	rs1800858
	c.337+9	G>A		0.24 (0.28)	rs2435351
	exon3		V125V	0.01 (nd)	rs1800859
	exon3		N199N	0.005 (nd)	rs55810667
	c.626-72/71	delCC		0.33 (0.34)	rs35906041
	c.1264-5	C>T		0.02 (0.009)	rs9282835
	exon7		A432A	0.31 (0.29)	rs1800860
	c.1648+84	A>G		0.19 (0.22)	rs3026750
	c.1648+88	insC		0.19 (0.22)	rs34827976
	exon11		G691S	0.22 (0.22)	rs1799939
	c.2284+47	C>T		0.28 (0.28)	rs760466
	c.2285-102	C>T		0.01 (nd)	
	c.2285-85	G>A		0.005 (nd)	rs3026766
	exon13		L769L	0.20 (0.22)	rs1800861
	exon14		S836S	0.03 (0.04)	rs1800862
	c.2608-24	G>A		0.20 (0.21)	rs2472737
	exon15		S904S	0.23 (0.26)	rs1800863
	c.2801+54	A>T		0.005 (nd)	rs3026772
	c.2801+72	G>A		0.005 (nd)	rs3026773
	exon18		R982C	0.05 (0.03)	rs17158558

3'UTR RET9 <sup>2</sup>	c.3219+15	C>T	0.17 (0.15) <sup>4</sup>	rs2075912
	c.3219+128	C>T	0.005 (-)	
	c.3219+166	A>T	0.20 (0.22) <sup>4</sup>	rs2075913
	c.3219+731	C>T	0.15 (0.15) <sup>4</sup>	rs2565200
3'UTR RET51 <sup>2</sup>	c.3345+29	A>C	0.005 (-)	
	c.3345+95	C>T	0.17 (0.22) <sup>4</sup>	rs17028
	c.3345+388	G>A	0.23 (0.13) <sup>4</sup>	rs3026782
	c.3345+576	G>A	0.005 (0.003)	
	c.3345+600	T>A	0.15 (0.19)	rs2742240
	c.3345+1046	G>C	0.01 (0.003)	
	c.3345+1116	T>C	0.20 (0.24)	rs2435355
	c.3345+1506	A>G	0.17 (0.19)	rs2742241
	c.3345+1582	G>A	0.005 (-)	
	c.3345+1590	G>A	0.04 (0.03)	

<sup>1</sup> positions are given according to the ECR sequence (see Table 1) ;

<sup>2</sup> c. positions are given according to NM\_020975, except for RET9 3'UTR (NM\_020630) ;

<sup>3</sup> frequency of the homozygous variant ;

<sup>4</sup> frequency in controls according to Pilot-CEU ;

<sup>5</sup> p = 0.002 ;

(-) absent in controls (370 to 444 chromosomes) ;

(nd) not determined

## Analysis of *GDNF*

Sequencing of *GDNF* coding and non-coding sequences identified 7 referenced SNPs and 6 unreported variants (Table 3B). Variant frequencies in the fetuses were not different from those in controls. Therefore, this study did not provide any indication of the involvement of this gene in the etiology of KDD.

**Table 3B** Polymorphic variants in the *GDNF* sequence

	Position	Variation in non coding sequence	Variation in coding sequence	Frequency of the minor allele in KDD fetuses (controls)	SNP referenced in NCBI
5' <sup>1</sup>	ECR1/405	G>A		0.07 (0.11) <sup>3</sup>	rs2975100
	ECR1/2	C>A		0.07 (0.22) <sup>4</sup>	rs2075680
	c.1-229	insGCC		0.01 (-)	
Exons <sup>2</sup>	c.150	T>C		0.24 (0.33) <sup>1</sup>	rs2973033
	c.351+17	C>T		0.005 (-)	
	Exon3		R143R	0.02 (0.03) <sup>5</sup>	rs36010631
3'UTR <sup>1</sup>	ECR3/57	G>T		0.005 (nd)	
	ECR3/93	T>A		0.005 (nd)	rs45535335
	ECR3/363	A>T		0.005 (nd)	rs45611430
	ECR3/450	G>A		0.02 (0.02)	
	ECR3/529	C>T		0.01 (nd)	rs58787312
	ECR3/795	G>A		0.007 (-)	
	ECR3/988	T>C		0.05 (0.03)	

<sup>1</sup> positions are given according to the ECR sequence (see Table 1) ;

<sup>2</sup> c. positions are given according to NM\_000514 ;

<sup>3</sup> frequency in controls according to Pilot-CEU ;

<sup>4</sup> frequency in controls according to JBCI-allele ;

<sup>5</sup> frequency in controls according to AGI\_ASAP population ;

(-) absent in controls (296 to 452 chromosomes) ;

(nd) not determined

### **CNV analysis at *RET*, *GDNF* and other genes linked to the RET/GDNF signalling**

Finally, as CNVs could be responsible for an altered gene expression, we quantified the *RET* and *GDNF* loci in 41 of the fetuses, based on genomic data generated by DNA hybridization on Illumina HumanOmni1 chips. We extended this analysis to several other genes involved in the RET/GDNF pathway, namely the GDNF co-receptor encoding gene *GFRA1*, regulators of the expression of RET and/or GDNF (*GDF11*, *PAX2*, *EYA1*, *SIX1*, *SIX2*, *SIX4*, *HOXA11*, *HOXD11*, *FOXC1*, *GATA3*), repressors of the pathway (*SPRY1*, *ROBO2*, *SLIT2*), the activator *SOX9* and target genes *ETV4* and *ETV5*. [32-34] For each gene, we analysed not only the gene itself but also surrounding sequences extending up to neighbouring genes on each side, in order to be sure to include regulatory sequences. In addition to two intronic CNVs, in *SLIT2* and *EYA1*, each identified in 4 fetuses, we characterized a 1 kb heterozygous deletion spanning the *GDNF* non-coding exon 1 in one case. These three CNVs have been reported in controls (<http://projects.tcag.ca/variation/>), suggesting that they are polymorphic variations. There was no CNV in any of the other tested genes.

### **DISCUSSION**

The pattern of expression of RET and GDNF in fetal kidneys, the severe disruption of kidney development in *Ret* or *Gdnf* knockout mice, as well as large amounts of data generated from cellular and organ culture models, support the major role of the RET/GDNF signalling in control of branching morphogenesis during kidney development. [17,18,20-22,35-39] Moreover, several *RET* mutations associated with MTC and/or HSCR have been shown to impair branching in a three-dimensional kidney cell culture model, [26,40] in agreement with a role of RET in kidney morphogenesis. It was thus expected that mutations in *RET* and *GDNF* could be responsible for kidney development defects in human. Accordingly, heterozygous *RET* mutations were previously reported in 30% of a small series of 29 fetuses with BRA or URA and one heterozygous *GDNF* mutation in a fetus with URA. [29] However, our results, based on analysis of a larger series of 105 cases including 90 fetuses with either BRA or URA and contralateral RHD or MCDK do not confirm the high frequency of *RET* mutations in fetuses with renal agenesis or other severe abnormality of kidney development. Discrepancy between the two studies could be explained by differences in the ethnic origin of the patients and/or by a bias due to the small number of patients in the study of Skinner *et al.*. We only report 7 potential mutations in the *RET* coding sequence (6.6%), and no mutation in the *GDNF* coding sequence. We did not analyse the sequence of the GDNF co-receptor encoding gene *GFRA1* in this study because no mutation has ever been reported in this gene,

neither in KDD nor in HSCR.[27,29,41] As reported,[29] the *RET* mutations that we identified in KDD fetuses were heterozygous, distributed all along the gene and most of them were missense variants. Only one nonsense mutation was identified and absence of tissue available for RNA and protein extraction precluded analysis of the impact of this mutation on the expression of the gene. As for HSCR, heterozygosity of the mutations suggests a mechanism based on haploinsufficiency, leading to a dysregulation of the RET signalling in kidney. However, one cannot exclude that some of the missense variants might be activating mutations with both gain-of-function and loss-of-function effects, as suggested for some of the mutations previously described.[29] While Skinner *et al.* described the presence of two or three mutations in some fetuses, none of the fetuses in our series presented with several mutations.[29] No mutation was common to both studies and only the L56M variant has previously been reported in a HSCR patient.[42] Absence of splicing defect in the kidney from the fetus with the T451T variant suggests that this neutral variant is a rare polymorphism rather than a causative mutation. Moreover, heterozygosity of the P992P variant in a fetus from a consanguineous family and his father suggests that the kidney development defect in this family is probably not linked to this *RET* variant. Finally, while Skinner *et al.* did not trace the inherited vs. *de novo* origin of the mutations in their fetuses, we demonstrate that the missense mutations R57Q and D567N as well as the W1056X nonsense mutation were inherited from healthy fathers. Although we cannot eliminate the hypothesis that somatic mosaicism could explain the absence of phenotype in the father, these results likely indicate that, even if these *RET* mutations impair kidney development, other genetic or epigenetic events affecting the RET/GDNF signalling must cooperate.

In addition to the 7 *RET* variations in the coding sequence, a total of 48 SNPs in coding and non-coding *RET* and *GDNF* sequences, as well as 21 new rare variants in non-coding sequences were identified in our series of fetuses (4 to 22 *RET* variants per fetus; 0 to 4 *GDNF* variant per fetus). This includes *RET* SNP variants A432A in exon 7 and G691S in exon 11, that have been reported as associated with CAKUT,[43,44] and *RET* variants rs1864411, rs1864410, rs2435357 and rs2506004 in intron 1 that have been reported as part of a HSCR susceptibility haplotype.[45,46] Based on the observation that allele frequencies in our cases were similar to controls, our data do not support a role in KDD for any of these variants. Four previously unreported variants in *RET* 5'-ECR regions were identified in a total of six patients and not in controls. However, the absence of clustering of these variants in one specific ECR is not in favour of a role in the dysregulation of RET expression. Moreover,

discordant genotypes in the 3 pairs of fetuses belonging to families with recurrent KDD suggest that *RET* is not the culprit gene in these patients.

Interestingly, we identified one polymorphism in *RET* intron 1 (ECR6/271, rs2506012) with significant over-representation of the minor G allele in KDD fetuses. Analysis of transcription factor binding on the ECR6 sequence using the Genomatix (<http://www.genomatix.de>) and the Mapper (<http://mapper.chip.org>) softwares predicted creation of a site for the aryl hydrocarbon receptor nuclear translocator (ARNT) transcription factor by this variant. ARNT is a protein that is involved in translocation of the aryl hydrocarbon receptor (AHR) to the nucleus, following induction by ligands like dioxin and polycyclic aromatic hydrocarbons. Interestingly, ARNT and its receptor AHR are both expressed during kidney development (<http://www.gudmap.org/>) and activation of AHR has been reported to inhibit branching morphogenesis of metanephric kidneys.[47] Thus, this polymorphism could confer an increased susceptibility to environmental factors and could result in an altered regulation of *RET*. ARNT has also been identified as the beta subunit of the heterodimeric transcription factor HIF1 (hypoxia-inducible factor 1). As HIF1A is co-expressed with ARNT in cap mesenchyme and UB (<http://www.gudmap.org/>), an altered regulation of *RET* by HIF1 can also be proposed. While analysis of a second series of samples confirmed the difference in allele frequencies between KDD fetuses and controls, it resulted in a decrease in the level of significance ( $P=0.002$  to  $P=0.01$ ). Thus, analysis of a larger series of patients will be necessary to unambiguously draw conclusions. Moreover, due to the low frequency of this variant in the fetuses, its potential contribution to kidney development defect can only account for a small proportion of patients.

Quantification of *RET* transcript in KDD vs. control kidney samples revealed down-expression in the three KDD samples. There are several possible explanations for this result, including sample bias, cause or consequence of the altered differentiation process. One of the mechanisms involved in regulation of gene expression is through binding of micro-RNAs to 3'UTR sequences. Indeed, variations in 3'UTR and mutations in the micro-RNA encoding genes, resulting in an altered gene regulation, have been reported in several pathologies.[48-50] In one case, the variation was a common polymorphism, significantly over-represented in patients vs. controls.[51] In the *RET* gene, a variant located in the 3'UTR has been reported to confer protection from HSCR.[52] However, sequencing of *RET* and *GDNF* 3'UTRs in KDD fetuses did not allow us to identify new variants nor variants with a higher frequency in patients vs. controls. When the micro-RNAs that regulate *RET* expression during kidney development are identified, it will be worthwhile screening for mutations in these sequences.

Another genetic mechanism that could affect RET and/or GDNF expression and consequently kidney development is gene copy number variations. The only CNV of potential interest that we identified was a deletion spanning a CpG rich region and the non-coding exon 1 of GDNF. Although this CNV has been reported in normal controls (<http://projects.tcag.ca/variation/>), suggesting that it is a polymorphism, it could result in a decreased GDNF expression. However, it was present in only one fetus and thus cannot be considered as a significant event in KDD. Moreover, CNV analysis of 17 other genes encoding regulators or targets of the RET/GDNF pathway including the GDNF co-receptor encoding gene *GFRA1*, did not allow us to identify any CNV affecting these genes.

Altogether, this study clearly shows that *RET* and *GDNF* genomic alterations are not significantly associated with renal agenesis / hypodysplasia / multicystic dysplastic kidney in humans, suggesting that they play a minor role in KDD. These results are an important issue to be taken into account for genetic diagnosis of these defects. Interestingly, redundant receptor tyrosine kinase signalling, notably including FGF10/FGFR2, as well as a balance between positive and negative regulation of this signalling network, rather than RET/GDNF per se, was suggested to be the central pathway regulating branching for kidney development.[53] Regulatory or target genes common to these redundant signalling events could be worthwhile testing as new candidate genes to explain kidney development defects.

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**Competing interest** None

**Patients consent** obtained

**Ethics approval** This study was conducted with the approval of the the Comité de Protection des Personnes pour la Recherche Biomédicale Ile de France 2



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