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## 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), *KAL1* (Kallman syndrome), *FRAS1* and *FREM1* (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 GFRα1 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 dysmorphy, 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[1] | 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 |

Positions on chromosomes are given according to the NCBI36/hg18 built ;
[1] % 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 AGI_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[1] | Variant | Consequence | Exon | Phenotype | Features of the variants |
|---|---|---|---|---|---|
| c.166 C>A | p.L56M | Missense | 2 | BRA and uterine agenesis | Possibly damaging[2] |
| c.170 G>A | p.R57Q | Missense | 2 | MCDK | Benign[2]<br>Present in healthy father |
| c.1353 G>A | p.T451T | Neutral | 7 | URA/MCDK | Potential effect on splicing[3] |
| c.1699 G>A | p.D567N | Missense | 9 | BRA | Probably damaging[2]<br>Present in healthy father |
| c.2359 G>A | p.V787I | Missense | 13 | URA/RHD | Probably damaging[2] |
| 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 |

[1] c. positions are given according to the coding sequence of NM_020975
[2] identified with PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/);
[3] 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[1] | 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 |
| | ECR2/230 | delTCAC | | 0.005 (-) | |
| | ECR2/504 | C>T | | 0.03 (0.02) | |
| Intron 1 ECRs[1] | 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 | | **0.07 (0.02)**[5] | rs2506012 |
| | ECR7/27 | C>T | | 0.21 (0.19)[4] | rs2435357 |
| | ECR7/244 | C>A | | 0.21 (0.18)[4] | rs2506004 |
| Exonic and flanking intronic sequences[2] | c.73+53 | G>A | | 0.15 (0.10)[3] | 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)[3] | |
| | 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[2] | c.3219+15 | C>T | | 0.17 (0.15)[4] | rs2075912 |
| | c.3219+128 | C>T | | 0.005 (-) | |
| | c.3219+166 | A>T | | 0.20 (0.22)[4] | rs2075913 |
| | c.3219+731 | C>T | | 0.15 (0.15)[4] | rs2565200 |
| 3'UTR RET51[2] | c.3345+29 | A>C | | 0.005 (-) | |
| | c.3345+95 | C>T | | 0.17 (0.22)[4] | rs17028 |
| | c.3345+388 | G>A | | 0.23 (0.13)[4] | 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) | |

[1] positions are given according to the ECR sequence (see Table 1) ;
[2] c. positions are given according to NM_020975, except for RET9 3'UTR (NM_020630) ;
[3] frequency of the homozygous variant ;
[4] frequency in controls according to Pilot-CEU ;
[5] 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'[1] | ECR1/405 | G>A | | 0.07 (0.11)[3] | rs2975100 |
| | ECR1/2 | C>A | | 0.07 (0.22)[4] | rs2075680 |
| | c.1-229 | insGCC | | 0.01 (-) | |
| Exons[2] | c.150 | T>C | | 0.24 (0.33)[1] | rs2973033 |
| | c.351+17 | C>T | | 0.005 (-) | |
| | Exon3 | | R143R | 0.02 (0.03)[5] | rs36010631 |
| 3'UTR[1] | 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) | |

[1] positions are given according to the ECR sequence (see Table 1) ;
[2] c. positions are given according to NM_000514 ;
[3] frequency in controls according to Pilot-CEU ;
[4] frequency in controls according to JBCI-allele ;
[5] 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

## REFERENCES


1. Sanna-Cherchi S, Ravani P, Corbani V, Parodi S, Haupt R, Piaggio G, Innocenti ML, Somenzi D, Trivelli A, Caridi G, Izzi C, Scolari F, Mattioli G, Allegri L, Ghiggeri GM. Renal outcome in patients with congenital anomalies of the kidney and urinary tract. *Kidney Int* 2009;**76**:528-33.
2. Heidet L, Decramer S, Pawtowski A, Moriniere V, Bandin F, Knebelmann B, Lebre AS, Faguer S, Guigonis V, Antignac C, Salomon R. Spectrum of HNF1B mutations in a large cohort of patients who harbor renal diseases. *Clin J Am Soc Nephrol* 2010;**5**:1079-90.
3. Kochhar A, Orten DJ, Sorensen JL, Fischer SM, Cremers CW, Kimberling WJ, Smith RJ. SIX1 mutation screening in 247 branchio-oto-renal syndrome families: a recurrent missense mutation associated with BOR. *Hum Mutat* 2008;**29**:565.
4. Orten DJ, Fischer SM, Sorensen JL, Radhakrishna U, Cremers CW, Marres HA, Van Camp G, Welch KO, Smith RJ, Kimberling WJ. Branchio-oto-renal syndrome (BOR): novel mutations in the EYA1 gene, and a review of the mutational genetics of BOR. *Hum Mutat* 2008;**29**:537-44.
5. Ulinski T, Lescure S, Beaufils S, Guigonis V, Decramer S, Morin D, Clauin S, Deschenes G, Bouissou F, Bensman A, Bellanne-Chantelot C. Renal phenotypes related to hepatocyte nuclear factor-1beta (TCF2) mutations in a pediatric cohort. *J Am Soc Nephrol* 2006;**17**:497-503.
6. Weber S, Moriniere V, Knuppel T, Charbit M, Dusek J, Ghiggeri GM, Jankauskiene A, Mir S, Montini G, Peco-Antic A, Wuhl E, Zurowska AM, Mehls O, Antignac C, Schaefer F, Salomon R. Prevalence of mutations in renal developmental genes in children with renal hypodysplasia: results of the ESCAPE study. *J Am Soc Nephrol* 2006;**17**:2864-70.
7. Krug P, Moriniere V, Marlin S, Koubi V, Gabriel HD, Colin E, Bonneau D, Salomon R, Antignac C, Heidet L. Mutation screening of the EYA1, SIX1 and SIX5 genes in a large cohort of patients harboring branchio-oto-renal syndrome calls into question the pathogenic role of SIX5 mutations. *Hum Mutat* 2010.
8. Alazami AM, Shaheen R, Alzahrani F, Snape K, Saggar A, Brinkmann B, Bavi P, Al-Gazali LI, Alkuraya FS. FREM1 mutations cause bifid nose, renal agenesis, and anorectal malformations syndrome. *Am J Hum Genet* 2009;**85**:414-8.
9. Biason-Lauber A, Konrad D, Navratil F, Schoenle EJ. A WNT4 mutation associated with Mullerian-duct regression and virilization in a 46,XX woman. *N Engl J Med* 2004;**351**:792-8.
10. Botzenhart EM, Bartalini G, Blair E, Brady AF, Elmslie F, Chong KL, Christy K, Torres-Martinez W, Danesino C, Deardorff MA, Fryns JP, Marlin S, Garcia-Minaur S, Hellenbroich Y, Hay BN, Penttinen M, Shashi V, Terhal P, Van Maldergem L, Whiteford ML, Zackai E, Kohlhase J. Townes-Brocks syndrome: twenty novel SALL1 mutations in sporadic and familial cases and refinement of the SALL1 hot spot region. *Hum Mutat* 2007;**28**:204-5.
11. Cain JE, Islam E, Haxho F, Chen L, Bridgewater D, Nieuwenhuis E, Hui CC, Rosenblum ND. GLI3 repressor controls nephron number via regulation of Wnt11 and Ret in ureteric tip cells. *PLoS One* 2009;**4**:e7313.
12. Georgopoulos NA, Koika V, Galli-Tsinopoulou A, Spiliotis BE, Adonakis G, Keramida MK, Sgourou A, Koufogiannis KD, Papachatzopoulou A, Papavassiliou AG, Kourounis G, Vagenakis GA. Renal dysgenesis and KAL1 gene defects in patients with sporadic Kallmann syndrome. *Fertil Steril* 2007;**88**:1311-7.

13. Pitera JE, Scambler PJ, Woolf AS. Fras1, a basement membrane-associated protein mutated in Fraser syndrome, mediates both the initiation of the mammalian kidney and the integrity of renal glomeruli. *Hum Mol Genet* 2008;**17**:3953-64.
14. Van Esch H, Groenen P, Nesbit MA, Schuffenhauer S, Lichtner P, Vanderlinden G, Harding B, Beetz R, Bilous RW, Holdaway I, Shaw NJ, Fryns JP, Van de Ven W, Thakker RV, Devriendt K. GATA3 haplo-insufficiency causes human HDR syndrome. *Nature* 2000;**406**:419-22.
15. Sanna-Cherchi S, Caridi G, Weng PL, Dagnino M, Seri M, Konka A, Somenzi D, Carrea A, Izzi C, Casu D, Allegri L, Schmidt-Ott KM, Barasch J, Scolari F, Ravazzolo R, Ghiggeri GM, Gharavi AG. Localization of a gene for nonsyndromic renal hypodysplasia to chromosome 1p32-33. *Am J Hum Genet* 2007;**80**:539-49.
16. Weber S, Landwehr C, Renkert M, Hoischen A, Wuhl E, Denecke J, Radlwimmer B, Haffner D, Schaefer F, Weber RG. Mapping candidate regions and genes for congenital anomalies of the kidneys and urinary tract (CAKUT) by array-based comparative genomic hybridization. *Nephrol Dial Transplant* 2010.
17. Chi X, Michos O, Shakya R, Riccio P, Enomoto H, Licht JD, Asai N, Takahashi M, Ohgami N, Kato M, Mendelsohn C, Costantini F. Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis. *Dev Cell* 2009;**17**:199-209.
18. Meyer TN, Schwesinger C, Bush KT, Stuart RO, Rose DW, Shah MM, Vaughn DA, Steer DL, Nigam SK. Spatiotemporal regulation of morphogenetic molecules during in vitro branching of the isolated ureteric bud: toward a model of branching through budding in the developing kidney. *Dev Biol* 2004;**275**:44-67.
19. de Graaff E, Srinivas S, Kilkenny C, D'Agati V, Mankoo BS, Costantini F, Pachnis V. Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. *Genes Dev* 2001;**15**:2433-44.
20. Jain S, Encinas M, Johnson EM, Jr., Milbrandt J. Critical and distinct roles for key RET tyrosine docking sites in renal development. *Genes Dev* 2006;**20**:321-33.
21. Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* 1996;**382**:70-3.
22. Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* 1994;**367**:380-3.
23. Angrist M, Bolk S, Thiel B, Puffenberger EG, Hofstra RM, Buys CH, Cass DT, Chakravarti A. Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease. *Hum Mol Genet* 1995;**4**:821-30.
24. Attie T, Pelet A, Edery P, Eng C, Mulligan LM, Amiel J, Boutrand L, Beldjord C, Nihoul-Fekete C, Munnich A, et al. Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. *Hum Mol Genet* 1995;**4**:1381-6.
25. Margraf RL, Crockett DK, Krautscheid PM, Seamons R, Calderon FR, Wittwer CT, Mao R. Multiple endocrine neoplasia type 2 RET protooncogene database: repository of MEN2-associated RET sequence variation and reference for genotype/phenotype correlations. *Hum Mutat* 2009;**30**:548-56.
26. Arighi E, Popsueva A, Degl'Innocenti D, Borrello MG, Carniti C, Perala NM, Pierotti MA, Sariola H. Biological effects of the dual phenotypic Janus mutation of ret cosegregating with both multiple endocrine neoplasia type 2 and Hirschsprung's disease. *Mol Endocrinol* 2004;**18**:1004-17.
27. Pini Prato A, Musso M, Ceccherini I, Mattioli G, Giunta C, Ghiggeri GM, Jasonni V. Hirschsprung disease and congenital anomalies of the kidney and urinary tract (CAKUT): a novel syndromic association. *Medicine (Baltimore)* 2009;**88**:83-90.

28. Lore F, Di Cairano G, Talidis F. Unilateral renal agenesis in a family with medullary thyroid carcinoma. *N Engl J Med* 2000;**342**:1218-9.
29. Skinner MA, Safford SD, Reeves JG, Jackson ME, Freemerman AJ. Renal aplasia in humans is associated with RET mutations. *Am J Hum Genet* 2008;**82**:344-51.
30. Wang K, Li M, Hadley D, Liu R, Glessner J, Grant SF, Hakonarson H, Bucan M. PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. *Genome Res* 2007;**17**:1665-74.
31. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 2002;**3**:285-98.
32. Little M, Georgas K, Pennisi D, Wilkinson L. Kidney development: two tales of tubulogenesis. *Curr Top Dev Biol* 2010;**90**:193-229.
33. Lu BC, Cebrian C, Chi X, Kuure S, Kuo R, Bates CM, Arber S, Hassell J, MacNeil L, Hoshi M, Jain S, Asai N, Takahashi M, Schmidt-Ott KM, Barasch J, D'Agati V, Costantini F. Etv4 and Etv5 are required downstream of GDNF and Ret for kidney branching morphogenesis. *Nat Genet* 2009;**41**:1295-302.
34. Reginensi A, Clarkson M, Neirijnck Y, Lu B, Ohyama T, Groves AK, Sock E, Wegner M, Costantini F, Chaboissier MC, Schedl A. SOX9 controls epithelial branching by activating RET effector genes during kidney development. *Hum Mol Genet* 2011.
35. Jain S, Knoten A, Hoshi M, Wang H, Vohra B, Heuckeroth RO, Milbrandt J. Organotypic specificity of key RET adaptor-docking sites in the pathogenesis of neurocristopathies and renal malformations in mice. *J Clin Invest* 2010;**120**:778-90.
36. Jijiwa M, Fukuda T, Kawai K, Nakamura A, Kurokawa K, Murakumo Y, Ichihara M, Takahashi M. A targeting mutation of tyrosine 1062 in Ret causes a marked decrease of enteric neurons and renal hypoplasia. *Mol Cell Biol* 2004;**24**:8026-36.
37. Majumdar A, Vainio S, Kispert A, McMahon J, McMahon AP. Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* 2003;**130**:3175-85.
38. Shakya R, Watanabe T, Costantini F. The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev Cell* 2005;**8**:65-74.
39. Srinivas S, Wu Z, Chen CM, D'Agati V, Costantini F. Dominant effects of RET receptor misexpression and ligand-independent RET signaling on ureteric bud development. *Development* 1999;**126**:1375-86.
40. Degl'Innocenti D, Arighi E, Popsueva A, Sangregorio R, Alberti L, Rizzetti MG, Ferrario C, Sariola H, Pierotti MA, Borrello MG. Differential requirement of Tyr1062 multidocking site by RET isoforms to promote neural cell scattering and epithelial cell branching. *Oncogene* 2004;**23**:7297-309.
41. Myers SM, Salomon R, Goessling A, Pelet A, Eng C, von Deimling A, Lyonnet S, Mulligan LM. Investigation of germline GFR alpha-1 mutations in Hirschsprung disease. *J Med Genet* 1999;**36**:217-20.
42. Hofstra RM, Wu Y, Stulp RP, Elfferich P, Osinga J, Maas SM, Siderius L, Brooks AS, vd Ende JJ, Heydendael VM, Severijnen RS, Bax KM, Meijers C, Buys CH. RET and GDNF gene scanning in Hirschsprung patients using two dual denaturing gel systems. *Hum Mutat* 2000;**15**:418-29.
43. Yang Y, Houle AM, Letendre J, Richter A. RET Gly691Ser mutation is associated with primary vesicoureteral reflux in the French-Canadian population from Quebec. *Hum Mutat* 2008;**29**:695-702.
44. Zhang Z, Quinlan J, Hoy W, Hughson MD, Lemire M, Hudson T, Hueber PA, Benjamin A, Roy A, Pascuet E, Goodyer M, Raju C, Houghton F, Bertram J, Goodyer

P. A common RET variant is associated with reduced newborn kidney size and function. *J Am Soc Nephrol* 2008;**19**:2027-34.

45. Burzynski GM, Nolte IM, Bronda A, Bos KK, Osinga J, Plaza Menacho I, Twigt B, Maas S, Brooks AS, Verheij JB, Buys CH, Hofstra RM. Identifying candidate Hirschsprung disease-associated RET variants. *Am J Hum Genet* 2005;**76**:850-8.
46. Pelet A, de Pontual L, Clement-Ziza M, Salomon R, Mugnier C, Matsuda F, Lathrop M, Munnich A, Feingold J, Lyonnet S, Abel L, Amiel J. Homozygosity for a frequent and weakly penetrant predisposing allele at the RET locus in sporadic Hirschsprung disease. *J Med Genet* 2005;**42**:e18.
47. Falahatpisheh MH, Ramos KS. Ligand-activated Ahr signaling leads to disruption of nephrogenesis and altered Wilms' tumor suppressor mRNA splicing. *Oncogene* 2003;**22**:2160-71.
48. Mencia A, Modamio-Hoybjor S, Redshaw N, Morin M, Mayo-Merino F, Olavarrieta L, Aguirre LA, del Castillo I, Steel KP, Dalmay T, Moreno F, Moreno-Pelayo MA. Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. *Nat Genet* 2009;**41**:609-13.
49. Simon D, Laloo B, Barillot M, Barnetche T, Blanchard C, Rooryck C, Marche M, Burgelin I, Coupry I, Chassaing N, Gilbert-Dussardier B, Lacombe D, Grosset C, Arveiler B. A mutation in the 3'-UTR of the HDAC6 gene abolishing the post-transcriptional regulation mediated by hsa-miR-433 is linked to a new form of dominant X-linked chondrodysplasia. *Hum Mol Genet* 2010;**19**:2015-27.
50. Yang Z, Kaye DM. Mechanistic insights into the link between a polymorphism of the 3'UTR of the SLC7A1 gene and hypertension. *Hum Mutat* 2009;**30**:328-33.
51. Yang Z, Venardos K, Jones E, Morris BJ, Chin-Dusting J, Kaye DM. Identification of a novel polymorphism in the 3'UTR of the L-arginine transporter gene SLC7A1: contribution to hypertension and endothelial dysfunction. *Circulation* 2007;**115**:1269-74.
52. Griseri P, Lantieri F, Puppo F, Bachetti T, Di Duca M, Ravazzolo R, Ceccherini I. A common variant located in the 3'UTR of the RET gene is associated with protection from Hirschsprung disease. *Hum Mutat* 2007;**28**:168-76.
53. Michos O, Cebrian C, Hyink D, Grieshammer U, Williams L, D'Agati V, Licht JD, Martin GR, Costantini F. Kidney development in the absence of Gdnf and Spry1 requires Fgf10. *PLoS Genet* 2010;**6**:e1000809.