A Common RET Variant Is Associated with Reduced Newborn Kidney Size and Function

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ABSTRACT
Congenital nephron number varies five-fold among normal humans, and individuals at the lower end of this range may have an increased lifetime risk for essential hypertension or renal insufficiency; however, the mechanisms that determine nephron number are unknown. This study tested the hypothesis that common hypomorphic variants of the RET gene, which encodes a tyrosine kinase receptor critical for renal branching morphogenesis, might account for subtle renal hypoplasia in some normal newborns. A common single-nucleotide polymorphism (rs1800860 G/A) was identified within an exonic splicing enhancer in exon 7. The adenosine variant at mRNA position 1476 reduced affinity for spliceosome proteins, enhanced the likelihood of aberrant mRNA splicing, and diminished the level of functional transcript in human cells. In vivo, normal white newborns with an rs1800860(1476A) allele had kidney volumes 10% smaller and cord blood cystatin C levels 9% higher than those with the rs1800860(1476G) allele. These findings suggest that the RET(1476A) allele, in combination with other common polymorphic developmental genes, may account for subtle renal hypoplasia in a significant proportion of the white population. Whether this gene variant affects clinical outcomes requires further study.


Human kidney development begins at approximately 5 wk gestation, when RET-bearing cells of the descending nephric duct encounter glial-derived neurotrophic factor (GDNF) released by metanephric mesenchyme at somite 24.1 In response to the trophic effects of GDNF, a ureteric bud sprouts from each nephric duct and Arborizes within the lateral mesenchyme. Signals from each ureteric bud branch tip induce adjacent metanephric stem cells to form individual nephrons, which fuse to the tree-like collecting system. Because new nephron formation ends at approximately 36 wk gestation, the extent of branching nephrogenesis by this time determines nephron endowment for life.

Interestingly, nephron number varies widely (0.3 to 1.3 million nephrons per kidney) among normal humans.² Although once dismissed as a benign reflection of human diversity, Brenner et al.³ proposed that individuals born at the low end of the nephron endowment spectrum may have increased risk for developing “essential” hypertension and renal insufficiency later in life. They hypothesized that signals driving compensatory hypertrophy of overworked nephrons cause glo-
merulosesclerosis and a cycle of subtle, slowly progressive renal dysfunction. Recent evidence supports this theory; an autopsy study by Keller et al. showed that German adults with essential hypertension had 47% fewer nephrons per kidney than well-matched normotensive control subjects. As predicted, hypertensive patients had hypertrophic glomeruli (glomerular volume 233% of control subjects) and increased glomerulosclerosis (5.5% of glomeruli versus 0% in control subjects). Other evidence suggests that racial differences in congenital nephron number might also explain the relatively high incidence of ESRD in Aboriginal versus white populations. At autopsy, Aboriginal individuals have 23% fewer glomeruli (683,174 per kidney) than white individuals (885,318 per kidney; \( P < 0.04 \)).

Little is known about the factors that set nephron number, but the GDNF/RET signaling pathway seems to play a central role. The RET gene (NM_020630) encodes a 1072 amino acid transmembrane tyrosine kinase receptor (NP_065681) expressed at the tips of the branching ureteric buds during fetal kidney development; homozygous Ret knockout mice are anephric, and heterozygotes have a 22% reduction in nephron number at 15 d of age. In this study, we hypothesized that hypomorphic variants of the RET gene might be prevalent in “normal” humans, contributing to suboptimal nephron number and subtle renal hypoplasia in a significant portion of the population.

**RESULTS**

We scanned the NCBI dbSNP database (see Appendix) and identified two common variants (minor allele frequency >10%) of the RET gene coding region that might alter its tyrosine kinase receptor function during kidney development. In exon 11, an A/G substitution at mRNA position 2251 (rs1799939) occurs in approximately 16% of white alleles, changing gly691 to ser691. In exon 7, an A/G substitution at mRNA position 1476 (rs1800860) occurs in 25% of white alleles and lies within an exonic splice enhancer (ESE) sequence (Figure 1A). The critical position of this substitution suggests that it might modify pre-mRNA splicing. We used ESEfinder to calculate the effect of the rs1800860 polymorphism on binding to proteins (SF2/ASF, SC35) of the mRNA spliceosome (Table 1). In each case, the 1476(A) allele reduces RET affinity score below the predicted threshold for effective binding to these components of the splicing machinery.

To confirm that the minor RET\(_{1476}(A)\) allele alters normal mRNA splicing, we studied three RET-expressing human cell lines: (1) SK-N-BE(2) neuroblastoma cells (1476\(_{A}/A\)); (2) SH-SYSY neuroblastoma cells (1476\(_{G}/A\)); and (3) Wit49 Wilms’ tumor cells (1476\(_{G}/G\)). RET transcripts were amplified with nested primers spanning exon 6 through exon 15. Only the predicted wild-type 1252-bp transcript is evident in Wit49 (1476\(_{G}/G\)) cells, whereas an aberrant 190-bp transcript is noted in the two cell lines bearing one or more 1476\(_{A}\) alleles (Figure 1B). The 190-bp transcript was not seen in any of four other 1476\(_{G}/G\) cell lines (data not shown). Sequencing of the 1252-bp band in the heterozygous (rs1800860) SH-SYSY cell line demonstrated that normal splicing of exon 7 can occur with either nucleotide at mRNA position 1476 (Figure 2); however, when the minor transcript in SK-N-BE(2) or SH-SYSY cells was sequenced, we also noted aberrant splicing from exon 7a to exon 14b with a heterogeneous 18-bp intervening sequence which consisted of nucleotides continuing beyond 1476\(_{A}\) into exon 7b (upper sequence) or nucleotides from exon 14b upstream of mRNA position 2500 through 2518 (down sequence). This suggests aberrant splicing between sites after the ESE in exon 7 and two alternative cryptic splice sites (position 2500 and 2518) in exon 14b.

![Figure 1](image)

**Figure 1.** 1476\(_{A}\) SNP in human RET mRNA is associated with an aberrant 190-bp transcript. (A) Genomic structure of the human RET gene, indicating the 1476\(_{G}/A\) SNP within an ESE of exon 7. (B) Nested primers spanning exon 6 to exon 17 were used to amplify RET transcripts in reverse-transcribed RNA extracted from various human cell lines. Only the predicted wild-type 1252-bp transcript is evident in the homozygous (1476 \(_{G}/G\)) Wit49 Wilms’ tumor cell line (lane 3); however, an aberrant 190-bp transcript is noted in heterozygous (1476\(_{G}/A\)) SH-SYSY neuroblastoma cells (lane 2) and in homozygous 1476\(_{A}/A\) SK-N-BE(2) neuroblastoma cells (lane 1). The aberrant 190-bp transcript was not seen in four other 1476\(_{G}/G\) Wilms’ tumor, renal cell carcinoma, and ovarian carcinoma cell lines (data not shown). (C) Sequence analysis of the aberrant 190-bp band demonstrates fusion of RET exon 7a to exon 14b with a heterogeneous 18-bp intervening sequence which consisted of nucleotides continuing beyond 1476\(_{A}\) into exon 7b (upper sequence) or nucleotides from exon 14b upstream of mRNA position 2500 through 2518 (down sequence).

![Image](image)
Predicted effect of SNP rs1800860 on affinity score for binding of RET mRNA to core spliceosome components. We used ESEfinder software to predict the effect of an adenine versus guanine nucleotides at position 1476 of the human RET transcript on binding to two core protein components of the spliceosome (SF2/ASF and SC35). The matrix score for SF2/ASF is reduced from 3.08 for the ancestral CAGGC sequence to 0.50 for the less common CAGGCAT variant (threshold for normal splicing efficiency ~ 1.96). Similarly, affinity for the SC35 component is reduced from 3.85 to 2.10 (threshold ~ 2.40). Based on these predictions, we hypothesized that the rs1800860 A allele (1476A) might lead to aberrant RET mRNA splicing.

Table 1. Predicted effect of SNP 1476G/A on affinity score for binding of SR protein

<table>
<thead>
<tr>
<th>Allele</th>
<th>Motif SF2/ASF</th>
<th>Score</th>
<th>Motif SC35</th>
<th>Score</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>CAGGCA</td>
<td>0.503</td>
<td>ΔTTTCA</td>
<td>2.095</td>
</tr>
<tr>
<td>G</td>
<td>CAGGCGT</td>
<td>3.081</td>
<td>GTTTCA</td>
<td>3.847</td>
</tr>
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</table>

Figure 2. Sequence of wild-type human RET exon 7 (NM_020630). After reverse transcription of mRNA from heterozygous (rs1800860) SH-SY5Y cells, exon 7 was amplified by PCR and the normal 1252-bp amplicon was sequenced. The presence of either G or A at position 1476 demonstrates that normal splicing of RET exon 7 can occur with both alleles.

Figure 3. The aberrant del(7b-14a) RET transcript lacks key functional domains. CLD1 through 4, CRD, transmembrane segment (TM), juxtamembrane segment (JM), and two cytoplasmic tyrosine kinase domains (TK1 and TK2) are displayed adjacent to the corresponding exon structure of the wild-type RET transcript (WT). To the left is the aberrant del(7b-14a) transcript associated with 1476(A) alleles; deletion of extracellular domains for ligand (GDNF) and co-receptor (GFRα1), transmembrane segments, and a large portion of TK1 is predicted, rendering the transcript nonfunctional.

DNA extracted from cord blood was used to genotype each infant for the rs1800860 (1476G/A) and rs1799939 (2251G/A) SNP. Genotype distribution for each SNP conformed to the Hardy-Weinberg equilibrium (P = 0.39 and 0.47, respectively); SNP frequencies in our cohort (Table 2) were similar to those reported in the CAUC1 and CEU populations (NCBI dbSNP database; see Appendix). RET2251(G/A) SNP was associated with neither renal volume nor cord cystatin C; however, total kidney volume factored for body surface area (KidVol/BSA) in newborns bearing one or more 1476(A) alleles was 9.7% smaller than that in newborns with the homozygous 1476(G/G) genotype (P = 0.009). The 1476(A) allele was also associated with 9.2% increase in cord blood cystatin C concentration (P = 0.002), suggesting a comparable reduction in functional renal mass15 (Table 2).

The 1476(A) allele was widely distributed in our population; combined renal volume in infants with a 1476(A) allele
ranged from 16.06 to 44.48 ml versus 15.01 to 50.33 ml in infants who were homozygous for the more common 1476(G) allele. Thus, the association between 1476(A) allele and renal volume cannot be attributed to a few infants with very small kidneys. Similarly, the distribution of left/right kidney volume ratios was similar in newborns with one or more 1476(A) alleles (0.93 ± 0.22 SD) versus newborns homozygous for the 1476(G) allele (0.96 ± 0.18 SD) (P > 0.05). Thus, the association between 1476(A) and renal volume was not due to a few infants with unilateral renal hypoplasia.

To confirm that the RET1476(A) allele compromises expression of wild-type receptor mRNA, we examined allele-specific mRNA expression in the heterozygous SH-SY5Y and G401 cell lines. RET exon 7 was amplified from genomic DNA and cellular mRNA, using high-sensitivity sequencing technology to quantify each allele-specific amplicon as described by others. mRNA expression from the 1476(A) allele was substantially reduced in both SH-SY5Y (by 25%) and G401 (by 50%) cells, compared with the expression level from the 1476(G) allele (Figure 6).

Our previous studies demonstrated an association between a common (18.5% of white individuals) human PAX2 haplotype (AAA) and a 10% reduction in newborn renal size. Interestingly, RET transcription is regulated by PAX2 gene dosage. When we analyzed our cohort for both genes, renal volume in the 17 of 136 newborns carrying both the RET1476(A) and PAX2(AAA) minor alleles was 23% lower than that in infants with the RET1476(G/G), PAX2(GGG) haplotype (Figure 7).

**DISCUSSION**

During renal development, the branching ureteric bud expresses high levels of the transcription factor PAX2. Brophy and colleagues recently showed that, among its many functions, PAX2 directly activates transcription of genes for both RET, a tyrosine kinase receptor, and its ligand, GDNF. Epithelial cells expressing RET receptors cluster at the tip of each ureteric bud branch as it undergoes branching morphogenesis. When activated by GDNF from nearby mesenchyme, the RET receptor heterodimerizes with GFRα1, stimulating cell proliferation, migration, and survival via several intracellular signals, including the RAS/mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT, and RAC1/JUN NH(2) terminal kinase pathways. Loss of RET, GDNF, or GFRα1 results in renal agenesis, because of inhibition of ureteric bud growth and branching.

Because RET integrity is critical for branching nephrogenesis, we hypothesized that heterozygous RET mutations might partially compromise the extent of ureteric bud branching during development, leading to suboptimal nephron number. In mutant mice, heterozygous null alleles reduce nephron number by approximately 22% at postnatal day 15. Thus, a heterozygous hypomorphic RET allele such as the 1476(A) variant should fit at the milder end of this spectrum and might plausibly produce the observed 10% decrease in newborn nephron number.

Mouse studies suggest a graded relationship between kidney size and the level of RET function; Ret knockout mice are anephric, whereas severely hypomorphic Ret alleles such as Tyr1062Phe or RetDN produce severe renal hypoplasia. Thus, only a subtle effect on kidney size would be expected for a RET polymorphism predicted to have only a modest effect on total RET mRNA level; however, the relationship between kidney volume and nephron number is difficult to establish in mice; nephrogenesis continues for at least 2 wk after birth and potentially overlaps with a period of postnatal compensatory...
significant effect on KidVol/BSA (newborns with one or more 1476(A) alleles was 9.2% higher than in homozygous 1476(G/G) babies (those reported for the CAUC and CEU populations in the NCBI dbSNP database. Total renal volume adjusted for body surface area (KidVol/BSA) in newborns

...ternary sites in exon 14. When this occurs, exons containing 1476 of this ESE increases the risk for aberrant splicing to alternative splice site in exon 8. An adenine nucleotide at position 1476 of exon 7, which modifies the fidelity of mRNA splicing to the normal 1476(G/G) newborns (n = 46) was 90.3% of that for infants with the major RET1476(G)/PAX2GGG alleles (n = 41; P = 0.01). Similarly, KidVol/BSA among newborns with one or more hypomorphic PAX2AAA alleles (n = 14) was 89.5% of RET1476(G)/PAX2GGG newborns (P = 0.04). In the subset of newborns bearing both hypomorphic RET1476(A) and PAX2AAA alleles (n = 17), KidVol/BSA was only 77% of that in wild-type RET1476(G)/PAX2GGG infants (P = 0.0067).

Figure 6. Comparison of allele-specific mRNA expression in rs1800860(G/A) heterozygous G401 and SH-SY5Y cells. Peak area ratio of G/A alleles was measured in cDNA and genomic DNA from two cell lines heterozygous for the rs1800860 SNP. The G/A allelic ratio (2.09 ± 0.12) in cDNA was 2.1 times greater than the ratio (1.01 ± 0.02) in genomic DNA from G401 cells (*P = 0.004). Similarly, G/A ratio was 1.4 times greater in cDNA (1.41 ± 0.14) than in genomic DNA (0.98 ± 0.03) for SH-SY5Y cells (**P = 0.03).

hypertrophy. For example, Clarke et al.10 noted that heterozygous Ret(+/-) postnatal day 15 mice had a 22% reduction in nephron number but only a 10% reduction in kidney volume (NS). To establish that newborn kidney size was a valid surrogate for nephron number in humans, we measured glomerular number and kidney weight in newborn infants who died before 3 mo of age. This showed a strong correlation between renal mass and nephron number in the newborn period.

In this study, we identified a common SNP within the ESE of exon 7, which modifies the fidelity of mRNA splicing to the normal splice site in exon 8. An adenine nucleotide at position 1476 of this ESE increases the risk for aberrant splicing to alternative sites in exon 14. When this occurs, exons containing the crucial GFRα1 binding site, transmembrane, and first tyrosine kinase domains all are deleted, undoubtedly rendering the RET receptor dysfunctional. On the basis of our studies in cultured heterozygous human cells, the presence of a 1476A allele reduces wild-type mRNA expression by approximately 38%; in a heterozygous cell, this would amount to an overall reduction of functional RET transcript by 19%. Arguably, branching morphogenesis of the ureteric bud during fetal kidney development might be reduced in proportion to this reduction of RET expression. In our cohort of normal white infants from Montreal, the 1476(A) minor RET allele, infants with one or more 1476(A) alleles exhibited a 9.7% reduction in newborn kidney volume (normalized for body surface area) and 9.2% reduction in newborn renal function. Thus, the degree of renal hypoplasia observed in infants (9 to 10%) with

Table 2. Association between SNP and newborn kidney volume or cord blood cystatin C

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Genotype Frequencies (%)</th>
<th>KidVol/BSA (ml/m²)</th>
<th>Cystatin C (mg/L)</th>
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</thead>
<tbody>
<tr>
<td>rs1800860</td>
<td>GG</td>
<td>46.3</td>
<td>139.4 ± 33.0</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>47.1</td>
<td>126.3 ± 24.7</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>6.6</td>
<td>123.0 ± 22.3</td>
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<td></td>
<td>GA + AA</td>
<td>53.7</td>
<td>125.9 ± 24.3</td>
</tr>
<tr>
<td>rs1799939</td>
<td>GG</td>
<td>56.3</td>
<td>131.3 ± 31.0</td>
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<tr>
<td></td>
<td>GA</td>
<td>40.0</td>
<td>134.1 ± 27.5</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>3.7</td>
<td>132.1 ± 25.3</td>
</tr>
<tr>
<td></td>
<td>GA + AA</td>
<td>43.7</td>
<td>133.9 ± 27.1</td>
</tr>
</tbody>
</table>

Association between RET SNPs and newborn kidney volume or cord blood cystatin C. Normal term Caucasian newborns (136) from Montreal were genotyped for the rs1800860 (1476G/A) and rs1799939 (2251G/A) RET SNPs by sequence analysis of PCR amplicons. Genotype frequencies in our cohort are similar to those reported for the CAUC and CEU populations in the NCBI dbSNP database. Total renal volume adjusted for body surface area (KidVol/BSA) in newborns bearing one or more 1476(A) alleles was 9.7%, less than that of homozgyous 1476(G/G) newborns (P = 0.009). Umbilical cord blood cystatin C concentration in newborns with one or more 1476(A) alleles was 9.2% higher than in homozgyous 1476(G/G) babies (P = 0.002). The presence of a 2251(A) allele had no significant effect on KidVol/BSA (P = 0.60) or umbilical cord cystatin C (P = 0.91) compared with newborns with the homozygous 2251(G/G) genotype.

aP = 0.009.
bP = 0.002.
one or more 1476(A) alleles is roughly commensurate with the reduction in functional RET mRNA expression (19%) measured in vitro.

To put this in a clinical perspective, the report of Keller et al. suggested that adults with essential hypertension are born with 47% fewer nephrons compared with those who remain normotensive. Thus, the effect of the 1476(A) allele could account for only one fifth of this clinically relevant congenital nephron deficit. Clearly, additional genes are involved in setting nephron number during development.

Heterozygous null mutations of RET have been reported in humans with Hirschsprung disease. If arborization of the ureteric bud is proportional to the level of RET expression during renal development, then one might expect that congenital nephron number in Hirschsprung disease should be reduced. There are reports of unilateral renal aplasia and various renal malformations in Hirschsprung disease, but nephron number has not been carefully assessed. By the time patients with Hirschsprung disease are identified, there has been ample time for compensatory renal hypertrophy to erase the initial relationship between kidney size and congenital nephron number. Thus, in the report of Keller et al., describing 50% reduction in nephron number among people with essential hypertension, adult kidney mass was similar to that in normal control subjects. In contrast, we measured renal volume and function at birth before the relationship can be masked by postnatal compensatory hypertrophy. Indeed, our findings from autopsied newborns suggested that kidney weight correlates with congenital nephron number (adjusted for age) for up to 3 mo.

Ureteric bud cells express high levels of PAX2 during renal development; homozygous Pax2 mutant mice are anephric, and heterozygous Pax2 null mutants exhibit significant renal hypoplasia. We recently identified a fairly common (allele frequency 0.2) hypomorphic human PAX2^{AAA} allele, which reduces PAX2 expression by 40% compared with the major wild-type PAX2^{GGG} allele; thus, total PAX2 mRNA in a heterozygous mouse is approximately 80% of normal. Total newborn kidney volume among infants with one or more PAX2^{AAA} alleles was approximately 10% smaller than in PAX2^{GGG}/PAX2^{GGG} infants. Fifteen percent of the newborn cohort in this study were compound PAX2^{AAA}/RET^{1476A} heterozygotes. In this subgroup, newborn KidVol/BSA was 23% smaller than in infants with homozygous wild-type RET^{1476G}/PAX2^{GGG} alleles. This corresponds very nicely with the observations of Clarke et al., who found additive effects of compound heterozygosity for Pax2 and Ret alleles on nephron number in mutant mice. Thus, together, the two hypomorphic RET and PAX2 alleles could account for up to half of the nephron deficit (48% of control) associated with essential hypertension reported by Keller et al. On the basis of HapMap linkage data in the white (CEPH) population, the 1476(A) SNP is not in high genetic linkage disequilibrium with most other portions of the RET gene; however, to rule out the unlikely possibility that some other site within the RET gene is responsible for reduced kidney size, we screened for association with 22 other haplotype-tagging SNPs spanning all major linkage blocks and 10 kb to either side of the coding sequence. No other haplotype-tagging SNPs were significantly associated with either kidney size or cystatin C level (data not shown).

Interestingly, we identified a second RET SNP (rs1799939) that alters a glycine at amino acid position 691 to a serine (RET^{2251G/A}); however, this SNP was not associated with newborn kidney size or cystatin C. Because the G/A substitution lies within a linker region between the RET transmembrane and tyrosine kinase domains, the 2251A isoform may retain sufficient signaling activity to permit normal nephrogenesis. In conclusion, we identified a polymorphic variant (RET^{1476A}) of the human RET receptor that increases the risk for aberrant mRNA splicing and causes decreased expression of the functional wild-type allele. The RET^{1476A} is associated with a reduction (approximately 10%) of newborn kidney volume and an increase (approximately 9%) in umbilical cord cystatin C. Because kidney size and function were assessed at birth, before the period of postnatal hypertrophy, these measurements likely reflect congenital nephron endowment. In mice, homozygous RET mutations block ureteric bud outgrowth, and heterozygous RET null mutations have been shown to interfere with optimal nephrogenesis. Similarly, we previously showed that heterozygous PAX2 mutations interfere with ureteric bud branching and reduce congenital nephron number in mice; a common polymorphic variant of PAX2 causes 10% reduction in human newborn kidney volume. Among the 15% of normal newborns who inherit both a hypomorphic RET^{1476A} and a hypomorphic PAX2^{AAA} allele, kidney volume is reduced by 23% of wild-type controls. Clarke et al. also noted a synergistic effect of Pax2 and Ret mutations on ureteric bud branching and nephrogenesis in embryonic mouse kidney explants. Our observations suggest a model in which common polymorphic variants of genes involved in renal branching morphogenesis account for subtle renal hypoplasia in the normal human population.

**CONCISE METHODS**

**Cell Culture**

Human neuroblastoma cells [SK-N-BE(2) SH-SY5Y] and Wilms’ tumor cells (G401, Wit49) were obtained from ATCC (Manassas, VA). The cells were grown and maintained using standard medium and conditions according to ATCC protocols.

**Reverse Transcriptase–PCR Analysis**

Total RNA was isolated from cells using Qiagen RNeasy Mini-plus Kit with gDNA eliminator column (Qiagen, Mississauga, ON, Canada). Two-step reverse transcriptase–PCR was performed; first-strand cDNA was primed with random hexamers and TaqMan MultiScribe Reverse Transcriptase according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Nested PCR was performed with the cDNA; the PCR primers are listed in Supplemental Table 1.
Study Populations
For analysis of glomerular number, we studied autopsied right kidneys from 15 infants who died of congenital anomalies within the first 3 mo of life at the University of Mississippi Medical Center. Infants with morphologic abnormalities of the kidneys were excluded. These studies were approved by the institutional review board of the University of Mississippi Medical Center. All autopsies were performed and kidney tissues were used with the permission and informed consent of county coroners and next-of-kin.

For association studies, healthy white infants (n = 136) born to women with uncomplicated pregnancies were recruited with informed parental consent at the final prenatal clinical visit to the Royal Victoria Hospital (Montreal, QC, Canada). The study (PED-04-016) was approved by the Montreal Children’s Hospital Research Ethics Board. Mothers with twins, diabetes, intrauterine growth restriction, genetic abnormalities, renal malformations, hydronephrosis, or delivery at <36 wk and newborns with low birth weight (<2500 g) or low serum albumin were excluded.

Estimation of Total Glomerular (Nephron) Number
The right kidney was perfusion-fixed with 10% buffered formalin and then weighed. Kidneys were excluded from the study when the two kidneys in the one subject were unequal in size or showed macroscopic or microscopic evidence of pathology. Kidneys from 15 infants aged ≤3 mo were analyzed. Subject age, gender, race, nephron number, and kidney weight are shown in Supplemental Table 3.

After perfusion, kidneys were immersion-fixed in formalin and sent to Monash University for stereologic analysis of total nephron number (Nglomer) using the physical dissector/fractionator combination. This is an unbiased stereologic counting method with which all glomeruli are sampled and, thereby, counted with equal probability. Important with this method, glomeruli are counted irrespective of their size, shape, and location. Full details of this technique have been previously described in detail.30–32 The association between Nglomer and kidney weight was analyzed using Pearson product moment correlation.

Kidney Volume Measurement
Left and right kidney volumes were measured by ultrasonography in newborns within the first 48 h of life using the formula kidney volume = 4/3π (length/2) (height/2) (width/2). Body surface area was calculated as the square root of [length (cm) × weight (kg)/3600] according to Mosteller.33

Renal Function Determination
Serum cystatin C was used as a surrogate of GFR.34 Cord blood cystatin C was measured by nephelometry (normal newborn range 1.17 to 3.06 ± 0.26 mg/L [SD]).15

Coding SNP Collection
The NCBI dbSNP database was screened for common coding SNP in the human RET gene with minor allele frequency of >10% in white populations. One common nonsynonymous SNP changing an amino acid was identified, and one common synonymous SNP affecting an ESE was found using the RESCU-ESE program (see Appendix).

SNP Genotyping
Genomic DNA was isolated from cord blood with the FlexiGene DNA kit (Qiagen) according to the manufacturer’s protocol. For each infant, 15 ng of genomic DNA was used for multiplex genotyping, using Sequenom iPLEX PCR technology (Sequenom, San Diego, CA). This system involves extension of the PCR amplicon with modified nucleotides to distinguish SNP alleles by matrix-assisted laser desorption ionization–time of flight technology. Primers for SNP detection were designed using MassARRAY AssayDesign software (Sequenom, San Diego, CA).

Quantitative Allele Ratio Analysis
Two heterozygous cell lines (SH-SYSY and G401) were selected for allelic expression analysis. Total RNA and genomic DNA were isolated in triplicate from the cells. PCR and reverse transcriptase–PCR amplicons were sequenced in duplicate as described by Pastinen et al.17 Sequencing primer sequences did not contain any known SNP and were used to amplify each RNA and DNA sample in duplicate (the PCR primers and sequencing primer are summarized in Supplemental Table 1). PeakPicker software (see Appendix) was used for quantitative allele ratio analysis. This program normalizes nucleotide peak amplitude for the effect of surrounding bases, and normalized ratio values were calculated in genomic DNA and mRNA (cDNA).

Statistical Analysis
Data are presented as means ± SD. Deviation from Hardy-Weinberg equilibrium was calculated by the χ² test. Normality of data distributions for kidney volume and serum cystatin C were confirmed by tests of skewness (values of 0.501 and 0.482, respectively) and kurtosis (values of 0.216 and 0.133, respectively). Association between SNP genotypes and KidVol/BSA or cord blood cystatin C was assessed by two-tailed, independent-samples t test. The comparison of rs1800860 G/A allele expression ratios in DNA and RNA was analyzed with two-tailed t test. All data were analyzed with SPSS for Windows 11.0 (SPSS, Chicago, IL) and Microsoft Excel. The association between Nglomer and kidney weight was analyzed using Pearson product moment correlation.

APPENDIX
GenBank Accession Numbers
Human RET gene, NT_033985; human RET mRNA, NM_020630; human RET protein, NP_065681.

Web Sites
ESE finder software: http://rulai.cshl.edu/ESE/
RESCU-ESE program: http://genes.mit.edu/burgelab/rescue-ese/
HapMap CEU data: http://www.hapmap.org
Peakpicker software: http://genomequebec.mcgill.ca/EST-HapMap

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Studies were conducted with informed consent from subjects and the approval of the Montreal Children’s Hospital institutional review board (PED 04-016) and the institutional review board of the University of Mississippi Medical Center.

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DISCLOSURES
None.

REFERENCES

Supplemental information for this article is available online at http://www.jasn.org/