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Targeted sequencing of 96 renal developmental microRNAs in 1213 individuals from 980 families with congenital anomalies of the kidney and urinary tract

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ORIGINAL ARTICLE

ABSTRACT

Background. Congenital anomalies of the kidney and urinary tract (CAKUT) are the most common cause of chronic kidney diseases in children and young adults, accounting for ∼50% of cases. These anomalies represent maldevelopment of the genitourinary system and can be genetically explained in only 10–16% of cases by mutations or by copy number variations in protein coding sequences. Knock-out mouse models, lacking components of the microRNA (miRNA) processing machinery (i.e. *Dicer*, *Drosha*, *Dgcr8*), exhibit kidney malformations resembling human CAKUT.

Methods. Given the *Dicer*-null mouse phenotype, which implicates a central role for miRNAs gene regulation during kidney development, we hypothesized that miRNAs expressed during kidney development may cause CAKUT in humans if mutated. To evaluate this possibility we carried out Next-Generation sequencing of 96 stem-loop regions of 73 renal developmental miRNA genes in 1248 individuals with non-syndromic CAKUT from 980 families.

Results. We sequenced 96 stem-loop regions encoded by 73 miRNA genes that are expressed during kidney development in humans, mice and rats. Overall, we identified in 31/1213 individuals from 26 families with 17 different single nucleotide variants. Two variants did not segregate with the disease and hence were not causative. Thirteen variants were likely benign variants because they occurred in control populations and/or they affected nucleotides of weak evolutionary conservation. Two out of 1213 unrelated individuals had potentially pathogenic variants with unknown biologic relevance affecting miRNAs *MIR19B1* and *MIR99A*.

Conclusions. Our results indicate that mutations affecting mature microRNAs in individuals with CAKUT are rare and thus most likely not a common cause of CAKUT in humans.

Keywords: CAKUT, microRNA, miRNA, stem-loop

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) comprise a heterogeneous group of clinical conditions including unilateral renal agenesis, renal hypodysplasia, ureteropelvic junction obstruction, primary megaureter and vesicoureteral reflux [1]. CAKUT represent the most frequent group of all birth defects recognized in the neonatal period [2], and account for 40–50% of children and young adults on dialysis [3]. CAKUT arise starting at 4 weeks of gestation in humans, when the ureteric bud and the metanephric mesenchyme

begin to develop co-dependently to eventually form the bladder trigone, the ureters and the kidneys. These embryologic events require a tightly regulated gene expression at both the DNA level (mediated by transcription factors) and RNA level, including the microRNA (miRNA) gene silencing machinery. Mutations affecting the required transcription factors, such as HNF1B, PAX2 or TBX18, cause CAKUT in humans and mice [4–7]. However, despite great research efforts, >80% of all CAKUT cases still cannot be explained by mutations in known disease-causing genes that encode proteins. Recently, it was shown that a depletion of miRNAs in different nephrogenic cell lineages in mouse models resembles human CAKUT [8–11].

Following the discovery of miRNAs in the early 1990s, great insight has been gained regarding their biological function [12]. miRNAs are small non-coding RNA molecules of about 22 nucleotides, encoded by >1000 miRNA genes. They function in post-transcriptional regulation of gene expression. miRNAs are transcribed by RNA polymerase II into pri-miRNAs and subsequently processed by several key enzymes to become single-stranded mature miRNAs. Mature miRNAs bind complementary to a multitude of target motifs in mRNAs, which induces degradation of the target. For instance, the mature sequence of miRNA-99a (13-aacccguagauccgaucuugug-34) binds to 131 different validated target genes (http://mirtarbase.mbc.nctu.edu.tw) and thereby recruits the RNA-induced silencing complex (RISC) degrading the target mRNA.

The conditional knock-out of the RISC component Dicer in cells of either nephron lineage (*Six2Cre*) or the ureteric bud-derived collecting duct system (*HoxB7Cre*) leads to early termination of nephrogenesis, cystic hypodysplasia and hydronephrosis [11, 13]. To rule out miRNA-independent effects of Dicer, Bartram *et al.* analyzed the knock-out of two different miRNA processing components (*Drosha* and *Dgcr8*) in tubular epithelial cells in the kidney and developing genitourinary tract (*KspCre*). In line with the Dicer knock-out, the *Drosha*/*Dgcr8* knock-outs lead to a reduced number of nephrons, ureteropelvic junction obstruction, hydronephrosis and early death due to renal failure [8, 9].

It is noteworthy that these mouse models all exploit the Cre/loxP system to delete all miRNAs in nephrogenic target tissues, a tool that does not dissect contributions of individual miRNAs. However, there is a growing body of evidence that somatic mutations affecting single miRNA genes are sufficient to cause disease. For instance, an N-ethyl-N-nitrosourea induced (ENU-induced) point mutation in MIR96 has been shown to cause progressive hearing loss in mice [14] and, recently, it was also reported that a germline deletion of the miRNA-17–92 cluster causes Feingold Syndrome 2 in humans (OMIM #614326) [15]. Consequently, it seems possible that miRNAs, which are specifically expressed in nephrogenic tissues during the development of the genitourinary system, cause CAKUT in humans if mutated. To evaluate this possibility, we investigated in a large number of patients with CAKUT ($n = 1248$) [16] for mutations in 96 stem-loop regions of 73 miRNA genes, that are jointly expressed in nephrogenic tissues in humans, mice and rats, as summarized recently by Saal and Harvey (Supplementary Figure S1 and Supplementary Table S1) [17].

MATERIALS AND METHODS

Human subjects

Following informed consent, we obtained clinical data, blood samples and pedigrees from individuals with CAKUT. Approval for research on humans was obtained from the Boston Children's Hospital and University of Michigan Institutional Review Board. The diagnosis of CAKUT was made by (pediatric) nephrologists or urologists based on standardized clinical and renal ultrasound criteria [18, 19]. Clinical data were obtained using a standardized questionnaire (http://www.renalgenes.org). Mutations in the following 26 genes known to be mutated in isolated CAKUT in humans were excluded by high-throughput multiplex PCR and next generation sequencing prior to this study: *BMP4, BMP7, CDC5L, CHD1L, EYA1, GATA3, HNF1B, PAX2, RET, ROBO2, SALL1, SIX1, SIX2, SIX5, SOX17, UMOD, UPK3A*, *FRAS1*, *FREM2*, *GRIP1*, *FREM1*, *ITGA8*, *GREM1*, *SLIT2*, *SRGAP1* and *TBX18* [6, 16, 18, 20]. *HNF1B* deletions were excluded by quantitative PCR in individuals with the CAKUT phenotype of renal hypodysplasia [18].

Candidate miRNA selection

Candidate miRNAs were selected based on published miRNA expression profiles in nephrogenic tissues as summarized recently by Saal and Harvey [17]. In brief, miRNAs expressed in renal developmental tissues in humans (144 miRNAs), mice (148 miRNAs) and rats (141 miRNAs) were cross-compared for miRNAs expressed in all three species. The selected miRNAs were considered miRNAs with conserved expression in the developing genitourinary system ('kidney miRNA') and included in this study (73 miRNAs) (Supplementary Figure S1 and Supplementary Table S1) [17].

Targeted miRNA sequencing

For 73 'kidney miRNA' genes, comprising 96 miRNA stem-loop regions, 96 target specific primer pairs were designed using PrimerZ software (Supplementary Table S1) [17, 21]. The amplicon size ranged from 190 to 309 bp. Targeted amplification and Next-Generation (NGS) was done as described previously by our group [22, 23]. NGS was carried out using an Illumina MiSeq V2 instrument (2×250 bp paired reads). Seventy-six amplicons (79.2%) had a coverage >100× and eight amplicons (8.3%) were covered <10× (Supplementary Table S1). All identified variants were confirmed by Sanger sequencing in genomic DNA. Segregation analysis was performed if parental DNA was available.

Bioinformatics

NGS data alignment and variant detection was done using CLC Genomics Workbench 4.9 software. Variants were considered if consistent with the following criteria: Variant present in the stem-loop region of a selected renal miRNA, absent from dbSNP132-common database, NGS coverage >10× and PhyloP nucleotide conservation score >2 (Supplementary Table S2). Retained variants were annotated using SeattleSeq Annotation (http://snp.gs.washington.edu/SeattleSeqAnnotation141).

RESULTS

We conducted NGS-based targeted sequencing as previously described [23]: 1213/1248 DNA samples (97.2%) and 88/96 targets (91.7%). Our cohort of 1213 individuals from 980 different families with CAKUT originated from Eastern Europe (43.9%), Western Europe (40.3%), the Middle East (8.4%), South Asia (5.4%), East Asia (0.9%) and Africa (0.7%) (Table 1). There were 651 male (53.7%) and 562 female (46.3%) individuals that passed our quality control (Supplementary Table S1). The most common CAKUT phenotype was vesicoureteral reflux ($n = 503$), followed by ureteropelvic junction obstruction ($n = 276$). A total of 492 individuals were considered as having familial CAKUT according to clinical questionnaires in our cohort. For detailed cohort characteristics see Table 1.

Failed samples ($n = 35$) and failed targets (*MIR10A*, *MIR1241*, *MIR1243*, *MIR196A2*, *MIR200B*, *MIR23A*, *MIR242*, *MIR320C1*) were excluded from this study (Supplementary Table S1). Detected variants were filtered to isolate very rare (MAF <1%) and novel variants altering evolutionary conserved nucleotides located within miRNA stem-loop regions and confirmed by Sanger sequencing (Supplementary Table S2). We decided to only consider variants within miRNA stem-loop regions because stem-loop regions are evolutionarily highly conserved DNA elements and thus of functional relevance, similar to protein coding sequences. In the 1213 samples that passed quality control, we identified 31 individuals with 17 different single nucleotide variants affecting 16 different miRNA genes. We did not find two or more different potentially pathogenic variants in the same miRNA. Two out of 17 variants were found to be potentially pathogenic (Table 2) because they had never been reported in any public variant database and they altered evolutionarily conserved nucleotides (down to *Danio rerio*). Individual A528-21 with right renal agenesis had a C to A substitution in the gene *MIR19B1* that affected the sequence of the mature miRNA 19b (hsa-miR-19b-1-5p) (Table 2). Another individual, A1137-21, with severe vesicoureteral reflux and right kidney ptosis, had a C to T substitution in the gene *MIR99A* that affected the sequence of the mature miRNA 99a (hsa-miR-99a-5p) (Table 2). For both individuals, we could not reestablish contact with their families for segregation analyses. Additionally, we found 13 variants that we consider probably benign, because they were either present in public variant databases at a low frequency (<1%) or they altered weakly conserved nucleotides (Supplementary Table S3). Two additional variants present in four unrelated families did not segregate with the disease and hence could be excluded from being causative (Supplementary Table S3).

Table 1. Demographic and phenotypic composition of 1213 individuals from 980 families with CAKUT

Characteristic	Value	Percentage (%)
Gender		
Female	562	46.3
Male	651	53.7
Family history		
Yes	492	40.6
No	721	59.4
Ethnicity		
Eastern European	533	43.9
Western European	489	40.3
Middle Eastern	102	8.4
South Asian	65	5.4
East Asian	11	0.9
African	8	0.7
Others	5	0.4
CAKUT phenotype		
Vesicoureteral reflux	503	36.8
Ureteropelvic junction obstruction	276	20.2
Hypodysplasia	131	9.6
Duplex system	99	7.3
Unilateral renal agenesis	96	7.0
Multicystic dysplastic kidney	46	3.4
Ureterovesical junction obstruction	45	3.3
Renal ectopia	42	3.1
Posterior urethral valves	41	3.0
Hydronephrosis	39	2.9
Horseshoe	24	1.8
Multiple cysts	23	1.7
2 diagnosis[1]	130	10.7
3 diagnosis[1]	11	0.9
Extrarenal phenotype	167	13.8

[1]Multiple different CAKUT phenotypes were present in the same patient.

DISCUSSION

In summary, we screened a large cohort of 1213 individuals with CAKUT for single nucleotide variants or small insertion/deletions affecting evolutionarily conserved nucleotides within the stem-loop region of 96 miRNAs with conserved expression in the developing genitourinary system. We detected 15 different variants in 14 miRNA genes of most likely benign nature and two novel variants in the genes *MIR19B1* and *MIR99A*, which may be potentially pathogenic (Table 2). The strength of this study is that we could show mere absence of potentially damaging variants in the most promising renal developmental miRNAs from a large CAKUT cohort. In our opinion this indicates that (i) the examined miRNAs are well conserved by evolution and (ii) that point mutations most likely do not play an important role in human CAKUT. As for the two

Table 2. Potentially pathogenic variants detected in 96 miRNA stem-loop regions in 1213 individuals with CAKUT

Gene	Mature miRNA	Genomic variant	*Mm*	*Gg*	*Xt*	*Dr*	EVS	1000G	Family	Individual	State	Origin	Phenotype
MIR19B1	hsa-miR-19b-1-5p	chr13:92003212C>A	C	C	C	C	Unknown	Unknown	A528	−21	het	Macedonia	Rt renal agenesis
MIR99A	hsa-miR-99a-5p	chr21:17911433C>T	C	C	C	C	Unknown	Unknown	A1137	−21	het	Macedonia	VUR, rt kidney ptosis

Genomic variation is stated according to GRCh37/hg19 genomic reference positions.
Mm, *Mus musculus*; *Gg*, *Gallus gallus*; *Xt*, *Xenopus tropicalis*; *Dr*, *Danio rerio*; EVS, exome variant Server; 1000G, 1000 Genomes Project; het, heterozygous; rt, right; VUR, vesicoureteral reflux.

individuals with potentially pathogenic variants in *MIR19B1* or *MIR99A*, we do not have sufficient evidence to claim any causative role of the two variants. This study has several limitations. First, we used a candidate gene approach, which is naturally biased and misses causative mutations in miRNAs that were not included in this study. Certainly, despite our data, we cannot exclude a monogenic CAKUT spectrum disorder caused by mutations in one of the growing number of identified miRNA genes. Second, our genetic approach does not detect copy number variations and large genetic rearrangements and thus such mutations cannot be excluded. In future studies, it seems very reasonable to consider miRNAs in unbiased sequencing approaches (i.e. whole genome or exome plus non-coding RNAs) because in CAKUT there are still a large number of cases that cannot be genetically solved by mutations in coding genes. The past two decades have taught us that human CAKUT is a group of diseases of immense genetic heterogeneity [19]. In human CAKUT, more than 30 known monogenic causes fail to explain 70–80% of cases [6]. The role of environmental risk factors and epigenetics for human CAKUT is poorly understood [24]. To our knowledge, no studies addressing the role of miRNAs in human CAKUT have been published so far. In conclusion, although the depletion of the entirety of all miRNAs in nephrogenic tissues abrogates proper kidney development [8–11], our results suggest that mutations in 96 promising candidate miRNAs do not play a major role in the pathogenesis of human CAKUT.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

None declared.

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