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ORIGINAL ARTICLE

Copy number variation analysis identifies *MIR9-3* and *MIR1299* as novel miRNA candidate genes for CAKUT

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Abstract

Background Congenital anomalies of the kidney and urinary tract (CAKUT) represent a frequent cause of pediatric kidney failure. CNVs, as a major class of genomic variations, can also affect miRNA regions. Common CNV corresponding miRNAs (cCNV-miRNAs) are functional variants regulating crucial processes which could affect urinary system development. Thus, we hypothesize that cCNV-miRNAs are associated with CAKUT occurrence and its expressivity.

Methods The extraction and filtering of common CNVs, identified in control samples deposited in publicly available databases gnomAD v2.1 and dbVar, were coupled with mapping of miRNA sequences using UCSC Genome Browser. After verification of the mapped miRNAs using referent miRBase V22.1, prioritization of cCNV-miRNA candidates has been performed using bioinformatic annotation and literature research. Genotyping of miRNA gene copy numbers for *MIR9-3*, *MIR511*, and *MIR1299*, was conducted on 221 CAKUT patients and 192 controls using TaqMan™ technology.

Results We observed significantly different *MIR9-3* and *MIR1299* gene copy number distribution between CAKUT patients and controls (Chi-square, $P=0.006$ and $P=0.0002$, respectively), while difference of *MIR511* copy number distribution showed nominal significance (Chi-square, $P=0.027$). The counts of less and more than two of *MIR1299* copy numbers were more frequent within CAKUT patients compared to controls ($P=0.01$ and $P=0.008$, respectively) and also in cohort of patients with anomalies of the urinary tract compared to controls ($P=0.016$ and $P=0.003$, respectively).

Conclusions Copy number variations of miRNA genes represent a novel avenue in clarification of the inheritance complexity in CAKUT and provide potential evidence about the association of common genetic variation with CAKUT phenotypes.

Keywords CAKUT · miRNA · CNV · Association study

Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT) encompass a spectrum of structural and functional abnormalities occurring in approximately 1:500 live-born children [1]. In addition to its high incidence, CAKUT is the most frequent cause of pediatric kidney failure [2]. While the exact etiology of CAKUT remains incompletely understood, point mutations and rare copy number variants (rCNVs) currently explain 20 to 25% of CAKUT cases [3, 4] underscoring the substantial role of genetic factors. Additionally, a significant enrichment of microRNA (miRNA) genes in rCNVs associated with CAKUT was observed [5]. Common CNVs (cCNVs), as a major class of genomic variations, are also found to harbor miRNA genes [6]. The regulatory role of miRNAs located in cCNV regions is enriched in processes involved in transduction of external signals, leading to a range of cellular responses such as growth, differentiation, inflammation, and apoptosis [7]. All the mentioned processes are highly involved in CAKUT development as well [8–10]. Changes in miRNA dosage affecting development of mammalian cells and mice have been documented [11, 12]. Recently, the potential functional role of CNV-mediated variation of miRNA dosage proposed miRNAs

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as candidate variants in a genotype-phenotype association study [6]. However, the association of copy number variation of miRNA genes with CAKUT is still unknown.

Based on the aforementioned facts, we hypothesize that miRNA gene copy number variation is associated with CAKUT occurrence and its expressivity. We aimed to conduct a pipeline which employs the extraction of reliable cCNVs from multiple databases and mapping of miRNA sequences onto these regions, to identify miRNA genes with copy number variation (Fig. 1). Subsequently, both literature and bioinformatic sources have been used to prioritize cCNV-miRNAs for the miRNA gene copy number association study regarding CAKUT phenotypes. In such a manner, we have made a valuable contribution by identifying the associations of miRNA gene copy number variation with CAKUT occurrence and phenotypic variability for the first time.

Material and methods

Patients and controls

The genetic association study included a total number of 221 CAKUT patients (144 males/77 females) and 192 controls (72 males/120 females). Count and frequency of different primary phenotypes are presented in Table 1. Diagnosis was based on ultrasonography before and after birth, and confirmed by intravenous urography, radioisotope renography (99mTcDTPA), and MRI. Reflux was diagnosed on voiding cystourethrography (VCUG). The primary nature of obstructive megaureter (OMU) and primary vesicoureteral reflux (VUR) was confirmed through the exclusion of secondary causes, such as neurogenic and non-neurogenic voiding dysfunction, or bladder outlet obstruction. The patients participating in the study were admitted to the University Children's Hospital, Department of Nephrology, Belgrade, Serbia.

Due to the phenotypic diversity of CAKUT phenotypes, the patients were stratified into groups as follows:

- Patients with congenital anomalies of the urinary tract (VUR, ureteropelvic junction obstruction (UPJO),

Table 1 Primary phenotypes of CAKUT cases

Males, *n* (%)	144 (65.2)
Females, *n* (%)	77 (34.8)
CAKUT phenotypes	
Vesicoureteral reflux (VUR), congenital (%)	68 (30.8)
Ureteropelvic junction obstruction (UPJO) (%)	56 (25.3)
Obstructed megaureter (OMU) (%)	28 (12.7)
Megaureter (MU) (%)	7 (3.2)
Posterior urethral valves (PUV) (%)	4 (1.8)
Ureterovesical junction obstruction (UVJO) (%)	1 (0.5)
Renal agenesis (RA) (%)	14 (6.3)
Multicystic dysplastic kidney (MCDK) (%)	2 (0.9)
Ectopic kidney (%)	6 (2.7)
Renal hypoplasia, unilateral (%)	2 (0.9)
Hydronephrosis congenital (%)	10 (4.5)
Renal dysplasia, unilateral (%)	2 (0.9)
CAKUT* (%)	21 (9.4)

Data are presented as count (frequencies, %)

CAKUT, congenital anomalies of the kidney and urinary tract

*Patients with diagnosis of CAKUT whose definitive phenotype is to be obtained through medical follow-up

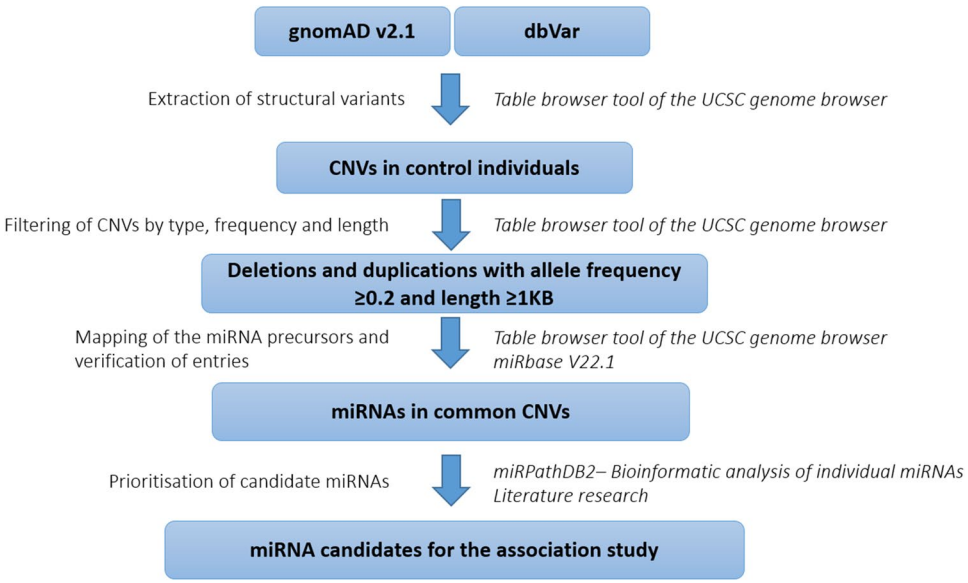

Fig. 1 The pipeline for identification of cCNV-miRNAs and prioritization of candidate miRNAs for the association study

OMU, megaureter (MU), posterior urethral valves (PUV), and ureterovesical junction obstruction (UVJO))
- Patients with obstructive anomalies of the urinary tract (UPJO, OMU, PUV, and UVJO)
- Patients with congenital anomalies of the kidney (renal agenesis (RA), renal hypoplasia, renal dysplasia, congenital hydronephrosis, multicystic dysplastic kidney (MCDK), and ectopic kidney)

Unrelated adult participants (mean age ± SD = 37.1 ± 8.9) undergoing annual medical check-up at the Occupational Medical Center, Vinča Institute of Nuclear Sciences, National Institute of the Republic of Serbia, University of Belgrade, without any congenital anomaly, kidney failure, chronic inflammatory disease, or diabetes mellitus were recruited as controls.

All participants in this study were unrelated Caucasians of Serbian origin. The Ethical University Research Committee approved the study. Blood for isolation of genomic DNA was obtained at the time of clinically indicated venipuncture from all participants. Adult participants and children's parents gave written informed consent.

A funnel pipeline for identification of candidate miRNA genes with copy number variation

To come up with meaningful miRNA gene candidates with copy number variation, we performed a funnel pipeline which took into account all known cCNV regions as a first step. After filtering these regions based on the inclusion criteria (length and allele frequency), we identified high-confidence cCNVs which have been investigated as potential carriers of miRNA genes (Fig. 1). Mapping of miRNA sequences onto filtered cCNV regions indicated which miRNA genes have copy number variation. The CNV itself served simply as a proxy for confirming the existence of miRNA gene copy number variability. To control for potential genetic elements in the extracted CNV regions which could introduce cis effects and thus mask the association of miRNA gene copy numbers with CAKUT, we made an inclusion criterion demanding that miRNA corresponding CNV must not overlap known CAKUT-associated genomic disorder regions and must not contain CAKUT-associated highly penetrant genes. If the miRNA gene appeared on this single locus in the genome, it was an advantage. Besides fulfilling all these criteria, the miRNAs were also reviewed in the most recent miRbase and investigated bioinformatically to identify their regulated pathways.

Access to the gnomAD v2.1 and dbVar databases and miRNA mapping

Identification and extraction of all structural variants and their frequencies, identified in control samples and deposited in publicly available databases—gnomAD v2.1 and dbVar—were conducted using the Table Browser tool of the UCSC genome browser (https://genome.ucsc.edu/). Data were last accessed on 29.12.2020. All the manipulations were performed using Hg19 assembly coordinates.

Table Browser parameters for the extraction of data from the gnomAD v2.1 database included: Mammal, Human, Hg19; Group:Variation; Track: gnomAD SV; Table: gnomAD Controls SV; Output: gnomAD Controls SV. The database consisted of individuals not selected as "case" in a case control study of common disease (5192 samples). In total, 270,000 structural variants were extracted. The data were subsequently filtered to meet the specific parameter criteria (alternative allele frequency for deletion or duplication > 0.2; structural variant length > 1 KB), which resulted in 2026 cCNVs. For our sample size, we have calculated > 80% power to detect at least 2.5-fold variation at the minor allele frequency of 0.2. Additionally, to harmonize the extracted information from the two employed databases of structural variation (gnomAD and dbVAR) for a more comprehensive analysis, we have placed the cutoff to 0.2 due to the categorization of the dbVAR entries as < 0.2, 0.2–0.5, and > 0.5. After application of the inclusion criteria, miRNA genes were mapped to the selected CNVs. miRNA mapping was conducted using the UCSC Table Browser (https://genome.ucsc.edu/). The following parameters were applied: Mammal, Human, Hg19, Group: Genes and Gene predictions; Track: sno/miRNA; Table: wgRNA. In total, 16 miRNA genes were mapped, and information about location and type of CNV is presented in Table 2 and Supplementary File 1.

Table Browser parameters for the extraction of data from the dbVar Common database included Mammal, Human, Hg19; Group:Variation; Track: dbVar Common; Table: Curated European. The data were filtered and only variants that meet the previously described parameter criteria were retained, which resulted in 1454 cCNVs. After miRNA mapping conducted by the same configuration criteria as for the cCNVs extracted from the gnomAD database, a total of 6 miRNAs were identified (Table 2 and Supplementary File 1). Out of these 6 miRNAs, only one miRNA (hsa-miR-3118-5) was not detected in a previous analysis performed on gnomAD Controls SV data.

Selection of the mapped miRNA for the association study

Jointly identified miRNAs employing gnomAD SV and dbVar Common databases were additionally verified using

Table 2 Identified miRNA candidates with copy number variation

miRNA gene	Chr	SV type	SV name	Chrom start	Chrom end	SV freq	Population	Genes affected
hsa-mir-663b	chr2	DUP	DUP_2_6167	132853576	133213563	0.5042	European	ANKRD30BL, GPR39
hsa-mir-1324	chr3	DUP	DUP_3_8930	75678999	75701000	0.7143	European	N/A
hsa-mir-4273*	chr3	DEL	DEL_3_33722	75767999	75796000	0.3593	European	ZNF717
hsa-mir-548i-3*	chr8	DEL	DEL_8_89370	7938549	7984800	0.485	European	N/A
hsa-mir-548i-3	chr8	DEL	DEL_8_89371	7942087	7950583	0.8454	European	N/A
hsa-mir-1302-9	chr9	DUP	DUP_9_26157	27999	39000	0.6735	European	N/A
hsa-mir-1299	*chr9*	*DUP*	DUP_9_27309	*67340080*	*70089613*	0.8917	European	*N/A*
*hsa-mir-511**	*chr10*	*DEL*	DEL_10_108289	*18126899*	*18135000*	0.386	European	*MRC1*
hsa-mir-3172*	chr14	DEL	DEL_14_141125	32953303	32954345	0.3457	European	AKAP6 (intronic)
hsa-mir-3179-1	chr16	DUP	DUP_16_41455	14988999	15125000	0.8399	European	NPIPA1, NOMO1, PDXDC1
hsa-mir-3180-1	chr16	DUP	DUP_16_41527	16397999	16404000	0.6797	European	N/A
hsa-mir-1972-1	chr16	DUP	DUP_16_41455	14988999	15125000	0.8399	European	NPIPA1, NOMO1, PDXDC1
hsa-mir-3180-2	chr16	DUP	DUP_16_41527	16397999	16404000	0.6797	European	N/A
hsa-mir-3180-3*	chr16	DEL	DEL_16_152903	18462999	18502000	0.2513	European	RP11-1212A22.4
hsa-mir-3180-3	chr16	DUP	DUP_16_41637	18495999	18502000	0.7167	European	N/A
hsa-mir-1302-11	chr19	DUP	DUP_19_46400	59999	72000	0.628	European	N/A
hsa-mir-3118-5*	chr21	DEL	DEL_21_178683	15012799	15034700	0.4045	European	POTED

Italicized miRNAs are selected for the case-control association study

SV type, type of structural variation (deletion/duplication); *SV name*, gnomAD v2.1 accession; *Chrom start*, starting position of the cCNV; *Chrom end*, ending position of the cCNV; coordinates are extracted from genome assembly hg19; *Genes affected*, genes affected by the corresponding cCNV

*miRNA genes identified in both gnomAD and dbVar databases

miRbase V22.1 (http://www.mirbase.org/) (Supplementary File 1). The verification established that hsa-miR-3172 is actually a "dead input" because it represents a fragment of transport RNA. We identified two *MIR511* genes (*MIR511-1* and *MIR511-2*) located in two CNV regions. In the latest miRbase database, these two miRNAs map to a single locus and the annotation was corrected to *MIR511*.

Furthermore, for the bioinformatic interpretation of individual miRNAs, we used the more dominant mature miRNA strand to reduce the noise during bioinformatic interpretation. Using the integrative algorithm miRPathDB2 (https://mpd.bioinf.uni-sb.de/), pathways from multiple databases (KEGG, Gene Ontology, Reactome, and Wiki Pathways) have been extracted as those containing significantly more targets of certain miRNA than expected by chance (Supplementary File 2). The enriched pathways were reviewed to confirm that the candidate miRNAs could be involved in pathways associated with CAKUT (such as development of urogenital system, morphogenesis, development of muscle, neurodifferentiation). The additional inclusion criteria demanded that miRNA corresponding proxy CNV must not overlap known CAKUT-associated genomic disorder regions and must not contain CAKUT-associated genes. If the candidate miRNA appears on one genomic locus, this was considered an advantage due to the higher dose dependence.

Detection of the candidate miRNA copy number variation and data analysis

DNA extraction and copy number quantification

Genomic DNA (gDNA) of all samples was extracted from 3 mL peripheral blood collected with EDTA, by standard proteinase K/phenol extraction method. The quality and quantity of extracted gDNA were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Verifying the exact gDNA input of 5 ng/μL for the copy number quantitation, an experiment was done using Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

Quantification of miRNA gene copy numbers was performed on the Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The genotyping was done using TaqMan™ Copy Number Assays (Thermo Fisher Scientific, Waltham, MA, USA) for the three selected miRNAs: *MIR9-3* Assay ID: Hs05330221_cn, *MIR511* Assay ID: NC_511_CD2W9JZ and *MIR1299* Assay ID: NC_9.14_CD7DRAN). The custom TaqMan assays have been designed in proximity to the *MIR511* and *MIR1299* genes of up to 2 KB, to ensure the maximum efficiency of the assays and to exclude potential interference due to the

SNP-rich regions. Simultaneously, a copy number reference assay (TaqMan™ Copy Number Reference Assay, human, RNase P, Cat.no.: 4403328) was included in a duplex real-time polymerase chain reaction (PCR) performed in technical triplicates.

Relative quantification analysis of gDNA target regions using the Real-Time PCR data from pre-designed TaqMan® Copy Number Assay (Hs05330221_cn) and Custom TaqMan® Copy Number Assays (NC_511_CD2W9JZ and NC_9.14_CD7DRAN) was performed by framing the parameters: Manual Ct Threshold = 0.2, Automatic Baseline = ON, with included conservative method for omitting the outliers. Dataset containing Ct values for Copy Number Assays and Reference Assay for each sample was subsequently imported into CopyCaller® Software (CopyCaller® Software v2.1, Applied Biosystem, Foster City, CA, USA) for post-PCR data analysis of the copy number quantitation experiment.

The copy number analysis was performed using the no-calibrator algorithm (expected most frequent sample copy number = 2) per individual plate. Copy number calculated genotypes, copy number predicted genotypes, confidence, and *Z*-score were extracted for further statistical analysis using Statistica 8.0 software package (StatSoft, Inc. 2007, Tulsa, OK, USA). Samples with calculated candidate miRNA gene copy numbers were divided into three groups: low copy number (< 2 gene copies), 2 gene copies, and high copy number (> 2 gene copies).

Statistical analysis

The statistical analysis of all data was performed using the Statistica 8.0 software package (StatSoft, Inc. 2007, Tulsa, OK, USA). Differences in copy number genotype frequency distributions between controls and patients and between subgroups of patients were assessed by Chi-square test. As a measure of strength of association between the studied copy number frequency and CAKUT, the odds ratio (OR) was used with its 95% confidence interval (CI). To reduce the chance of obtaining false-positive results (type I errors), the Bonferroni correction was used, and results were considered statistically significant if $P \leq 0.016$.

Results

Candidate miRNA genes for association analysis of copy number variation with CAKUT

Based on the aforementioned inclusion criteria involving cCNV miRNA mapping and bioinformatical analysis (Supplementary Files 1 and 2), we have selected *MIR1299* and *MIR511* as miRNA genes with copy number variations which have been associated with pathways involved in CAKUT (Supplementary File 2). A significant body of literature demonstrates potent molecular activity of the miR-1299 [13–16] and miR-511 [17, 18], thus making these miRNAs reliable candidates for the association study in CAKUT. In addition, we have intentionally selected one known copy number candidate, *MIR9*-3 which has not been identified in cCNVs defined by our stringent criteria. However, it was associated with relevant biological processes in CAKUT bioinformatically while literature data supported its previous investigation in copy number association studies [19, 20].

Copy number variation of candidate miRNA genes in patients with CAKUT and controls

MIR9-3 copy number variation showed significant association with CAKUT ($P = 0.006$) (Table 3). High copy number (> 2 gene copies) was detected only in patients. Consequently, we could not calculate an odds ratio for high copy

Table 3 cCNV-miRNA copy number frequencies in CAKUT patients and controls

Genes	CN	Controls (%)	CAKUT (%)	P^a	OR (95% CI)	P^b
MIR9-3	< 2	11 (5.8)	4 (2.2)	**0.006**	0.37 (0.11–1.18)	0.91
	= 2	179 (94.2)	170 (93.9)		0.94 (0.39–2.26)	0.90
	> 2	0 (0)	7 (3.9)		N/A	N/A
MIR511	< 2	4 (2.6)	12 (7.5)	0.027	3.08 (0.96–9.81)	0.056
	= 2	141 (90.4)	144 (90)		0.96 (0.45–2.02)	0.91
	> 2	11 (7)	4 (2.5)		0.33 (0.10–1.09)	0.07
MIR1299	< 2	5 (3.3)	21 (11.1)	**0.0002**	**3.72 (1.36–10.15)**	**0.01**
	= 2	145 (94.8)	149 (79.3)		**0.21 (0.09–0.46)**	**0.0001**
	> 2	3 (1.9)	18 (9.6)		**5.29 (1.52–18.40)**	**0.008**

Data are presented as count (frequency, %). Bonferroni correction was applied and *P* values were considered significant if the value was less than 0.05/3 = 0.016

CAKUT, congenital anomalies of the kidney and urinary tract; *CN*, miRNA gene copy number; *OR*, odds ratio; *CI*, confidence interval; P^a, Chi-square test; P^b, logistic regression

number association between controls and patients. Logistic regression analysis showed no association of low *MIR9-3* copy number (< 2 gene copies) with CAKUT, $P^b = 0.91$ (Table 3).

MIR511 copy number variation showed significant association with CAKUT ($P = 0.027$); however, after the Bonferroni correction, the association was not considered significant (Table 3). Logit models showed no statistically significant association of *MIR511* with high copy number (OR = 0.33; 95% CI = 0.10–1.09; $P = 0.07$) and low copy number (OR = 3.08; 95% CI = 0.96–9.81; $P = 0.056$) with the risk of CAKUT occurrence.

MIR1299 copy number variation showed significant association with CAKUT ($P = 0.0002$) (Table 3). *MIR1299* copy number variation was associated with an increased risk of CAKUT, with OR = 3.72 (95% CI = 1.36–10.15; $P = 0.01$) and OR = 5.29 (95% CI = 1.52–18.40; $P = 0.008$) for low and high copy number, respectively (Table 3). For these two analyses, we obtained a study power of 63.9% and 68.8%, respectively, for the *P* value cutoff 0.016.

In the case control study which included subgroup of patients with congenital anomalies of the urinary tract (consisted of UPJO, OMU, MU, VUR, PUV, and UVJO), in comparison to the controls, the association remained significant for *MIR9-3* ($P = 0.002$) and *MIR1299* ($P = 0.0001$) using Chi-square test (Table 4). In the logistic regression analysis, a statistically significant association is only found for *MIR1299* copy number variations. Logit models showed an increased risk for the occurrence of CAKUT, with OR = 3.58 (95% CI = 1.26–10.17; $P = 0.016$) and OR = 6.50 (95% CI = 1.84–22.96; $P = 0.003$) for low and high copy numbers of the *MIR1299* gene, respectively (Table 4). For these two analyses, we have achieved study power of 55.6% and 79.8%, respectively, for the *P* value cutoff 0.016.

miRNA gene copy number variation in patients with CAKUT with regard to phenotype

Considering the phenotype stratification of CAKUT patients, we analyzed congenital anomalies of the kidney versus congenital anomalies of the urinary tract and we did not find significant association with miRNA gene copy number variation (Table 5). In a separate association analysis, performed between patients with obstructive CAKUT and patients with congenital kidney anomalies compared to VUR patients, we found no statistically significant difference in the copy number frequencies of the candidate miRNA genes (Table 6). Statistical trends were observed in the variation of *MIR1299* copy number between obstructive CAKUT and VUR, with low copy number being more abundant in VUR (16.4% vs. 5.4%, $P^b = 0.05$) and referent 2 copies being less frequent in VUR (70.5% vs. 85.1%, $P^b = 0.04$) as presented in Table 6.

Discussion

We have employed a pipeline aiming to identify and validate miRNAs with common copy number variation, potentially associated with CAKUT by using existing genomic data and bioinformatical analysis. For validation purposes, a case-control association study of the three miRNA genes (*MIR9-3*, *MIR511*, and *MIR1299*) was performed regarding the number of miRNA gene copies in CAKUT patients and controls from a Serbian population. We showed a significant association of *MIR9-3* and *MIR1299* gene copy numbers with CAKUT.

Previously, rare CNVs were identified as a common CAKUT risk factor in multiple high-throughput screening studies [4, 21, 22]. In addition, the significant enrichment of miRNA genes in rare CNVs associated with CAKUT was

Table 4 cCNV-miRNA copy number frequencies in patients with congenital anomalies of the urinary tract and controls

Genes	CN	Controls (%)	Congenital anomalies of the urinary tract (%)	P^a	OR (95% CI)	P^b
MIR9-3	< 2	11 (5.8)	3 (2.3)	**0.002**	0.38 (0.10–1.40)	0.14
	= 2	179(94.2)	121(92.4)		0.74 (0.30–1.81)	0.51
	> 2	0 (0)	7 (5.3)		N/A	N/A
MIR511	< 2	4 (2.6)	8 (6.8)	0.03	2.76 (0.81–9.46)	0.10
	= 2	141 (90.4)	108 (91.5)		1.15 (0.49–2.67)	0.74
	> 2	11 (7)	2 (1.7)		0.23 (0.05–1.05)	0.06
MIR1299	< 2	5 (3.3)	15 (10.8)	**0.0001**	**3.58 (1.26–10.17)**	**0.016**
	= 2	145 (94.8)	108 (77.7)		**0.19 (0.08–0.44)**	**0.0001**
	> 2	3 (1.9)	16 (11.5)		**6.50 (1.84–22.96)**	**0.003**

Data are presented as count (frequency, %). Congenital anomalies of the urinary tract: UPJO, OMU, MU, VUR, PUV, and UVJO. Bonferroni correction was applied and *P* values were considered significant if the value was less than 0.05/3 = 0.016

CN, miRNA gene copy number; *OR*, odds ratio; *CI*, confidence interval; P^a, Chi-square test; P^b, logistic regression

Table 5 cCNV-miRNA copy number frequencies regarding major CAKUT cohorts

Genes	CN	Congenital anomalies of the kidney (%)	Congenital anomalies of the urinary tract (%)	P^a	OR (95% CI)	P^b
MIR9-3	< 2	0 (0)	3 (2.3)	0.27	N/A	N/A
	= 2	32 (100.0)	121 (92.4)		N/A	N/A
	> 2	0 (0)	7 (5.3)		N/A	N/A
MIR511	< 2	3 (12.0)	8 (6.8)	0.14	1.88 (0.46–7.72)	0.38
	= 2	20 (80.0)	108 (91.5)		0.37 (0.11–1.21)	0.10
	> 2	2 (8.0)	2 (1.7)		5.04 (0.66–38.31)	0.11
MIR1299	< 2	5 (15.2)	15 (10.8)	0.55	1.48 (0.49–4.43)	0.48
	= 2	26 (78.8)	108 (77.7)		1.07 (0.42–2.71)	0.89
	> 2	2 (6.0)	16 (11.5)		0.49 (0.11–2.30)	0.37

Data are presented as count (frequency, %). Congenital anomalies of the kidney: RA, renal hypoplasia, renal dysplasia, congenital hydronephrosis, MCDK, and ectopic kidney. Congenital anomalies of the urinary tract: UPJO, OMU, MU, VUR, PUV, and UVJO. Bonferroni correction was applied and *P* values were considered significant if the value was less than 0.05/3 = 0.016

CN, miRNA gene copy number; *OR*, odds ratio; *CI*, confidence interval; P^a, Chi-square test; P^b, logistic regression

Table 6 cCNV-miRNA copy number frequencies in CAKUT patients regarding phenotype groups

Genes	CN	Obstructive CAKUT (%)	Congenital anomalies of the kidney (%)	VUR (%)	P^a	OR (95% CI)	P^b	P^c	OR (95% CI)	P^d
MIR9-3	< 2	2 (3.1)	0(0)	1 (1.6)		0.71 (0.21–2.41)	0.57		N/A	N/A
	= 2	58 (89.2)	32 (100)	61 (95.3)	0.43	1.56 (0.77–3.18)	0.21	0.46	N/A	N/A
	> 2	5 (7.7)	0 (0)	2 (3.1)		0.62 (0.27–1.45)	0.26		N/A	N/A
MIR511	< 2	3(4.5)	3 (12.0)	5 (10.42)		1.57 (0.74–3.33)	0.22		0.85 (0.18–4.00)	0.84
	= 2	63 (94.0)	20 (80.0)	42 (87.5)	0.45	0.67 (0.34–1.30)	0.23	0.46	1.75 (0.47–6.57)	0.39
	> 2	1 (1.5)	2 (8.0)	1 (2.08)		1.18 (0.29–4.87)	0.81		0.24 (0.02–2.96)	0.26
MIR1299	< 2	4 (5.4)	5 (15.2)	10 (16.4)		15.8 (1.00–3.42)	0.05		1.10 (0.34–3.59)	0.88
	= 2	63 (85.1)	26 (78.8)	43 (70.5)	0.07	0.64 (0.42–0.99)	0.04	0.54	0.64 (0.23–1.77)	0.39
	> 2	7 (9.5)	2 (6.0)	8 (13.1)		1.20 (0.70–2.07)	0.50		2.33 (0.46–11.98)	0.30

Data are presented as count (frequency, %). Obstructive CAKUT: UPJO, OMU, PUV, and UVJO. Congenital anomalies of the kidney: RA, renal hypoplasia, renal dysplasia, congenital hydronephrosis, MCDK. and ectopic kidney. Bonferroni correction was applied and *P* values were considered significant if the value was less than 0.05/3 = 0.016

CN, miRNA gene copy number; *OR*, odds ratio; *CI*, confidence interval; P^a, Chi-square test (VUR vs. obstructive CAKUT); P^b, logistic regression for VUR vs. obstructive CAKUT; P^c, Chi-square test (VUR vs. congenital anomalies of the kidney); P^d, logistic regression for VUR vs. congenital anomalies of the kidney; *VUR*, vesicoureteral reflux

observed, while subsequent miRNA gene loss effect on target gene expression was confirmed [5]. Previously, changes in miRNA dosage that could influence the development in mammalian cells and mice have been documented [11, 12]. Recently, it was proposed that CNV-mediated variation of miRNA gene dosage should be considered as high-priority candidate variants in genotype-phenotype association studies [6]. miRNA dosage sensitive modulation of gene expression was also suggested in plants implying the conserved mechanism of miRNA copy number variation effect [23]. We have demonstrated in vitro that heterozygous deletion of the *MIR484* leads to upregulation of *MDM2*, *APAF1*, and downregulation of *NOTCH3*, genes that could have a substantial role in CAKUT development [5]. However, despite the discovery of the potential of rare CNVs to dysregulate miRNA genes, and the proposal of CNV-located miRNAs being potentially functional variants [6], the copy number association study of miRNA genes located in common CNVs was not performed in CAKUT.

MIR9 expression is associated with acute kidney injury (AKI) [24]. Zhu and colleagues found increased expression level of miR-9 in kidney tissues of glycerol-induced AKI in a murine model, while the inhibition of miR-9 exhibited renoprotective effects affecting the reduction of inflammatory response, oxidative stress, and vascular endothelial growth factor [24]. This is in line with our findings describing the enrichment of *MIR9-3* low copy number among controls while a high copy number characterized CAKUT patients.

In addition, a high copy number of *MIR9-3* which could increase miR-9 levels, associated with AKI, was not identified in controls. However, in another study, the miR-9-5p, a mature miRNA being developed from the *MIR9-3* gene, is shown to protect from kidney fibrosis in a mouse model of unilateral ureteral obstruction by reprogramming of the metabolic derangement and mitochondrial dysfunction affecting tubular epithelial cells [25]. It was also hypothesized that miR-9-5p could affect development of cysts in autosomal dominant polycystic kidney disease as an antifibrotic factor [26]. There is a need for further analysis to make a final resolution of *MIR9* connection with kidney and urinary tract defects. Low copy number of *MIR9* was previously associated with the susceptibility for acute anterior uveitis, which is also an inflammatory disease, and in the same study the authors proved that miRNA expression is directly affected by its gene dosage [20]. Our study lacks analysis of the *MIR9-3* expression level regarding the gene copy number, which represents one of the study limitations. Whether the association of *MIR9-3* gene copy number observed in our study expresses the effect through the gene dosage or it is mediated through another mechanism remains to be explored. Exploration of this mechanism especially concerns the *MIR9-3* gene which, unlike other tested miRNA genes in our study, belongs to a family of three independent precursor genes which encode the same mature miR-9 [27].

Although bioinformatical analysis has linked *MIR511* with CAKUT-associated pathways, there is a lack of experimental evidence of *MIR511* involvement in CAKUT currently. Previous studies have demonstrated that the upregulation of miR-511 reduced the p-PI3K and p-AKT protein levels in HCT116 and SW480 cells [17]. Increased activation of the AKT pathway is demonstrated in the formation of cysts in multicystic renal dysplasia [28]. Our findings regarding *MIR511* copy number are in accordance with described miRNA dosage effect [17], as we have found higher frequency of copy number losses and a lower frequency of copy number gains in CAKUT compared to controls. However, due to a lack of statistical significance, and a limited number of samples in our study, future studies should follow to exclude possibilities of the type II error.

We have identified an increased frequency of both high copy number and low copy number of the *MIR1299* gene in CAKUT patients. It is not unusual that both up- and downregulation of certain genes could lead to the same pathology, which was already described in the case of the *PKD1* gene where both upregulation and downregulation lead to polycystic kidney disease in animal models [29–32]. This suggests the importance of molecular homeostasis in the urinary system. miR-1299 has been mainly investigated in cancers, regulating cell proliferation, migration, and invasion [33]. In various malignancies, miR-1299 was proven to be a negative regulator of genes such as *NOTCH3* in ovarian cancer [34] or *MMP*s [35] and *CCND1* [36] in breast cancer. These genes have also been associated with CAKUT [5, 10, 37]. As a tumor suppressor in many tumor tissues [13–15] miR-1299 demonstrates a complex effect on a number of downstream targets, including EGFR/PI3K/AKT signaling pathway [14, 16]. Additionally, NF-kappa B signaling pathway, downstream of PI3K/Akt [38], was among the top enriched pathways in the union of miR-1299 and miR-9 targets according to DIANA-miRPath v3.0 tool. NF-kappa B signaling is a focal mediator of inflammation and involved in the pathogenesis of kidney inflammatory diseases [39]. We have identified a total of 13 genes (*NFKB1*, *TNFSF13B*, *ATM*, *MYD88*, *CCL19*, *PTGS2*, *CFLAR*, *ERC1*, *BIRC3*, *RELA*, *MAP3K7*, *XIAP*, and *BIRC2*) complementary regulated by miR-1299 and miR-9 in NF-kappa B pathway of which *ATM* and *MAP3K7* were common targets of both miRNAs.

CAKUT represents an umbrella term of phenotypes in which both kidneys and the urinary tract could be affected [40]. However, it has been shown that differences in phenotype inclusion can influence rare CNV detection [21, 40, 41]. For *MIR1299*, association analysis between controls and patients with congenital anomalies of the urinary tract was with increased study power (79.8% for gain of copy number) in comparison to the association analysis between controls and the entire CAKUT group (68.3% for gain of copy number). We have identified that there are differences in the frequency of the low copy number of *MIR1299* gene between subgroups of patients, specifically between patients with obstructive CAKUT and VUR. Although this association ($P = 0.05$) was not considered significant, according to our stringent criteria, the sample size could be the limiting factor, which ought to be addressed in further studies.

It should be noted that low-level mosaicism may develop from somatic mutations in healthy tissues with high turnover rates such as blood [42], which must be considered a limitation of this study due to the age difference between patients and controls. This could potentially lead to overestimation of miRNA gene copy number frequencies and thus introduce bias in obtained results. On the other hand, the investigated miRNA gene copy numbers followed the inherited (germline) CNVs which are shown to be more reliable and a better biomarker than somatic CNVs which could differ at the single-cell level and could be variable in response to external stimuli [43]. However, in this work, the robust confirmation of "age when sampled" does not impact the frequency of common CNVs, which was not performed. Regarding these shortcomings, future studies should aim to recruit age-matched children without CAKUT to clarify this major limitation. Ideally, genotyping of miRNA gene copy number variation of the parents of CAKUT patients should be also performed to improve the quality of CNVs [44], which should be considered in future studies.

Although it would be premature to predict the contribution of miRNA gene dosage to a complex disease such as CAKUT, the results presented here open an additional avenue in the current genetic diagnostic approaches, which at the moment have the potential to genetically resolve just 20% of CAKUT patients [45]. Significantly different miRNA expression levels have been found in tissue from patients with CAKUT compared to healthy control tissue, and the potential role of CNVs in their expression changes has been suggested previously [46]. Thus, common copy number variation affecting miRNA genes should be considered in the future when untangling the complex CAKUT inheritance. Further studies should in addition provide evidence about *MIR9*-3, *MIR511*, and *MIR1299* miRNA expression levels in tissues and cell models and their functional effect on CAKUT.

CNVs have gained interest over the past few years due to their potency to affect multiple genomic elements. In addition to their influence on gene expression and phenotypic variations, CNVs also play a significant role in shaping genetic diversity within populations [47]. A more profound analysis of gene expression patterns and the comprehensive functional exploration of CNV-located miRNAs will contribute to the expansion of our current knowledge regarding the molecular underpinnings of the diverse range of CAKUT phenotypes. While the whole-genome CNV analysis offers unprecedented datasets, the analysis of individual miRNA gene copy number variations represents a novel source for elucidating their precise role and understanding functional significance. In this study, we have performed an integrated prioritization involving data mining and bioinformatics for focused selection of cCNV miRNAs, as evaluated in the study. Future studies should consider the additional cCNV miRNAs identified in this study to further evaluate their association with CAKUT.

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Author contribution Ivan Zivotic: methodology, data analysis, writing–original draft and editing. Ivana Kolic: experimental procedures, formal analysis, editing of the manuscript. Mirjana Cvetkovic: sampling, clinical consultation, manuscript review. Brankica Spasojevic-Dimitrijeva: sampling, clinical consultation, manuscript review. Maja Zivkovic: conceptualization, writing–review and editing. Aleksandra Stankovic: conceptualization, writing–review and editing. Ivan Jovanovic: conceptualization, writing–review and editing, supervision, project administration.

Data availability All data generated and analyzed during this study are included in this published article.

Declarations

Ethics approval The Ethical Research Committee of University Children's Hospital in Belgrade approved the study. Adult participants and children's parents gave written informed consent.

Conflict of interest The authors declare no competing interests.

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