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Expression profiling of the AT2R mRNA in affected tissue from children with CAKUT

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Abstract

Objectives: Congenital anomalies of the kidney and urinary tract (CAKUT) are common causes of chronic renal failure in children. The angiotensin II receptor type 2 (AT2R) is one of proposed candidate genes for CAKUT, but the expression was never explored in humans. The aim was to establish the AT2R gene expression in human CAKUT concerning -1332A/G polymorphism, which might affect alternative splicing.

Design and methods: Forty-eight patients with CAKUT constitute the basis of this study. Genotyping for -1332A/G, RT-PCR for AT2R gene expression and confirmation sequencing were performed.

Results: The expression of Ex 1/2/3 and Ex 1/3 transcript splice variants of the AT2R mRNA were detected in human CAKUT tissue. The pattern was observed independently of A to G transition.

Conclusions: The expression of AT2R mRNA in human CAKUT was established for the first time and was not affected by -1332A/G polymorphism in children with CAKUT.
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Keywords: AT2R; Gene; mRNA; Expression; Polymorphism; CAKUT

Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT) are common causes of chronic renal failure in infants and young children. There is emerging evidence of how mutations alter gene expression during development to cause some of these disorders [1]. The molecular basis of CAKUT has not been conclusively explained. The most common theory suggests that angiotensin II receptor type 2 (AT2R) is essential for renal development. A spectrum of CAKUT malformation was detected in knockout animal experiments targeting AT2R gene [2]. The list of potential genetic causes of CAKUT based on knockout animal experiments including BMP4 [3], Foxc [4], L1 [5], Upk III [6], Pax2 [7], ROBO2 [8] is growing. The stages of renal development are tightly regulated, so to achieve a subtle coordination between proliferation, differentiation and apoptotic processes. The AT2R, abundantly expressed in embryonic stages, but scantily in adult tissues [9], plays a key role in these processes [10]. AT2R could be re-expressed under some pathophysiological conditions [11]. The molecular mechanisms underlying the regulation of AT2R expression are still waiting to be completely resolved.

The human AT2R gene consists of two short noncoding exons, two introns and exon 3, which contains the complete protein coding region [12]. The AT2R transcripts with exon composition 1/2/3 and 1/3 have been observed in human heart and human failing heart [13, 14]. If intronic A/G polymorphism in AT2R at position −1332 [2]/+1675 [15] might affect alternative splicing of human AT2R gene [2], or not [16] is not clear. The expression of AT2R gene was never explored in human tissue affected with CAKUT. The aim of the present

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work was establishing the presence and alternative splicing profile of AT2R mRNA in human tissue affected by congenital anomaly of urinary tract in regard to −1332 A/G AT2R polymorphism.

Methods

Patients

Forty-eight patients with diagnosis of CAKUT, who were previously selected for ureter corrective surgery, constitute the basis of this study. Diagnosis was based on ultrasonography before and after birth, and confirmed by IVU and radioisotope renography (^{99m}Tc-DTPA). Reflux is diagnosed on voiding cysto-urethrography (VCUG). They are classified into three groups: ureteropelvic junction (UPJ) obstruction, vesicoureteric junction (VUJ) obstruction or primary megaureter (MU) and primary vesicoureteral reflux (VUR). The primary nature of MU and VUR was confirmed through the exclusion of secondary causes such as neurogenic and non-neurogenic voiding dysfunction, or bladder outlet obstruction (e.g., posterior urethral valves). The patients' age range was 0.1–6 years.

Patients were admitted to the wards, participating in the study, from January 2002 to December 2004, in Urology and Nephrology Departments of University Children's Hospital, Belgrade, Serbia and Montenegro.

Blood samples from all participants were collected for isolation of genomic DNA. Genotyping of AT2R gene polymorphism -1332A/G, which was performed in order to define the genotype for further investigation of AT2R expression pattern in CAKUT patients with different genotypes.

A control adult uterine myometrium tissue ($n=10$) was used as a positive control tissue, since it is known as an adult tissue that expresses AT2R mRNA [17]. The myometrium specimens were obtained during the year 2003. from non-pregnant, premenopausal women who underwent a total hysterectomy for gynecological reasons at the Gynecology and Obstetrics Clinic, Clinical Center of Serbia, Belgrade, Serbia and Montenegro. Parts of human myometrium tissues were histologically confirmed to be normal. All participants were Caucasians, Serbian origin. The Ethical University Research Comity approved the study. Adult participants and children's parents gave written informed consent. The ACE inhibitors, AT1R blockers or any other drugs were not used in time of operation.

Procurement of tissue specimens

The ureter segment specimens and myometrium tissue samples were obtained with minimal manipulation of the sample immediately after operations, which were performed using standard surgical techniques. All tissue samples were snap-frozen in liquid nitrogen and stored at −70°C until isolation of RNA. The same collection, handling and processing protocol was used for both ureter and myometrium samples.

Determination of AT2R -1332A/G genotypes

Genomic DNA was isolated from whole blood samples collected with EDTA and purified by the proteinase K/phenol extraction method [18]. Genotyping of -1332A/G polymorphism was performed by sense AT2RS 5′-GGA AGG TAG AAC ATA CAT TAA ATG-3′ and antisense AT2RAS 5′-AGA GAA ACA GCA GCT AAA GAA TT-3′ primers and *Eco*RI restriction endonuclease (Fermentas, Lithuania) as previously described [19].

Gene expression

Reverse-transcription polymerase chain reaction (RT-PCR) was performed to determine the expression pattern of AT2R mRNA in human tissue with CAKUT of both, AA/A- and GG/G- genotypes. The intact tissue was homogenized and total RNA was extracted by Trizol reagent (Life Technologies) according to the manufacturer's instructions. Structural integrity of RNA was confirmed by formaldehyde gel electrophoresis. One microgram of RNA was treated with DNAse I (Fermentas, Lithuania) and reverse transcription was performed using First strand cDNA synthesis kit with oligo-dT18 primers (Fermentas, Lithuania) according to manufacturer's instructions. Mock reactions lacking RT were performed during the cDNA synthesis step in order to exclude genomic contamination. An aliquot of 1/10 of RT reaction was used to amplify AT2R gene in a 20 μL reaction. The specific cDNA sequence of two alternatively spliced AT2R mRNA transcripts was amplified by standard PCR (9700, Applied Biosystems) with denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 2 min for 40 cycles using sense (AT2R exon 1) primer: 5′ ATT CAA AgC ATT CTg CAg CC 3′ and antisense (AT2R exon 3) primer: 5′ TgC CAg AgA TgT TCA CAA 3′ [2]. The expected size of PCR products was 306 bp for the splice variant Ex 1/3 and 366 bp for the variant Ex 1/2/3. The 324 bp long transcript of the GAPDH gene (sense primer 5′ TCg Tgg AAg gAC TCA Tg 3′ and antisense primer 5′ AgT gTA gCC CAg gAT gCC 3′) [20] served as a control for equal RNA input and integrity. PCR products were separated on a 2% agarose gels and visualized by ethidiumbromide staining. Exactly the same protocol for RNA and cDNA was used for both ureters and positive control myometrium tissue.

Sequencing

The PCR amplicons of AT2R transcript variants Ex 1/2/3 and Ex 1/3 were gently scraped from silver stained polyacrylamide gel with 22-gouge needle pre-wet with PCR mix and separately reamplified with exon1 and exon3 primers. The reamplified PCR products containing only one transcript variant, Ex 1/2/3 or Ex 1/3, were purified and sequenced by MWG, Biotech, Germany.

Statistical analysis

Statistical analysis was performed using Statistica software package (Version 5, StatSoft, 1997). In all tests, differences with two-tailed alpha-probability $p \leq 0.05$ were considered as significant. In order to calculate the ratio of expression of two AT2R splice variants we semi-quantified signal as the product of band density and area after normalization to GAPDH, using NCBI Image software, Image J. The correlation of the signal intensity between transcripts Ex 1/2/3 and Ex 1/3 was calculated by Pearson's correlation test.

Results

The AT2R mRNA expression pattern in human tissue with CAKUT

The expression profile of AT2R gene was determined in all samples with sufficient RNA quality and quantity, as determined by formaldehyde gel electrophoresis, GAPDH 324 bp fragment amplification and spectrophotometric measurement of RNA concentration and $A_{260\ nm}/A_{280\ nm}$ ratio. Samples with degraded RNA and poor RNA yield (les than 100 ng/μL) were excluded from further analysis. There is a possibility that during surgical procedures some of the samples were not immediately snap frozen in liquid nitrogen since in clinic settings the patient safety and well-being is the primary concern. Thus, eighteen CAKUT samples and six myometrium samples were considered. They were representative of the whole series of patients considering prevalence of UPJ, VUJ, VUR (six of each), as well as presence of AT2R -1332A/G genotypes (both AA/A- and GG/G- were present in each sample subset). Still, in this study we considered CAKUT as one disease and for the analysis we collected the same tissue from all patients, the ureter tissue.

In human ureter samples affected by CAKUT we have detected the presence of both, Ex 1/2/3 and Ex 1/3 AT2R mRNA transcripts (Fig. 1). Importantly, the splice variant Ex 1/2/3 was detected in all study samples and the shorter splice variant Ex 1/3 was detected in 80% of study samples. None of the analyzed samples had only the short, Ex 1/3 transcript. In all CAKUT patients as well as in positive control myometrium samples ($n=6$), the expression of the longer splice variant Ex 1/2/3 was about twofold higher when compared to Ex 1/3 transcript. The expression ratio of Ex 1/2/3 and Ex 1/3 transcripts was comparable across two different tissues: human CAKUT and control myometrium tissues. The significant positive correlation was calculated for the expression of AT2R transcripts Ex 1/2/3 and Ex 1/3 ($r=0.73$, $p<0.0001$).

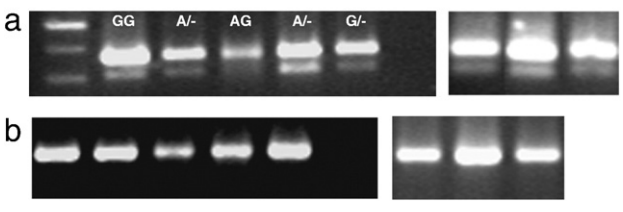

Fig. 1. (a) The human AT2R mRNA splice pattern of RT-PCR analysis demonstrating the presence of both splice variants: Ex 1/2/3 (upper, 366 bp band) and Ex 1/3 (lower, 306 bp band) in human tissue affected by CAKUT irrespectively of AT2R-1332 A/G genotypes (lanes 1–5) and human myometrium positive control tissue (lanes 7–9); (b) GAPDH (324 bp) served as a control for RNA input and quality. m-100 bp ladder (Fermentas) was used for 2% agarose electrophoresis.

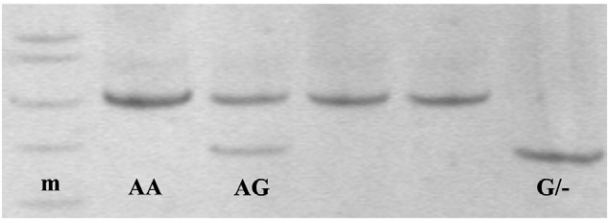

Fig. 2. Genotyping of AT2R −1332 A/G gene polymorphism, on 8% polyacrylamide gel. m-ΦX174 DNA/*Hinf*I ladder (Fermentas). AT2R −1332 A/G genotypes are indicated below lanes (AA, AG, G/−). Sizes of bands after digestion with *Eco*RI (Fermentas) are shown on the left.

The AT2R mRNA expression pattern in relation to-1332 A/G genotype in human CAKUT

Genomic DNA samples, isolated from Caucasian children with congenital anomaly of urinary tract were genotyped for -1332 A to G transition in intron 1 of AT2R gene (Fig. 2). In subjects with AA or A/- genotype we identified the presence of both Ex 1/2/3 (366 bp) and Ex 1/3 (306 bp) transcripts. Moreover, in subjects with GG or G/- genotype both transcripts were detected. The same pattern was observed in patients and control myometrium samples. The results were confirmed by sequencing. The identities of the sequencing products were confirmed by BLSATN, with 100% (228/228) and 98% identities (175/178) and 0% gaps (0/228 and 1/178) for Ex 1/2/3 and Ex 1/3 transcripts, respectively. The AT2R mRNA splice pattern was independent of the AT2R-1332 A/G genotype (Fig. 3).

Discussion

This is the first *ex vivo* study of AT2R on human tissue with any CAKUT malformation. We investigated the AT2R mRNA expression pattern in respect with −1332 A/G genotype in affected human tissue obtained from children with CAKUT. Two alternative splice variants were detected: Ex 1/2/3 and Ex 1/3 transcripts, where the splice variant Ex 1/2/3 was more abundant. This is in accordance with studies on human hearts [13, 14]. Expression ratio of Ex 1/2/3 and Ex 1/3 transcripts was comparable across two different tissues: CAKUT and control myometrium tissue, according to the same pattern of longer splice variant being approximately twofold higher expressed than the shorter one. No tissue-specific distribution and no splice pattern shift in diseased tissue were detected. This is in agreement with other studies on human hearts [14,16].

The observed AT2R mRNA splice pattern was independent of the AT2R-1332 A/G genotype in human CAKUT. Results similar to ours were obtained in the study of the samples of explanted human hearts, and transfected human HT1080 and rat

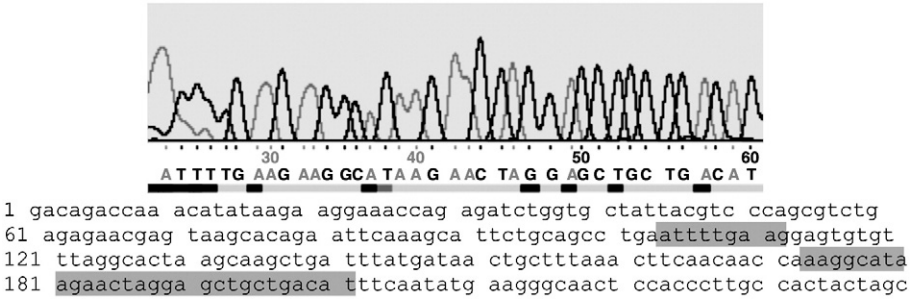

Fig. 3. Confirmation of sequence of the short AT2R mRNA transcript (Ex 1/3) detected by RT-PCR in human tissue with CAKUT, from patient with A/A genotype. The splice out of exon 2 is not caused by -1332A/G polymorphism. Ex 1/3 junction is highlighted (gray) in reference sequence NM_000686.4. Ref seq: Ex 1, 1–112 bp; Ex 2, 113–172 bp; Ex3 173–2899 bp.

PC12W cells [16]. This conclusion is in contrast to a previous study, based on the expression pattern of AT2R gene, examined in fibroblast cell lines from anonymous males [2]. Possible discrepancy could be caused by known effect of growth-dependent regulation of AT2R expression in cultured mammalian cells. The AT2R expression down-regulation, suggesting posttranscriptional regulation of AT2R expression, depending on cultivation was observed [21,22,23]. So far, AT2R expression has not been demonstrated in human primary cell cultures, and the relative instability of AT2R expression in isolated cells represents a significant limitation of *in vitro* investigations [23]. Knowledge about the function of the AT2R is limited to studies using animal models or transfected cell lines. This study and recent studies on human heart tissue [14,16] may contribute to the extension of knowledge about the regulation of AT2R expression *in vivo*. This is of great importance for further research in CAKUT, since the AT2R gene is one of the candidate genes for this disease family. It was previously suggested that AT2R has a role in CAKUT predominantly during development, by regulation of its expression in fetal kidney and urinary tract tissue [2]. Detection of its expression after birth in this study in human tissue affected by CAKUT suggests that AT2R may have a prolonged role in CAKUT etiology. This is in accordance with results on animal model of unilateral ureteral obstruction (UUO), which mimic CAKUT associated renal injuries after ureteral obstruction, where expression of both AT1R and AT2R was reported [24]. Other results based on animal models have been suggested reexpression of AT2R in pathological conditions involving tissue remodeling or inflammation, such as neointima formation, heart failure, wound healing or kidney damage [9,25,26,27]. These results and our finding of AT2R expression in human CAKUT may provide rationale for further investigation of the possible new pharmacological therapy through AT2R in CAKUT. Now, the conservative treatment besides surgical treatments includes ACE inhibitors to preserve tissue damage [28]. It was suggested that blockade of both AT1R and AT2R reduce inflammation in the kidney and may confer additive renal effects than either receptor agonist as monotheraphy [29,30].

CAKUT is family of diseases with a diverse anatomical spectrum. Some such anomalies are often concurrent [31]. The existence of different phenotypes, incomplete penetrance, variable aggregation, and clinical presentation makes uniform data collection difficult and introduces selection bias. Condition of children with renal maldevelopment or opstructive uropathy is often complicated by other urinary tract anomalies such as VUR and UPJ in the affected kidney and sometimes in the contralateral [32]. Uncertainty in the clinical classification resulted in the aggregation of different entities under the popular single-label acronym CAKUT: Congenital Anomalies of the Kidney and Urinary Tract [31]. There is also genetic support for the use of CAKUT, since mutations in a single gene can have pleiotropic effects on the development of the urogenital tract [33]. Since the most common kind of surgical treatment in children with CAKUT is corrective surgery of urinary tract, the human ureter tissue was selected as target tissue for examination of AT2R expression pattern in this study. This could be the limitation of the study, but in the age range of our children-patients the nephrectomy caused by CAKUT is rare and this is the one of the reasons for lack of the kidney samples in our study. Still, question remains as to whether particular genetic defects causing susceptibility to renal maldevelopment initially target urinary tract abnormalities or direct deregulated kidney development [34]. So, we deliberately selected the uniform tissue for expression analysis to improve the power of the study.

In conclusion, there is the lack of the expression studies in kidney and urinary tract with CAKUT *ex vivo* and according to our knowledge this is the first study investigating AT2R gene expression pattern in human CAKUT. We showed positive AT2R expression independent of the AT2R polymorphism genotype. These results will lead us to explore novel regulatory mechanisms, independent from alternative splicing, that could qualitatively or quantitatively affect human AT2R expression.

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