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ORIGINAL ARTICLE

Role of RAAS pathway gene polymorphisms in congenital uropathies

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Abstract

Aims: To study the prevalence of gene polymorphisms of RAS pathway genes in children with Congenital Anomalies of kidney and urinary tract (CAKUT) and evaluate their role in the outcome.

Material and Methods: A cross sectional study was done in 250 children (<14 yr) with CAKUT and 150 controls over a period of 3 years (2019–2021). Three diseases namely Posterior Urethral Valve (PUV), Vesico ureteric reflux (VUR) and Pelvic ureteric junction obstruction (PUJO) were selected. Polymorphism of 4 genes of RAAS pathway-AGT, AT2R, AT1R and ACE was assessed in blood samples of subjects. Polymorphism frequency was analysed with respect to clinical and radiological outcomes. Patients were followed over a period of 1 year to evaluate the role of gene polymorphisms in disease progression.

Results: While comparing the polymorphism frequencies in cases and controls, we found that disease alleles of all 4 genes were over represented in the case group and significant association was seen with 2 genes-AT2R (p = 0.03) and AT1R (p = 0.02). Multivariate analysis showed that odds of getting CAKUT were higher with following genotypes namely ACE DD (+0.5 times), AT2R (+0.4 times), AT1R AC (+1.6 times) and AGT CC (=0.6 times). Progressive deteriorators formed 22% of the cases, more seen in PUV patients. Sub group analysis of progressive deteriorators showed that AT2R G allele and ACE DD allele increased the odds of progressive deterioration by 7 and 14 times respectively. Cumulative effect of pathogenic alleles of different RAS genes showed that co existence of DD alleles with other alleles had the most serious outcomes, thus raising the possibility of synergism like a 'second hit'.

Conclusion: Almost 1/4th of children with CAKUT deteriorated despite getting the adequate treatment. Our studies found significant association of ACE and AT2R gene polymorphisms with incidence and progression of congenital uropathies.

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Graphical Abstract

Role of RAAS pathway gene polymorphisms in congenital uropathies

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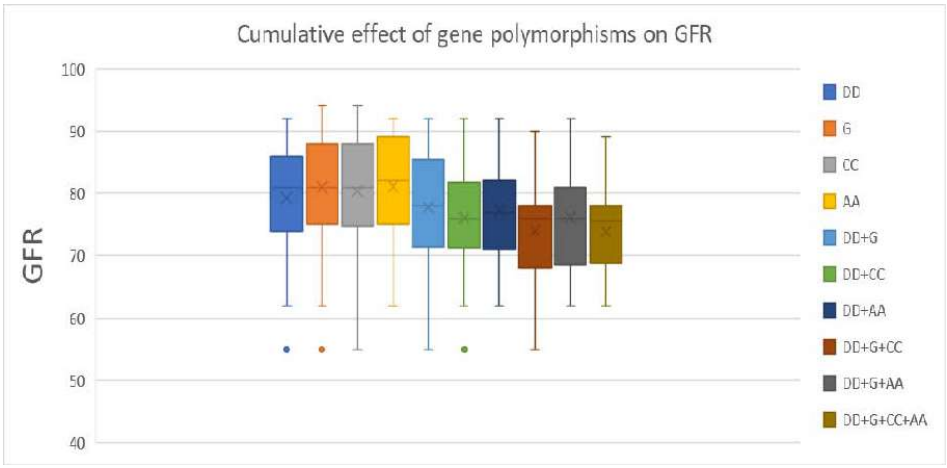

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Introduction

Congenital anomalies of Kidney and Urinary tract (CAKUT) includes a wide spectrum of anomalies including Kidney anomalies (agenesis, hypoplasia, dysplasia), ureteric anomalies (PUJ obstruction, duplex ureter), Vesico ureteric junction and many others [1]. Though the umbrella of CAKUT seems to be quite wide, many of the diseases are co existing and thus related in their pathogenesis.

Prevalence of CAKUT is 3-6 per 1000 live births accounting for almost 20-30% of congenital anomalies [2,3]. It is the most common cause of CKD (34–59%) and End stage renal disease in children (31%) [2,4–6]. Almost all children with ESRD require renal replacement therapy. CKD and the need for RRT in childhood lead to severe impairment of physical and psychosocial development [7-9]. All these consequences demand for intense experimental and clinical research for new diagnostic, preventive and therapeutic options to improve outcome of this disorder.

One intricate problem with this group of anomalies is that the renal damage starts during intra uterine period and goes on in some patients despite starting the best treatment at the correct time. A lot of research has been done in the past 2 decades to decode this mystery of progressive deterioration in CAKUT in children. Many risk factors including biochemical markers and genetic alterations have been proposed to predict early kidney injury in CAKUT. “ICMR Centre for Research on Congenital uropathies”, AIIMS, New Delhi has been working in this area for last 25 years. Multiple projects done have revealed the role of Plasma renin activity, Gene polymorphism, Urine bio markers- microalbuminuria, NGAL in pathogenesis of CAKUT [10–21].

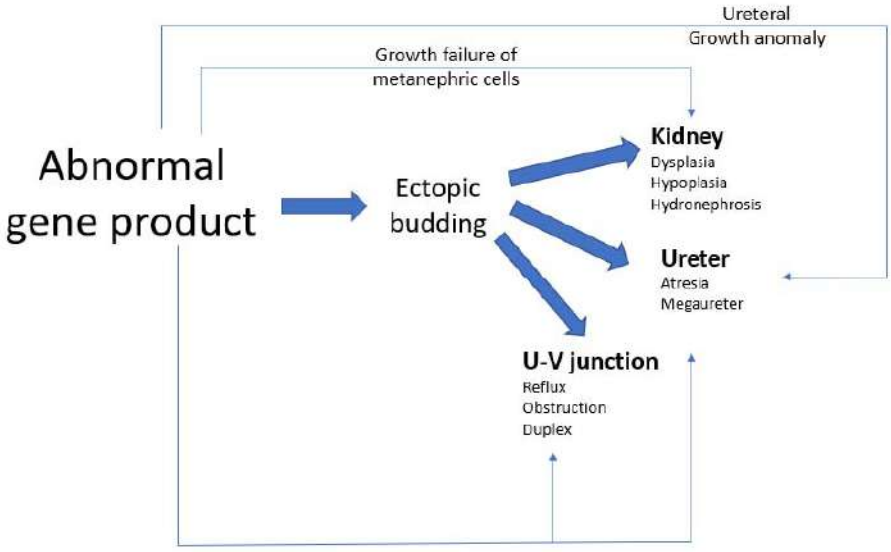

Figure 1: Overview of mechanism of CAKUT [1]
(Figure 1 is adapted from Kidney International, Vol.61 (2002), Ichikawa I, Kuwayama F, Pope IV JC, Stephens FD, Miyazaki Y. Paradigm shift from classic anatomic theories to contemporary cell biological views of CAKUT, pp.889-898, © 2002, with permission from elseiver)

Genetic predisposition to CAKUT is supported by concurrence of these anomalies with multi organ defects and frequency of familial cases (~10%) [22]. Around 2900 genes are involved in nephrogenesis but polymorphisms have been identified in less than 100 till now [23]. The notion that CAKUT may be caused by single gene mutations is suggested by 3 findings [1]. Familial aggregation of defects like VUR, MCDK, Duplex ureters [2] Monogenic mouse models exhibit CAKUT phenotypes [3] Human multi organ monogenic syndromes may include CAKUT phenotypes [22]. This notion was corroborated by discovery of >20 single nucleotide polymorphisms (SNPs) as a cause of CAKUT in humans [2,9,24–26].

Table 1. Important genes involved in human CAKUT are tabulated as below [22, 27]

S. No.	Gene	CAKUT
1	PAX2	Agenesis, hypoplasia, Renal coloboma syndrome, VUR
2	HNF1 β	Renal cysts/dysplasia and diabetes, Single kidney, Horse shoe kidney
3	BMP4	Renal hypo dysplasia, cleft lip, micro ophthalmia, duplex ureters
4	ACE	Renal tubular dysgenesis, hypodysplasia due to PUV, Pulmonary hypoplasia, skull abnormalities
5	AT2R	PUJO, Megaureter, MCDK, PUV
6	SIX5	Branchio oto renal syndrome
7	AGT	Renal tubular dysgenesis
8	AT1R	RTD, PUV
9	WNT4	Renal hypodysplasia, Mullerian aplasia, hyperandrogenism
10	ROBO2	VUR, Duplex ureters
11	CYP11B2	ESRD

Role of RAS (renin angiotensin system) is very important in nephrogenesis and progression of congenital uropathies. Developing mammalian metanephros expresses all components of RAS at various stages of embryogenesis. Mutations in genes encoding components of the RAS in mice cause diverse forms of CAKUT which include hydronephrosis, hypoplastic medulla & papilla,

marked thickening of renal arterial walls and vesico ureteric reflux [27]. Initial role of RAS in nephrogenesis was provided by findings that use of ACE inhibitors or AT-1 antagonists cause fetal anuria leading to oligohydramnios [28,29]. Genes like AT2R, AT1R have been implicated in normal embryogenesis too [23]. Genes like ACE I/D have been implicated in progression of various CAKUT [19,30].

Gene polymorphism means when there is variation of alleles in the population. It differs from the term mutation in that the frequency of variation is >1% in case of polymorphism. Multiple studies have been done to study gene polymorphism in adults with stroke, ESRD and pre eclampsia. Similar gene polymorphism studies in CAKUT have been done in the last 2 decades [31–37].

In this work, we have tried to analyse the role of RAAS genes polymorphism in prevalence and progression of CAKUT. Our aim behind this project was to find out genetic risk factors which will help in risk stratification in children with CAKUT.

Methods

A cross sectional study was carried out in Dept of Paediatric Surgery, AIIMS, New Delhi over a period of 3 years (2019–2021) in children with CAKUT. We chose 3 common congenital uropathies for our study i.e Posterior urethral valves (PUV), Vesico ureteric reflux (VUR) and PUJO (Pelvic ureteric junction obstruction). We chose 4 genes of RAAS pathway for our study- ACE (angiotensin converting enzyme), AT2R (Angiotensin type 2 receptor), AT1R (Angiotensin type 1 receptor) and AGT (angiotensinogen). Since AT2R gene is present on sex chromosome only male population was considered for comparison. 250 Children of <14yrs age were chosen for our study in a random manner from the group of our pediatric urology clinic patients. 150 children without CAKUT were chosen for comparing the prevalence of RAAS gene polymorphisms. These children were selected from our pediatric surgery ward who had non genito urinary illnesses. We also followed 250 children with CAKUT over a period of minimum 1 year to check for progressive deterioration in kidney functions. Deterioration in kidney functions was assessed in terms of ↓GFR, Increase in kidney scars, ↓ in slit renal function (PUJO). A subgroup analysis of gene polymorphisms was carried out in the group of progressive deteriorators.

Methodology for assessing gene polymorphism was as follows: Primers for specific mutations were designed after literature search and with the help of NCBI gene database.

Table 2: Primer sequences & Thermocycling protocols

	Primer sequences	Thermocycling protocol
ACE I/D	5'-CTGAGACCACTCCCATC-3' 5'-GATGTGGCCATCACATTCGTCAGAT-3'	940c for 2min ×cycles , 940c for 15s × cycles , 580c for 10s×cycles , 720c for 30s ×cycles
AT2R	5'-GGATGAACTTCGTTTTTCCTGTTT-3' 5'-GCAGTTATCATAAATCAGCTTGCTTAGT-3'	940c for 2min ×cycles , 940c for 15s × cycles , 590c for 10s×cycles , 720c for 30s ×cycles
AGT rs699	5'-GTG CTG TCC ACA CTG GCT CCC-3' 5'-AAGAACTGCACCTCCCGGCTGGATG-3'	940c for 2min ×cycles , 940c for 15s × cycles , 590c for 10s×cycles , 720c for 30s ×cycles
AT1R rs5186	5'-GCAGCACTTCACTACCAAATGAGCC-3' 5'-CTCATCTCCTGTTGCTCCTCTAACG-3'	940c for 2min ×cycles , 940c for 15s × cycles , 580c for 10s×cycles , 720c for 30s ×cycles

2 ml of peripheral blood sample was collected. DNA was extracted by Key gen extraction kit. Normal and mutant ARMS premixes were prepared according to previously published PCR guidelines. Premixes containing Taq polymerase were kept in thermocycler. PCR products were run by gel electrophoresis. Bands were assessed in UV light and depending on the base pair length, results were analysed.

Clinical and imaging details were taken from pediatric urology clinics records. GFR was measured by plasma sample method. Kidney scars were assessed in DMSA. Split renal function was assessed in PUJO patients with the help of LLEC scan. Outcome variables were gene polymorphism frequencies in CAKUT and non CAKUT patients and Base line GFR, renal scarring and Split renal function at presentation and 1 year. Progressive

deterioration was defined as GFR<60 or ↑se in renal scars or ↓se in SRF by ≥ 10%.

Data extraction was done using Microsoft Excel. Data analysis was done using Stata 12.0. Categorical variables were analysed using t-test and continuous variables were analysed using Pearson/Spearman co relation coefficient. Categorical variables were analysed using Chi-square test. Multi variate analysis was done by logistic regression. A p-value of <0.05 was considered statistically significant.

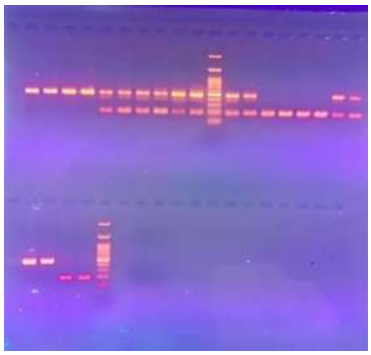

Figure 2. DNA bands on agarose gel under UV light

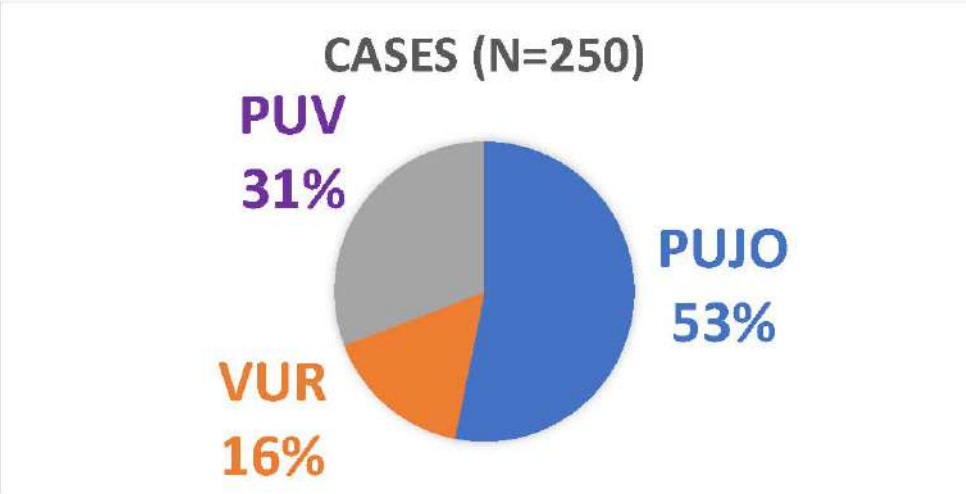

Figure 3: Distribution of 3 anomalies in cases

Results

Distribution of 3 anomalies in CAKUT population was as shown in Figure 3.

We checked for confounding factors like age, sex etc in CAKUT and non CAKUT children and found the 2 populations matching. Average age in CAKUT children was

To check the effect of sex of the child on gene polymorphism frequencies and renal outcomes variables, comparison was done within CAKUT population. No significant differences were found in males and females.

Then we compared gene polymorphism frequencies in CAKUT and non CAKUT children. G allele of AT2R gene, AC allele of AT2R, DD allele of ACE and AA allele of AGT were over represented in CAKUT population. Significant differences were noted in allelic frequencies of AT2R ($p = 0.03$) and AT1R ($p = 0.004$).

Table 3: Distribution of genotype frequencies in cases and controls

Genes	Genotype	CAKUT (*n*=250) (*n*=191 for AT2R)	Controls(*n*=150) (*n*=119 for AT2R)	*p* value
ACE I/D	DD	95	45	0.147
	II	106	65	
	ID	49	40	
AT1R	AA	101	59	0.004
	AC	114	51	
	CC	35	40	
AT2R	A	76	62	0.031
	G	116	57	
AGT	AA	60	46	094
	AC	100	65	
	CC	90	39	

Univariate and multivariate analysis was done to assess the odds of getting CAKUT with pathogenic alleles. It was found that ODDs of getting CAKUT increased with following allelic distribution- ACE DD (+0.5 times), AT2R G (+0.4 times), AT1R AC (+1.6 times) and AGT CC (+0.6 times).

Table 4: Odds ratio of prevalence of CAKUT w.r.t genotypes

	Genotype	Odds ratio (univariate analysis)	Adjusted odds (multivariate analysis)
ACE I/D	DD	1.29	1.90
	II	1	1.40
	ID	0.75	1
AT1R	AA	1	1.94
	AC	1.3	2.66
	CC	0.5	1
AT2R	A	0.6	–*
	G(ref)	1	
AGT	AA	1	1
	AC	1.17	1.15
	CC	1.76	1.6

*Multivariate analysis not possible as AT2R was checked in males only.

To assess the distribution of gene polymorphism with respect to 3 uropathies, we compared the allelic distributions in children with PUJO, PUV, VUR with respect to non CAKUT children. Children with PUJO had significant differences in allele distribution of AT2R gene (p=0.01). ACE gene allelic distribution was significantly different in children with PUV (p=0.03). In sub group of children with VUR, no significant differences were found.

After comparing CAKUT and non CAKUT children, sub group of CAKUT children was studied for renal function variables to find out the mischievous population of progressive deteriorators.

Baseline GFR at presentation was found to be in children with PUV (75ml/min/1.73m^2). Renal scars were found in 1/3rd of children with PUV and VUR. On the other hand, only 1/6th of PUJO population had renal scars.

When renal function variables were assessed over a period of 1 year, progressive deterioration was noted in 32% of PUV patients and 20–25% in PUJO and VUR patients.

Gene polymorphism was studied in this subgroup of progressive deteriorators and results were astonishing. There were significant differences in allelic distributions of all 4 genes- ACE (p<0.001), AT2R (<0.01), AT1R (<0.009), AGT (<0.001).

Table 5: Distribution of genotype frequencies in 'Progressive deteriorators'

	Genotype	Case (n=56) (n=51 for AT2R)	Controls(n=150) (n=119 for AT2R)	P -value
ACE I/D	DD	49	45	<0.0
	II	3	65	01
	ID	4	40	
AT2R	A	10	62	<0.0
	G	41	57	01
AT1R	AA	33	59	0.009
	AC	18	51	
	CC	5	40	
AGT	AA	10	46	<0.0
	AC	13	65	01
	CC	33	39	

ODDs of progressive deterioration with pathogenic alleles were assessed by multivariate analysis. We found that presence of DD allele and G allele increased the odds of progressive deterioration by 14 and 7 times respectively. While rest of the 2 genes increased the odds minimally.

Table 6: Odds of Progressive deterioration w.r.t genotypes

	Genotype	Odds ratio (univariate analysis)	Adjusted odds (multivariate analysis)
ACE I/D	DD	17	0.45
	II	1	1
	ID	0.44	14.2
AT1R	AA	1	1
	AC	0.74	0.72
	CC	2.89	0.43
AT2R	A	1	-*
	G	7.39	
AGT	AA	1	1
	AC	1.17	0.72
	CC	1.76	0.43

Cumulative effect of pathogenic alleles was assessed by comparing frequencies of pathogenic alleles with respect to GFR in children. And we found co-existence of DD allele (ACE gene) with other pathogenic alleles had led to further decrease in GFR.

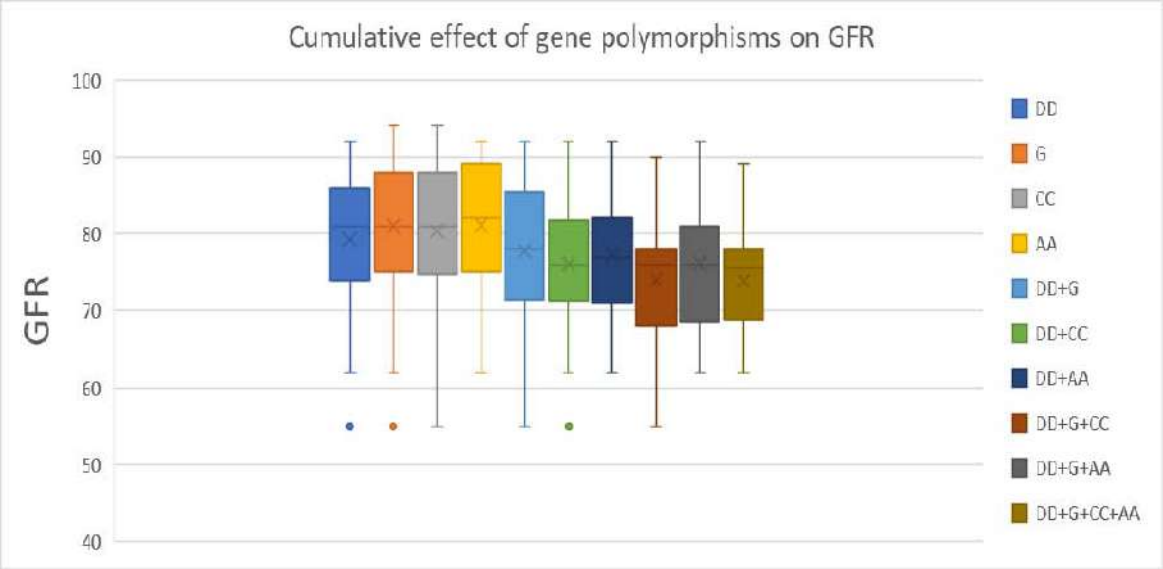

Figure 4: Cumulative effect of different pathogenic alleles

Discussion

Distribution of anomalies in our study was different than distribution mentioned in standard textbooks. Incidence of PUJO, PUV and VUR has been given as 1 in 1250, 1in 6000 and 1 in 100 (38). In our study, incidence of PUJO and VUR was lower than expectations i.e 53% and 16% respectively. Possible cause may be because they are diagnosed late as symptoms are vague and appear late in these 2 anomalies.

While comparing polymorphisms in total cases and controls, although the disease alleles of all 4 genes were over represented in CAKUT population but significant association was seen in 2 genes- AT2R and AT1R. Multivariate analysis showed that odds of getting CAKUT increased in all 4 pathogenic alleles – ACE DD (+0.5 times), AT2R G (+0.4 times), AT1R AC (+1.6times), AGT CC (+0.6 times).

AT2R gene polymorphism has been frequently found to be associated with kidney diseases in both adults and children. Hohenfeller et al, Rigoli et al and Nishimura et al have independently shown in their studies the significant association of AT2R with CAKUT in children [34,37]. High expression of AT2R place this gene amongst the list of important nephrogenic genes. Like other studies, association was seen in subgroup analysis of PUJO patients. But no significant association was seen in PUV or VUR patients. Narsimhan et al studied ACE I/D and AT2R(1332G) gene polymorphisms in 120 children of PUV in 2010. They found that ACE DD alleles and AT2R G alleles were significantly related individually to disease progression in CAKUT. They further that co existence of DD and G allele increased the risk to 3 times [39]. Our study has analysed 2 more genes -AT1R and AGT in a larger population. 2 more anomalies- PUJO and VUR have been included. GFR has been measured by plasma sampling method to give more accurate assessment of renal functions.

AT1R gene polymorphism has been found to be significantly associated with stroke, ESRD, preeclampsia in few studies in adult Caucasian patients (40-42). Our study revealed the role of AT1R AA alleles in prevalence of CAKUT overall and PUJO specifically. Allelic distribution differences were found to be significant in progressive deteriorators. Ours was the first study to study AT1R gen in CAKUT in Indian children thus setting the course for future generations.

Multiple studies by Bajpai et al, Rigoli et al, Livoti et al have reported the of DD allele ACE gene in progressive deterioration of kidney [19,34,43]. Similar results were seen in our study. But we noticed 3 new findings regarding this allele. Firstly, it was not associated with anomalies when their total incidence at presentation was analysed. But on follow up, they played an important role in renal damage thus emphasising the difference in 2 processes i.e nephrogenesis prenatally and progressive deterioration postnatally. Multi variate analysis to assess individual contribution of different genes in progressive deterioration showed that ACE DD allele increased the odds of progressive deterioration by 14 times. Peruzzi et al on the

contrary found higher incidence of II genotype in cases [30].

AGT gene: Lovati et al found involvement of AGT CC gene polymorphism in ESRD patients in their study [43]. We also found its role while analysing the sub group of progressive deteriorators but not while comparing the CAKUT and non CAKUT population overall.

While analysing the renal function variables, we found that children with PUV had the least GFR as compared to PUJO and VUR population. Progressive deterioration was seen in significant proportion i.e. 20% of children with CAKUT. It was more common in children with PUV.

While assessing the cumulative effect of pathogenic alleles of different RAS genes, co-existence of DD allele with other alleles had the most serious outcomes. Thus, there is fair possibility that DD allele of ACE is working like a second hit to increase the pace of deterioration in renal functions.

Limitations: Longer follow up period could have revealed more accurate temporal trends of progressive deterioration. It would have also helped in showing effects of surgical interventions. Parental studies could have shed light on penetrance and inheritance which is the essential information needed for genetic counselling. Despite a good sample size, 4 single nucleotide polymorphisms fell short of predicting whole of genetic risk factors. Wider genome analysis would become possible with advanced gene sequencing technologies giving a wholesome assessment of genetic etiology.

Pathogenesis of CAKUT is multifactorial so risk prediction studies need to be done to assess the individual contribution of genetic, anatomic, and biochemical factors. Gene polymorphism studies will help in better risk stratification and thus will lay the foundation of genetic counselling in future. To conclude, CAKUT is a very intricate spectrum of anomalies and needs multi pronged approach for monitoring- genetic studies, serial imaging and biochemical analysis. Preventing CAKUT may not be achievable presently but preventing the progression is definitely possible if we identify the high risk groups and renal damage at an early stage.

Statements and Declarations

Conflicts of interest

The authors declares that they do not have conflict of interest.

Funding

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