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Screening for Mutations in BMP4 and FOXC1 Genes in Congenital Anomalies of the Kidney and Urinary Tract in Humans

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Recent studies have demonstrated in mice that bone morphogenetic protein 4 (BMP4) and forkhead transcription factor 1 (FOXC1) are involved in the organogenesis of the kidney and urinary tract and that derangement of either gene, *BMP4* or *FOXC1*, leads to development of congenital anomalies of the kidney and urinary tract (CAKUT). In order to determine whether human CAKUT is associated with abnormalities in *BMP4* or *FOXC1*, we established a PCR-based methodology for the DNA sequence analysis of *BMP4* and *FOXC1* in humans. Our initial screening identified an insertion mutation in *FOXC1* with a triplet GGC in three of the seven patients with CAKUT. In the present study, no mutation was detected in the coding sequence of *BMP4*.

Key words : Congenital anomalies of the kidney and urinary tract; *BMP4*; *FOXC1*; sequence analysis.

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) occur in 0.5 % of all pregnancies, and more than half of those cases are identified during the perinatal period [1]. Despite recent advancement in prenatal diagnosis and early surgical interventions, CAKUT still accounts for approximately 30 % of primary etiologies that underlie pediatric cases of end-stage renal disease [1]. Thus, further understanding of CAKUT is essential for better diagnosis, management, and therapy of patients with CAKUT.

Recently, investigative efforts have been focused on the search for genetic abnormalities that are involved in human CAKUT. To date, a mutation in one allele of *PAX2*, a transcriptional regulator of the paired-box family widely expressed throughout the development of the excretory systems, has been identified in a family carrying renal-coloboma syndrome, a rare autosomal dominant syndrome characterized by optic nerve coloboma, renal anomalies and vesicoureteral reflux [2]. *EYA1*, a human homolog of *Drosophila melanogaster gene, eya*, is mutated in patients with dominantly inherited disorder, bronchio-oto-renal syndrome [3], which exhibits anomalies of the kidney and urinary tract such as duplex collecting system, renal hypoplasia/displasia and renal agenesis. Abnormalities in W*T-1*, the Wilm's tumor suppressor gene [4], are also linked with CAKUT. These genetic abnormalities, however, account for only a small fraction of CAKUT in humans, indicating that a majority of CAKUT involves either other genes singly or multiple genes concurrently [5, 6]. In this regard, the gene for angiotensin type 2 receptor, *AGTR2* has been shown to be a part of multiple genes [7].

Phenotypic analysis of genetically manipulated mice has shed light on the genetic nature

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of CAKUT. Mutations in *Pax2*, similar to the human counterpart, are associated with a variety of anomalies in the excretory system, including hypoplastic kidney and cystic kidney with hydroureter [8]. Mice with heterozygous mutation in bone morphogenetic protein 4 (BMP4), a member of transforming growth factor- β (TGF- β) superfamily of secretory signaling molecules [9], exhibit abnormal development of several organs, and some 50 % of those mice develop CAKUT with anatomical features including duplicate ureters, hydronephrotic urethrals, and hypo/dysplastic kidneys [10, 11]. Recently, we identified the specific roles of BMP4 in the regulation of the budding site and elongation of the ureter [12]. Mice with homozygous null mutation in *FOXC1*, a gene encoding a forkhead/winged helix transcription factor essential for cell fate determination, proliferation and differentiation during embryonic development [13], are born with abnormalities of the metanephric kidney, including duplex kidneys, double ureters, and hydroureters, with similar frequencies in males and females [14]. *FOXC1-/-* mutant mice also manifest multiple anomalies, such as hemorrhagic hydrocephalus and skeletal, ocular and cardiovascular defects, and die pre- or perinatally [15].

In keeping with the notion that both *BMP4* and *FOXC1* play critical roles in various stages of embyonic development in mice, studies have provided evidence that mutations in *BMP4* or *FOXC1* also cause maldevelopment of certain organs in humans.

In order to investigate the potential link of genetic abnormalities and CAKUT in humans, we developed PCR-based methods for DNA sequence analysis of *BMP4* and *FOXC1* in humans. In the present study, we describe our methodological approaches as well as the results of the initial screening in patients with CAKUT.

PATIENTS AND METHODS

Patients

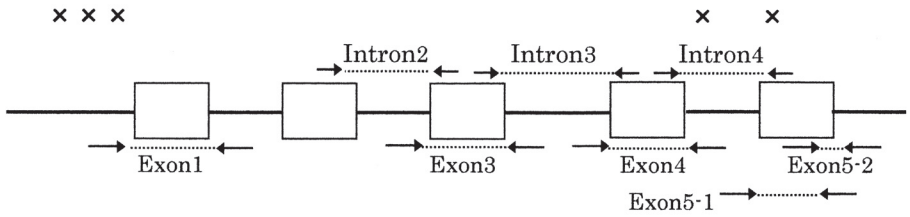
The present study enrolled seven patients with congenital anormalies in the kidney or urinary tract. Two of those were Japanese and the others were German. As described in detail in "Results and Discussion", the anatomical features of CAKUT of these patients were similar to or reminiscent of those previously described in mice with derangements in *BMP4 or FOXC1*. This study was approved by the ethics committee of Tokai University School of Medicine.

Methods

After written informed consent was obtained, either 10 ml or 5 ml of heparinized peripheral blood was obtained from adult and pediatric subjects, respectively, and mononuclear cells were isolated by gradient centrifugation. Genomic DNA was isolated from mononuclear cells by extraction with proteinase K/phenol/chloroform.

We developed PCR-based methods for sequence analysis of *BMP4* and *FOXC1*. *BMP4* consists of 5 exons (Fig. 1), of which, coding regions are exon 4 and 5. Since exon 3, 4, and 5 are shared by multiple mRNA species that are produced by alternate splicing [16], we designed 4 sets of primers to determine the DNA sequences of exon 3, 4, and 5 (Fig. 1). *FOXC1* is comprised of a single exon (Fig. 2) and its sequence has been documented. We designed 7 sets of primers that allow DNA sequence analysis of the entire coding region. Our strategy is described in detail in Results.

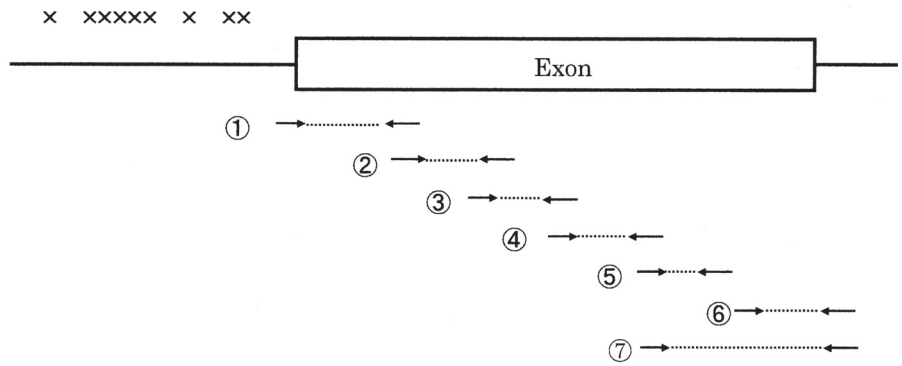
PCR was performed using either PLATINUM Taq polymerase High Fidelity (Invitrogen USA) for exon 1, 3, 4 and 5 of *BMP4* and primers 5, 6 and 7 for *FOXC1* or TaKaRa LA Taq with GC buffer (Takara Japan) for primers 1 through 4 for *FOXC1*. Reaction mixture contained, in a volume of 50 μl, 100 ng genomic DNA, 5 mM $MgSO_4$, 0.25 mM of each dNTP, 1X PCR enhancer (Invitrogen USA), 0.2 mM of each primer, and 2.5 units of PLATINUM Taq polymerase. For reaction with TaKaRa LA Taq polymerase, a 50 μl reaction mixture contained 100 ng genomic DNA, 1X GC buffer, 0.5 mM of each dNTP, 0.4 mM of each primer, and 2.5 units of LA Taq polymerase. The PCR amplification was performed 38 cycles, each of which consisted of denaturation at 95°C for 7 min, annealing for 1 min, and extension at 54°C for 10 min. Annealing temperature was decreased from the initial temperature of 67°C to 54°C by 1 degree per every cycle of amplification. PCR products were separated by electrophoresis in 0.8 % agarose gel and purified using Wizard SV Gel and PCR Clean-up system (Promega USA). Purified PCR products were directly sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Original



	Forward	Reverse
Intron2	AACATGGTGGGATTTCCTTTC	GTCCATGATTCTTGACAGCCAATC
Intron3	GATTGGCTGTCAAGAATCATGGAC	GCATTCGGTTACCAGGAATCATGG
Intron4	AACACCGTGAGAGCTTCCACCAC	TTCACTGGTCCCTGGGATGTTCTC

	Forward	Reverse
Exon1	AGAAAGGGAGGCGAGCGAGGGAGG	TCACAGCCTGTGACCAGCTTCTTC
Exon3	CACGTGACTCCGAGGGGCTGGAAG	GGATATTGTAAAGGAGGTCCGACG
Exon4	GCAGACACATTTCTATTTCTCCC	TTTCACTGCCCCTCTAGCCAGTCC
Exon5-1	AGCTATCTCTTGACTCTTCCATC	GTCCAGCTATAAGGAAGCAGTCTG
Exon5-2	ATCAGGCAGTCCTTGAGGATAGAC	CGAGCCCGAAGGAAAAGAGAAAGG

Fig. 1 Organization of human *BMP4* gene and the PCR primers developed in the present study. " → " and " ← " locate the positions of the forward and reverse primers, respectively. " × " indicates the location of single nucleotide polymorphism, SNP.



	Forward	Reverse
①	CCCGGACTCGGACTCGGC	ACTGGTAGATGCCGTTCAGG
②	CTCATCACCATGGCCATCCAGAAC	CTTGAGGTGCAGCCTGTCCTTCTC
③	GGCGCTTCAAGAAGAAGGAC	CTGAAGCCCTGGCTATGGT
④	AAGATCGAGAGCCCCGAC	CAGAAGGCCGGAGCTGAG
⑤	ACCATAGCCAGGGCTTCAG	CAGGTTGCAGTGGTAGGTCC
⑥	ACCATAGCCAGGGCTTCAG	TGGTTCAGGTACCACGAGGTGAGG
⑦	CAAGCCATGAGCCTGTACG	GGGTTCGATTTAGTTCGGCT

Fig. 2 Organization of human *FOXC1* gene and the PCR primers used in the present study. " → " and " ← " locates the positions of the forward and reverse primers, respectively. " × " indicates the location of single nucleotide polymorphism, SNP.

(AB Applied Biosystems USA). When the result of direct sequencing was unrevealing, PCR products were subcloned using TOPO cloning (Invitrogen USA) and sequenced. Possible sequence alterations were examined by comparing with the previously described sequences of *BMP4* and *FOXC1* available through DBGET Result Gene Bank. For analysis of DNA sequences, we used GENETYX-MAC 9.0.

RESULTS

Clinical features of patients

Features of CAKUT and other congenital anomalies seen in the present patients were as follows:

Case 1 patient (Japanese) had non-functioning right kidney and bilateral vesicoureteral reflux of Grade IV and I in the right and left kidney, respectively. Anomalies in multiple organs such as noncommunicating hydroencephalus in the right hemisphere, cysts in the right colloid plexus, cheilognathopalatoschisis, cloudy conjunctiva of the right eye, atrophy of the right optic nerves, micrognathia, cleft lip, deafness of the right ear, bilateral ulnar polydactyly, hypertrophy of the right first toe were observed.

Case 2 patient (Japanese) had a history of nephrostomy for bilateral incomplete double ureters without other anomalies.

Case 3-7 patients (German) had bilateral complete double ureters without other anomalies.

Strategy of PCR-based sequence analysis

Two *BMP4* mRNA species produced by alternate splicing contain either exon 1, 3, 4 and 5 or 2, 3, 4 and 5 of *BMP4* (Fig. 1). Thus, we chose to analyze the DNA sequences of the shared exons, 3, 4 and 5. Since the entire DNA sequence of *BMP4* was not available at the initiation of the present investigation, our strategy to amplify exon 3, 4 and 5 began with acquiring the sequences of intron 2, 3 and 4 by utilizing the reported sequences of mRNA and the intron-exon boundaries [16]. First, we amplified and sequenced the boundaries of exon 2 (3'-AACATGgtgggatttcctttc and aaatattccttttagGAGCCA-5'), exon 3 (3'-CTGTCAgtcagtagacacctg and cttccccctccccagAGACAC-5'), and exon 4 (3'-ACGAAGgtcagtctcattaac and cctaactgtgcctagAACTTC-5'). Then, intron 2, 3 and 4 were amplified and their sequences verified. Next, to amplify exon 3, 4 and 5, we designed 5 sets of primers at the introns (Fig. 1). From the determined sequences, we also designed primers for exon 1, which is contained in one of *BMP4* mRNA species.

FOXC1 consists of one exon, and the DNA sequence of the coding region has been described (Fig. 2). We designed 7 sets of primers and analyzed the entire sequence of the coding region (Fig. 2).

Obtained sequences of exon 3,4 and 5 of *BMP4* and the entire coding region of *FOXC1* were compared with the previously described seqeunces, NCBI U43842 and HS118B18, for *BMP4* and *FOXC1*, respectively.

Screening of patients with CAKUT

Our present analyses revealed no sequence abnormalities in *BMP4* in patients with CAKUT. However, in three patients, Case 1, 2 and 3, we identified a 3-bp insertion mutation in *FOXC1*. An insertion of GGC (Gly) occurred in the coding region of *FOXC1* between position 375 and 380 within the 6 GGC repeats (Fig. 3). This insertion is neither a missense mutation, nor does it cause a frame shift. It is not certain whether this insertion of glycine, a bipolar amino acid, would alter the tertiary structure of *FOXC1* protein and affect its DNA binding activity. Case 1 had non-functioning right kidney and vesicoureteral reflux in both kidneys, while Case 2 and 3 presented bilateral incomplete double ureters. Thus, except for the presence of ureteral anomalies, these three CAKUT patients share no apparent anatomical features. In order to ascertain the biological significance of our findings, we are currently extending our screening to an increasing number of patients with CAKUT as well as apparently healthy individuals with no documented history of CAKUT.

DISCUSSION

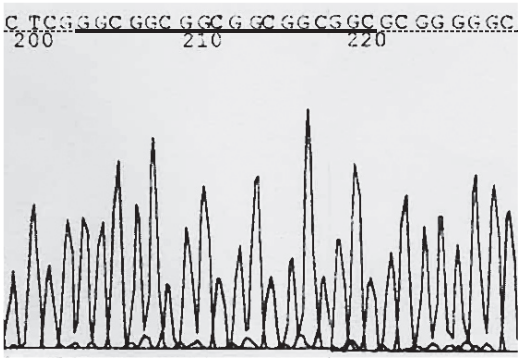
In the present screening on seven patients with CAKUT, we found an identical insertion mutation in *FOXC1* in three patients, whereas no abnormalities in the DNA sequence was apparent in *BMP4*. Our present studies were prompted by the recent findings that associate development of CAKUT in mice with alterations in *BMP4* or *FOXC1*. *BMP4* belongs to TGF- β superfamily of signaling molecules and plays a critical role during embryonic development, ranging from the establishment of the basic body plan

to morphogenesis of individual organs [9]. CAKUT arises in approximately half of mice with heterozygous mutation in *BMP4*, while homozygous null mutation in *BMP4* is lethal and mice die *in utero* [10]. Homozygous null mutation in *FOXC1*, a transcriptional factor, is associated with metanephric kidney abnormalities, namely duplex kidneys, double ureters, and hydroureters [14]. *FOXC1-/-* mutant mice die pre- or perinatally with multiple anomalies, such as hemorrhagic hydrocephalus and skeletal, ocular and cardiovascular defects [15]. These observations raise the possibility that the present results of our initial screening reflect, in part, either low penetrance or potential lethality of derangments in *BMP4 or FOXC1*.

In all likelihood, sequence alterations in *BMP4* or *FOXC1* would not necessarily account for all human CAKUT cases. In the light of the knowledge accumulating in mice, the results of our initial screening warrant further investigation for a potential association between the genetic abnormalities and the development of CAKUT. If abnormalities in these genes are found in multiple cases, common phenotypic features should be analyzed. It is possible that a cluster of anomalies or clinical manifestations, or a syndrome, is associated with abnormalities in *BMP4* or *FOXC1*. For example, mutations in *FOXC1* has been associated with maldevelopment of the anterior segment of the eye, and recent studies described four different *FOXC1* mutations in patients with Axenfeld-Rieger syndrome, a family of developmental diseases characterized by anterior segment abnormalities, glaucoma, and a variety of systemic manifestations [17].

There are five single nucleotide polymorphism (SNPs) in *BMP4* (Fig. 1) and nine SNPs in *FOXC1* (Fig. 2) (data available from the Human Genome Center, the Institute of Medical Science, the University of Tokyo through http://www.hgc.ims.u-tokyo.ac.jp). Since some of these SNPs can influence the activity of the coded protein, we are currently designing our strategy for analyzing SNPs. Analysis for the frequency of gene polymorphism in both CAKUT patients and the general population can also help delineate the potential association of these genes with CAKUT.

Normal sequence



Sequence with an insertion of GGC

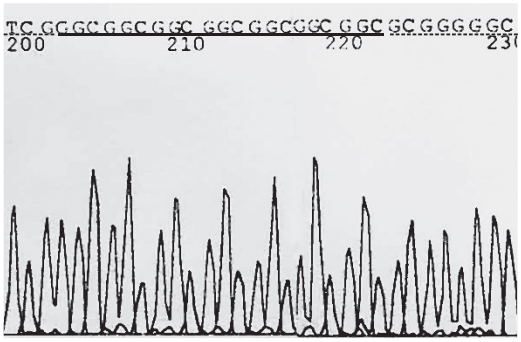


Fig. 3 An insertion of a triplet GCC within the 6 GCC repeats (underlined) found in *FOXC1* in Case 1, 2, and 3.

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