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The Gene Expression Profile of Cyst Epithelial Cells in Autosomal Dominant Polycystic Kidney Disease Patients

Jae Eun Lee, Min Ha Park and Jong Hoon Park*

Department of Biological Science, Sookmyung Womens University, Seoul 140-742, Korea

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Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder characterized by the formation of fluid-filled cysts in the kidney and progressive renal failure. Other manifestations of ADPKD include the formation of cysts in other organs (liver, pancreas, and spleen), hypertension, cardiac defects, and cerebral aneurysms. The loss of function of the polycystin -1 and -2 results in the formation of epithelium-lined cysts, a process that depends on initial epithelial proliferation. cDNA microarrays powerfully monitor gene expression and have led to the discoveries of pathways regulating complex biological processes. We undertook to profile the gene expression patterns of epithelial cells derived from the cysts of ADPKD patients using the cDNA microarray technique. Candidate genes that were differently expressed in cyst tissues were identified. 19 genes were up-regulated, and 6 down-regulated. Semi-quantitative RT-PCR results were consistent with the microarray findings. To distinguish between normal and epithelial cells, we used the hierarchical method. The results obtained may provide a molecular basis for understanding the biological meaning of cytogenesis.

Keywords: ADPKD, Expression profile, Microarray, RT-PCR

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a hereditary disease that occurs in one out of 1,000 humans (Michael *et al.*, 2000; Guanqing, 2001). ADPKD forms cysts in the kidney, liver, and pancreas, which result in cardiac defects (Boulter, 2001). In addition, ADPKD causes chronic renal failure in approximately 50% of patients by age 60. The disease is generally attributed to a mutation in PKD1 or PKD2 which encode polycystin-1 or 2 (van Adelsberg, 1999; Guanqing, 2000; Guanqing, 2001; Silvia *et al.*, 2001). ADPKD is caused by a mutation in PKD1 in about 85% of cases and a mutation in PKD2 in the remaining 15% (Michael *et al.*, 2000; Guanqing, 2001). Polycystin-2 is an integral membrane protein (968 amino acids) with 6 membrane spanning domain, and intracellular C-terminal contains a calcium-binding EF-hand (Hanaoka *et al.*, 2000). Polycystin-1 and 2 interact with the coiled-coil domain of intracellular C-terminal and thus form a calcium-permeable non-selective cation current. However, the mechanistics and functions of polycystin-1 and 2 are largely unknown.

Presently, cDNA microarray technologies are used to study disorders at the molecular level (Makaki *et al.*, 2001). The technique allows the characterization of the expression patterns of numerous genes in a single microarray (Schena *et al.*, 1995; Derisi *et al.*, 1996; Lockhart *et al.*, 1996; Quackenbush, 2002). Generally, the spots and background intensities of a microarray TIFF image file are analyzed and normalized to generate a gene expression pattern. Here, we used a cDNA microarray to identify the gene expression profile of cyst epithelial cells in ADPKD patients.

Material and Methods

Cell culture Kidney cyst tissues of two ADPKD patients were obtained from the Seoul Medical School. Epithelial cells were isolated from the cysts as follows. Cysts were washed with PBS solution, cut into pieces with a knife, and FBS added. Cells thus prepared were incubated at 37°C in DMEM medium. After 2-4 passages, cyst and normal renal epithelial cells (Cambrex Co. Beverly Hills, USA) were cultured in REGM medium (Cambrex Co.) at 37°C.

Total RNA extraction and hybridization Using Trizol reagent (Invitrogen, Carlsbad, USA), total RNA was extracted from the cultured cells. To prepare fluorescent labeled cDNA, RNA was incubated with oligo-dT, 5x reaction buffer, 0.1 M DTT, 10x dNTP,

*To whom correspondence should be addressed.
Tel: 82-2-710-9414; Fax 82-2-2077-7322
E-mail: parkjh@sookmyung.ac.kr

cy3-dUTP or cy5-dUTP fluorescent nucleotide (Amersham, Uppsala, Sweden), RNAsin, and Superscript reverse transcriptase (Invitrogen, Carlsbad, USA). Un-incorporated nucleotides in the prepared cDNA were removed using Bio-Spin column (Bio-Rad, Hercules, USA), and the cDNA was concentrated using a Microcon 30 column (Millipore, Bellercia, USA). After concentration of labeled cDNA, probe was added to poly (dA) yeast tRNA, human cot1 DNA and incubated at 99ºC for 5 min, and at room temperature for 5 min. The samples were mixed with hybridization buffer (Genomic solution, Cambridgeshire, USA) and used to treat a cDNA microarray chips containing 14,080 genes. Hybridization was performed at 56ºC for 14-16 h, and then the array was washed by placing them into washing solution containing 1 x SSC, and 0.1% SDS for 10 min at room temperature (Xia *et al.*, 2003).

DNA microarray scanning and analysis Microarray were scanned using an Arraywox scanner (Applied Precision Inc. Northwest Issaquah, USA), analyzed using ImaGene version 5.1 software (Biodiscovery, Segundo, USA), and normalized using Genesight 3.2 version software. Normalization was performed by using the subtract means and all genes. The data normalized by Genesight was compared using an M/A plot. Genes differentially expressed were identified by intensity differences, after subtracting the background intensity. Genes showing expression changes of at least 2-fold were selected, and these selected genes were clustered by the hierarchical method (Lee and Park, 2004).

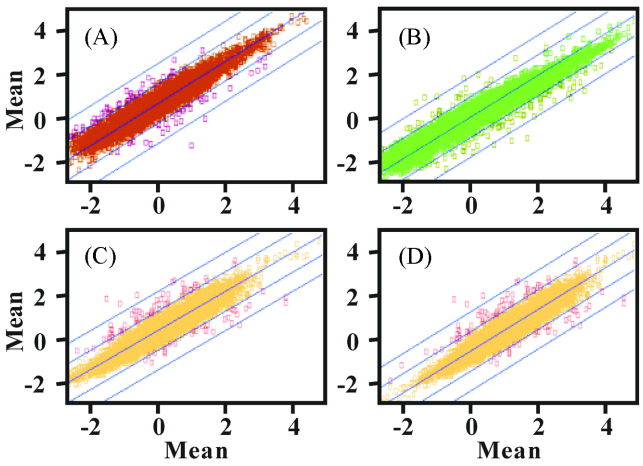

Fig. 1. The distribution chart of the mean ratio of the Cy3/Cy5 fluorescence intensity mean (log scale) of ADPKD patients microarray hybridization data. (A) and (B) Scatter plots for patient 1 performing each repeat experiment. (C) and (D) Scatter plots for patient 2 performing each repeat experiment.

RT-PCR Five μg of total RNA isolated from cultured cystic epithelial cells was used to synthesize cDNA by incubation with oilgo-dT, Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA), 0.1 M DTT, and 10x dNTP. Synthesized cDNA was diluted

Table 1. The list of 25 genes commonly up and down regulated in cyst epithelial cells

Acc #	Gene ID	Function	Fold
NT-002426	matrix metalloproteinase 12	cell motility	0.23
BC005809	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	unknown	0.3
NM_012133	coatomer protein complex, subunit gamma 2	vesicle transport	0.39
NM_006565	CCCTC-binding factor (zinc finger protein)	transcription factor activity	0.43
NM_005271	glutamate dehydrogenase 1	unknown	0.45
NM_001845	collagen, type IV, alpha 1	extracellular matrix structural constituent	0.48
NP_001785	cadhedrin 4, type 1, R cadhedrin	cell adhesion	1.26
NM_005429	vascular endothelial growth factor C	cell proliferation	2.08
NM_005926	microfibrillar-associated protein 1	extracellular matrix	2.1
NM_006265	RAD21 (*S. pombe*) homolog	apoptosis	2.15
NM_005269	glioma-associated oncogene homolog (zinc finger protein)	development	2.21
NM_173075	FE65-LIKE 2	intracellular signaling cascade	2.53
NM_000304	peripheral myelin protein 22	peripheral nervous system development	2.59
BC002934	predicted osteoblast protein	unknown	2.61
BC002439	tat-interacting protein (30kD)	apoptosis	2.63
NM_003463	protein tyrosine phosphatase type IVA, member 1	prenylated protein tyrosine phosphatase activity	2.63
AJ006291	leucine-rich protein mRNA	unknown	2.7
NM_001249	ectonucleoside triphosphate diphosphohydrolase 5	endoplasmic reticulum	2.81
NM_002354	tumor-associated calcium signal transducer 1	unknown	2.98
NM_000761	cytochrome P450, subfamily I (aromatic compound-inducible	endoplasmic reticulum	3.11
NM_002333	low density lipoprotein receptor-related protein 3	lipid transporter activity	3.12
NM_000211	integrin, beta 2 (ITGB2)	cell adhesion	3.78
NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	cell cycle	4.02
NM_080284	ATP-binding cassette, sub-family A (ABC1), member 6	transport	4.26
NM_000615	neural cell adhesion molecule 1	cell adhesion	4.31

and PCR was performed (Fatima *et al.*, 2002). GAPDH primer was used as the quantitative control. PCR products were subjected to electrophoresis and the intensities of bands were measured using Gelpro analyzer software (Media Cybernetics, Spring, USA).

Results

The over-expression of the PKD2 gene The transfection efficiency of the PKD2 gene was confirmed by RT-PCR and immunocytochemistry (data not shown). To identify the function of the PKD2 gene, we performed cDNA microarray analysis on epithelial cells derived from cysts of ADPKD patients (cyst epithelial cells). The results of our analysis using Genesight are shown in Fig. 1. The distribution showed a centralized pattern. Nineteen genes were up-regulated genes. These included neural cell adhesion molecule 1, which plays an important role in many neuro-development processes. Six genes were down-regulated genes, including matrix metalloproteinase 12, the product of which is one of the most abundant proteins in tissue re-modeling after injury (Table 1).

M-A plot M-A plot allows the detection of the intensity-dependent pattern of M; in log scale M represents $\log_2$R/G. A represents log(RG) (Yee *et al.*, 2002). Data were normalized using Lowess analysis (Christopher *et al.*, 2002). Presently, the most commonly used value for describing a genes expression level is the ratio in log scale. The $\log_2$ratio value, however, may be systematically dependent on the intensity. Locally weighted linear regression (Lowess) analysis has been proposed as a normalization method to remove intensity-dependent effects that affect the $\log_2$ ratio value (John, 2002). M/A data plots are shown in Fig. 2.

Table 2. Twenty-eight commonly up- or down-regulated genes obtained by hierachical clustering

Acc. #	Gene ID	Function	Fold
BC005809	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	unknown	0.3
BC000498	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	amino acid metabolism	0.37
XM_371951	coatomer protein complex, subunit gamma 2	vesicle transport	0.39
BC014267	CCCTC-binding factor (zinc finger protein)	transcription factor activity	0.43
BC010264	splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	pre-mRNA splicing factor activity	0.45
AL157499	homo sapiens mRNA; cDNA DKFZp434N2412	unknown	0.45
NM_139313	YME1 (S.cerevisiae)-like 1	unknown	0.45
NM_013290	GT198, complete ORF	unknown	0.46
NM_199248	calcium channel, voltage-dependent, beta 1 subunit	calcium ion transport	0.46
AF020774	homo sapiens hair and skin epidermal-type 12-lipoxygenase-related protein (ALOX12E) mRNA	unknown	0.47
NM_199187	keratin 18	intermediate filament	0.47
NM_006840	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2	receptor activity	0.52
NM_000712	biliverdin reductase A	electron transport	0.53
BC024026	transketolase (Wernicke-Korsakoff syndrome)	calcium ion binding	0.55
BC027946	FE65-LIKE 2	intracellular signaling cascade	2.53
NM_153322	peripheral myelin protein 22	peripheral nervous system development	2.59
NM_014888	predicted osteoblast protein	cytokine activity	2.6
XM_346206	tat-interacting protein (30kD)	apoptosis	2.62
BC023975	protein tyrosine phosphatase type IVA, member 1	prenylated protein tyrosine phosphatase activity	2.63
NM_201550	leucine-rich protein mRNA	unknown	2.7
NM_001249	ectonucleoside triphosphate diphosphohydrolase 5	endoplasmic reticulum	2.81
BC041789	albumin	unknown	3.04
BM433104	cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 2	endoplasmic reticulum	3.1
BC007408	low density lipoprotein receptor-related protein 3	lipid transporter activity	3.11
BC005861	integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1)	unknown	3.78
NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	cell cycle	4.02
NM_172346	ATP-binding cassette, sub-family A (ABC1), member 6	transport	4.25
BC014205	neural cell adhesion molecule 1	cell adhesion	4.31

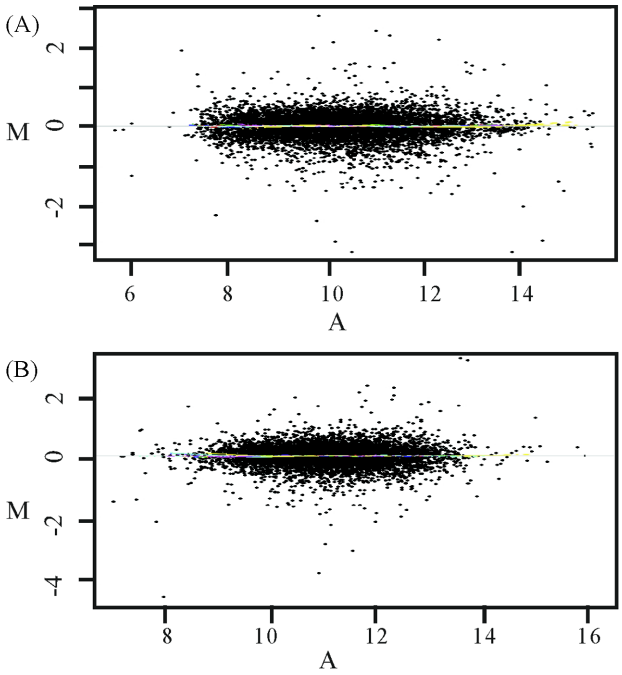

Fig. 2. M/A plot representation of ADPKD patients microarray hybridization data. (A) Scatter plots for patient 1 performing each repeat experiment. (B) Scatter plots for patient 2 performing each repeat experiment.

Clustering of DNA microarray data The subset of genes remaining after the removal of low quality genes was used to generate a dendrogram. Genes were grouped by complete hierachical clustering using Genesight 3.5 (Fig. 3), using the hierarchical clustering complete-link clustering algorithm (Herreo *et al.*, 2001). This was calculated by measuring two points located far apart in different clusters part. Even though differences in gene downregulation were beween patients, three genes (aspartate aminotransferase I, coatomer protein complex and CCCTC-binding factor) were identified by hierachical clustering.

RT-PCR A specific PCR fragment of selected genes was successfully amplified by RT-PCR using selected gene primers of the expected size. Three up-regulated genes and one down-regulated gene were detected by DNA microarray and by RT-PCR. Tumor-associated calcium signal transduction, NACM, and MMP-12 were up-regulated. In contrast, cadhedrin 4, type 1, and R-cadhedrin were down-regulated in epithelial cells derived from severe cysts (Fig. 4).

Discussion

Our data demonstrate the usefulness of microarray analysis

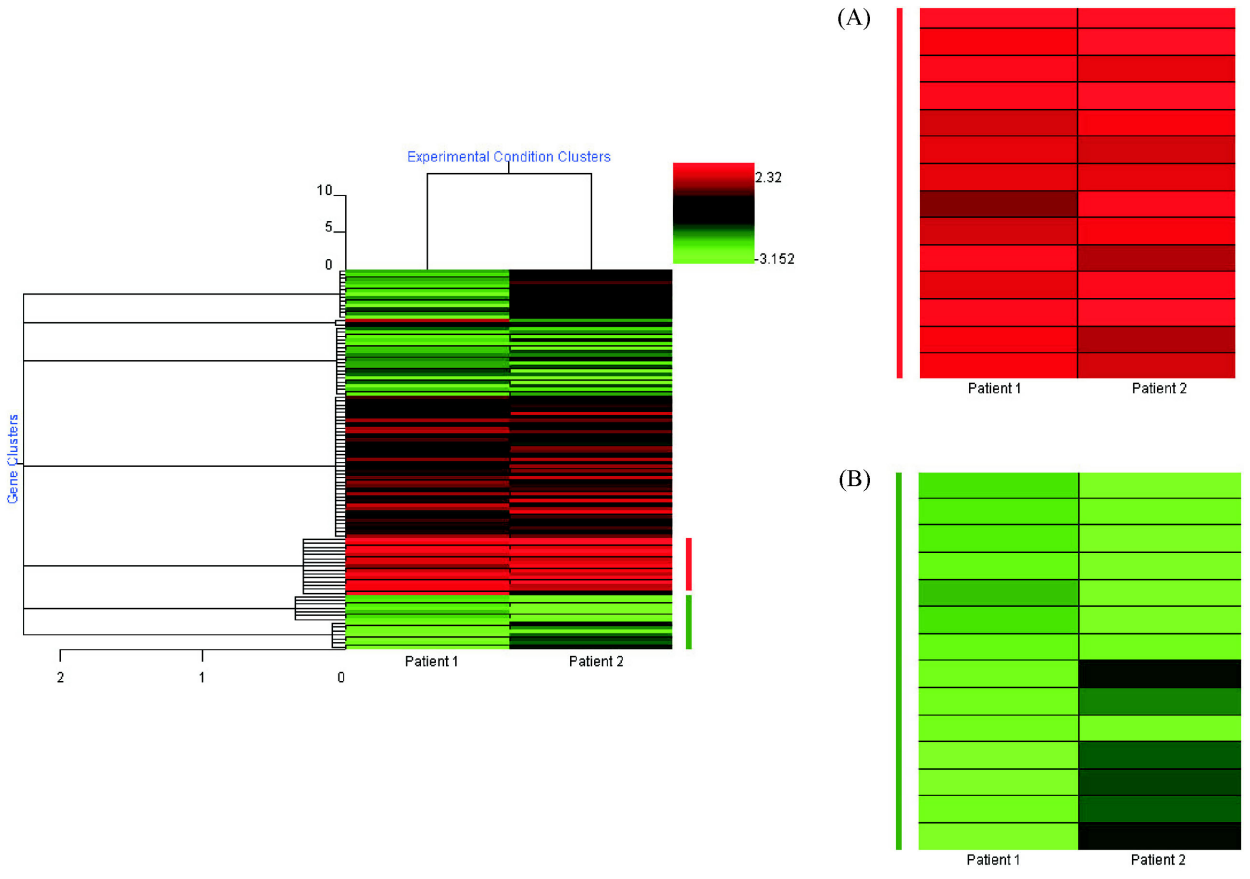

Fig. 3. Clusters of up- and down-regulated genes in two patients. (A) Up-regulated genes. (B) Down-regulated genes.

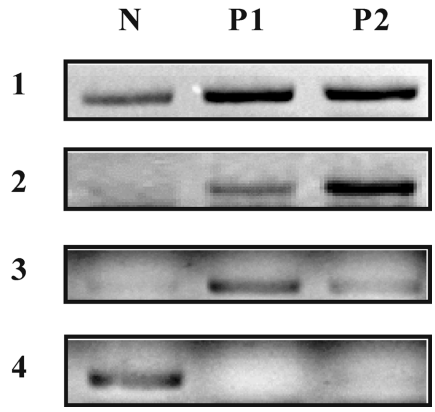

Fig. 4. The confirmation of candidate genes by RT-PCR. Lane N: Normal epithelial cell line, lane P1: ADPKD patient 1, lane P2: ADPKD patient 2, no 1: tumor-associated calcium signal transduction, no 2: neural cell adhesion molecule 1, no 3: cadhedrin 4, type 1, R-cadhedrin, no 4: matrix metalloproteinase 12.

for the determination of the gene expression pattern in human cyst epithelial cells. Apoptosis and differentiation in ADPKD tissue have been emerged, unlike normal tissue, but the pathway of cytogenesis was not characterized. To identify genes related to cytogenesis in ADPKD patients, we carried out cDNA microarray analysis of cyst epithelial cells derived from two patients. Microarray experiments were carried out on each sample three times. Image data was analyzed and clustered. Up- and down-regulated genes in epithelial cells were selected by comparing X and Y microarray data. Selected genes were also detected by semi-quantitative RT-PCR. Using the public GenBank database, neural cell adhesion molecule 1 (a cell adhesion molecule that plays important roles in many neuro-developmental processes), cadherin 4, type 1, R-cadherin (all calcium dependent cell adhesion molecules), matrix metalloproteinase 12 (involved in tissue re-modeling and injury), and tumor-associated calcium signal transducer 1 (a growth factor receptor) were identified from sequence data. All four genes may be related to cell proliferation. We analyzed whole cyst epithelial cell lines in this study and not heterogeneous cyst cells. Thus we potentially investigated aspects of cyst formation, which is important because further functional studies may yield the mechanism of cytogenesis.

In summary, we determined the global gene expression profiles of cyst epithelial cells in biopsy samples obtained from tissue by using cDNA microarrays, which may be useful to identify genes related to cytogenesis in ADPKD.

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