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Profiling of Gene Expression According to Cancer Stage in Clear Cell Type of Renal Cell Carcinoma

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Abstract

For toxicity model in the kidney, renal cell carcinoma (RCC) is one of the most important model to assess the structural and functional alterations. Most RCCs are sporadic, and environmental agents are suspected to play a role in the etiology of the disease. In this study, we discovered novel evidence for previously unknown gene expression patterns related to progression according to cancer stage in RCC. Four clear cell RCC tissue samples along with five corresponding patient-matched normal kidney tissue samples were obtained from patients undergoing partial or radical nephrectomy. To examine the difference of gene expression profile in clear cell RCC, radioactive cDNA microarrays were used to evaluate changes in the expression of 1,152 genes in a total. Using ^{33}P-labeled probes, this method provided highly sensitive gene expression profiles including drug metabolism, and cellular signaling. 29 genes were identified with expression levels that differed by more than 2.0 value of z-ratio, compared with that in control. Whereas expression of 38 genes were decreased by less than -2.0 value of z-ratio. In conclusion, this study has identified 67 gene expression alterations in clear-cell type of RCC. Most notably, genes involved in cell growth were up-regulated in stage I more than stage III whereas genes involved in signal transduction were down-regulated in which both stage I and stage III. The identified alteraions of gene expression will likely give in sight in to clear cell RCC and tumor progression.

Keywords: renal cell carcinoma, progression, gene expression profile, cDNA microarray

Renal Cell Carcinoma (RCC) is relatively rare, accounting for approximately 3% of malignancies worldwide and is increasing in incidence. Between one-third and one-half of the 30,000 patients diagnosed with renal cell carcinoma in the United States each year will die of their disease[1]. RCC is the 8th most common tumor in men and the 11th most common tumor in women, and its incidence (1-12 per 100,000) is rising steadily, by about 2-3%per year in industrialized countries[2]. Kidney cancer occurs most often in people between the ages of 50 and 70, and affects men almost twice as often as women.

Renal neoplasms include a variety of malignant and benign tumors, including several subtypes of renal cell carcinoma (RCC), oncocytoma, urothelial (transitional cell) carcinoma of the renal pelvis, and childhood Wilms tumor. Traditionally, this classification is based on morphologic features defined in the World Health Organization *International Histological Classification of Kidney Tumors*[3]. Histopathologically, RCC is a heterogeneous disease. The five distinct types of RCC include clear cell (70-80%), papillary (15-20%), chromophobe (45%), collecting duct (< 1%) and medullary cell (< 1%)[4]. The most common RCC, clear cell RCC, is believed to distinguish between common sporadic (95% of cases) and rare familial forms of RCC. In both cases, several chromosome abnormalities have been described, mostly the 3p25-26 region, in which the von Hippel-Lindau disease tumor suppressor gene resides[5].

Staging systems allow standardized classification of the anatomical extension of tumors. Over years their codification has been a fundamental activity in oncology, mainly for its prognostic relevance. In 1958 Flocks and Kadesky proposed the first classification of renal cell carcinoma according to the anatomical extension of tumors: tumors limited to the renal capsule (stage I), invasion of the renal pedicle and/or renal fat (stage II), regional lymph node involvement (stage III), and demonstrable distant metastasis (stage IV)[6].

Several environmental, medical, and genetic risk factors have been implicated in the etiology of this disease. Most RCCs are sporadic, and environmental agents are suspected to play a role in the etiology of the disease[7]. For toxicity model in the kidney, Renal cell carcinoma (RCC) is one of the most important model to assess the structural and functional altera-

tions. Although several events that contribute to metabolism are well-established consequences of sustained RCC, underlying mechanisms and relationships between RCC and metabolism by high-throughput approaches are ill-defined. Thus, our study was to determine the identification of novel targets for RCC related with metabolism by using cDNA microarray for understanding their therapeutic effects.

An important outcome is the identification of potential gene-based markers of renal metabolism and evidence that one can detect region-specific renal tissue using microarray technology[8]. Additional functional genomics studies may afford the opportunity to validate the proposed novel gene-based markers of renal metabolism, which in part may improve current sensitivity to assessing xenobiotic-induced carcinoma.

Recent developments in toxicogenomics have led to the development of DNA microarray technology, a tool of unprecedented power for the study of gene sequence, structure, and expression[9]. Using cDNA microarrays, the expression of thousands of genes can be monitored simultaneously and expression patterns in response to various biological stimuli compared with normal control can be observed. In this study, we carried out to discover novel evidence for previously unknown gene expression patterns related to metabolism in clear cell RCC tissue samples along with corresponding patient-matched normal kidney tissue samples.

Clinical Assessment

Clinical characteristics of clear cell RCC patients are presented in Table 1. No age- or sex-related differences in clear cell RCC and control patients were detected in any functional parameter measured (Spearman's correlation analysis).

Superimposed Image Analysis in Clear Cell RCC Patients

Radioactive hybridization was visualized by phos-

Table 1. General Characteristics of Subjects

Name	Sex/age	TNM	Stage	Type
금OO	M / 51	T1NoMo	Stage I	Clear cell
이OO	M / 63	T1NoMo	Stage I	Clear cell
조OO	F / 60	T1NoMo	Stage I	Clear cell
배OO	M / 55	T4N1Mo	Stage III	Clear cell

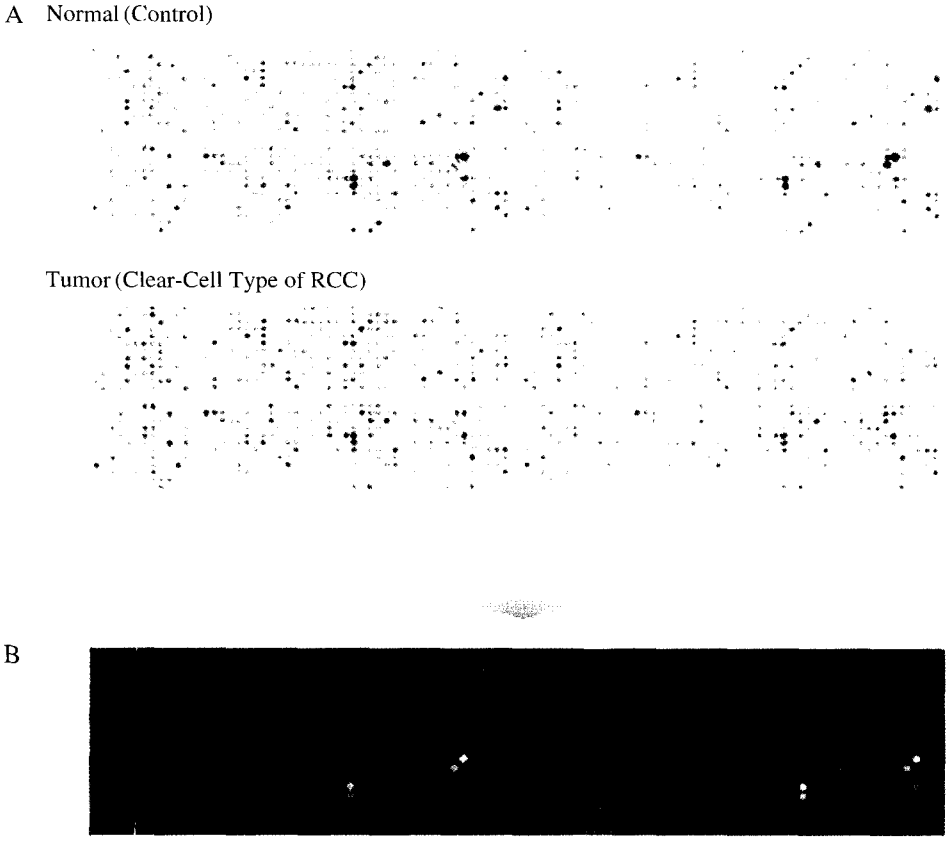

Fig. 1. Superimposed image. A, Representative cDNA microarrays of two independent hybridization experiments comparing cDNAs generated from controls (down) or from clear cell RCC patients (up). The cDNA microarray contained the two sets of 1,152 genes and printed in duplicate, and each duplicate is composed of eight individual subarrays. For example, two genes differentially expressed between controls and clear cell RCC patients are marked by circles (red, insulin-like growth factor binding protein 3; green, Tyrosine aminotransferase). B, Superimposed image of primary images of controls and clear cell RCC patients.

phoimager technologies. The primary image, that is the results of primary capture by phosphoimager, is shown in Figure 1A. This particular array was printed in duplicate and each duplicate was composed of eight individual subarrays. Visual inspection of the hybridization patterns readily identified a number of signals differentially expressed between normal and tumor tissue. Figure 1B is a superimposed image in which red color represents up-regulation and green represents down-regulation in clear cell RCC patients, and yellow represents genes of higher expression in clear cell RCC and controls, such as housekeeping genes. Analysis of the median densitometric signal intensity revealed that 67 genes differed between clear-cell RCC patients and controls by a Z-ratio of 2 at a descriptive $P \leq 0.05$. We marked two genes: two genes differentialy expressed between clear-cell RCC and control tissues (red, insulin-like growth factor binding protein 3 B; green, Tyrosine aminotransferase).

Gene Expression Profiles in Clear Cell RCC Patients

Using the cDNA expression array, we found that among 1,152 genes on the array membrane, 20% of total genes presented a quantifiable expression in clear-cell RCC. Gene expression profiles of interest were significantly up-regulated (29 genes) or down-regulated (38 genes) in clear cell RCC when compared with control patients. Genes showing highly altered expression levels were aligned in the order of the magnitude of altered expression in clear cell RCC. The up- and down-regulated genes are listed in Table 2 and 3. Gene expression profiles showed that 25 genes were up-regulated in stage I and 4 were up-regulated in stage III (Table 2); e.g., Insulin-like growth factor binding protein 3 (IGFB-3), insulin-like growth factor-binding protein 4 (IGFB-4), Protein tyrosine phosphatase, receptortype, mupolypeptide (PTPRA), ribosomal S6 kinase 1 (RSK1), vascular endothelial growth factor (VEGF), ProteinkinaseC (PKC), Arachidonate12-lipoxygenase (ALOX12and

Table 2. Up-regulated gene expression in clear-cell type of RCC

Up-Gene name	Z-ratio			
	T2	T3	T4	T5
Stage I				
BreastepithelialantigenBA46mRNA, completecds	8.83	7.87	3.68	1.96
Insulin-like growth factor binding protein 3	6.16	2.04	3.04	0.08
Fibronectin 1	1.50	7.08	2.53	0.15
ribosomal protein L7a; SURF3	2.52	4.67	1.74	1.63
Protein-tyrosinephosphatasemRNA, completecds	3.45	3.54	2.28	0.73
60S Ribosomal Protein L23 (RPL23: ribosomal protein L23)	3.04	4.05	1.65	1.42
GA-binding proteintranscriptionfactor, betasubunit2	1.52	6.17	3.03	−0.63
Protein tyrosine phosphatase, receptortype, mupolypeptide	4.03	4.12	0.85	−1.46
"Major histocompatibility complex, class II, DN alpha"	3.45	2.88	2.01	−0.39
GTP-binding protein Ran/TC4	2.23	2.94	2.99	−0.68
Protein tyrosine phosphatase, receptortype, alphapolypeptide	4.74	1.05	0.20	1.62
AnnexinI (lipocortinI)	1.33	2.51	3.03	0.03
Cyclin K (CPR4)	4.03	0.95	1.80	0.73
ribosomal S6 kinase 1 (RSK1)	2.34	2.18	2.72	−0.82
vascular endothelial growth factor	2.81	1.38	1.76	0.12
Phosphofructokinase, platelet	3.29	1.60	1.79	1.67
Proteinphosphatase 1, catalyticsubunit, alphaisoform	2.01	2.23	1.70	1.48
insulin-like growth factor-binding protein 4	1.66	3.32	0.87	0.45
Clk1mRNA, completecds	0.05	2.06	−0.56	−0.87
proteinphosphatasewithEF-hands-1 (PPEF-1)mRNA, completecds	2.17	0.74	1.95	−0.68
AP-2betatranscriptionfactor	2.39	0.42	2.28	0.39
Guanine nucleotide binding protein (G protein),	2.48	2.23	0.90	0.75
Guanine nucleotide binding protein betasubunit-likeprotein12.3	2.30	1.40	1.64	0.95
cytoplasmic beta-actin (ACTB)/Actin 1 (beta cytoskeletal actin)	1.74	3.05	0.30	0.66
Stage I and Stage III				
ProteinkinaseC, beta1	4.20	1.90	0.99	2.12
Stage III				
calmegin, completecds	−0.34	−0.89	0.13	2.93
Arachidonate12-lipoxygenase	0.13	−0.59	0.37	2.13
Proteinphosphatase2 (formerly2A)	1.57	0.48	1.70	2.08

Table 3. Down-regulated Gene Expression in Clear-cell Type of RCC

Down-Gene name	Z-ratio			
	T2	T3	T4	T5
Stage I				
TFIIBrelatedfactorhBRF (HBRF)mRNA, completecds	−1.00	−1.02	−5.12	−0.51
heat shock protein 40 homolog (HSP40 homolog); DNAJW	−0.58	−0.85	−3.65	−0.68
K-rasoncogeneproteinmRNA, completecds	−0.73	−1.40	−3.11	0.20
Possible GTP-binding protein HSR1	−0.05	−0.54	−3.99	−0.65
Feline sarcoma (Snyder-Theilen) viral (v-fes)/	−1.37	−0.22	−2.63	−0.72
chemokine (C-C motif) receptor 7	−1.24	−0.48	−1.93	−0.15
Stage I and Stage III				
Tyrosine aminotransferase (TAT)	−6.22	−4.80	−7.70	−11.15
Apoptosis-associated tyrosine kinase	−5.46	−4.48	−6.66	−9.58
BCL-2 binding athanogene-1; glucocorticoid receptor-associated protein RAP46	−4.46	−2.91	−6.62	−8.69
Interleukin 9 receptor	−5.46	−3.63	−6.84	−7.05
Superoxide dismutase 1, soluble	−6.31	−4.60	−6.82	−6.17
Endothelial transcription factor GATA2	−1.61	−1.35	−3.21	−4.91
OS-9precurosormRNA, completecds	−2.46	−2.32	−0.73	−4.30
"Janus kinase 3 (a protein tyrosine kinase, leukocyte)"	−3.05	−1.82	−2.92	−3.43
Stage III				
Thyroid stimulating hormone receptor	2.31	−0.87	−2.23	−4.31
Androgen receptor	−1.04	−0.16	−2.19	−3.92
Bruton agamma globulinemia tyrosine kinase	−1.67	−0.89	−3.04	−3.71
GTP binding protein (ARL3)mRNA, completecds	0.46	1.08	0.33	−2.73
ESTs, Weakly similar to glycinereceptoralpha-1 chain	−0.27	−0.09	−0.49	−2.52
ESTs, Highlysimilartoprotein-tyrosinephosphatasemeg1	−0.29	−0.56	−0.34	−2.50
Mitogen-activated protein kinase P38 beta (MAP kinase P38 beta)	0.16	0.22	0.25	−2.43
RAD51 (S. cerevisiae) homolog C	0.44	1.60	0.25	−2.15
Nuclear factor I/C (CCAAT-binding transcription factor)	−1.10	−0.45	−1.22	−2.10
Glutamate receptor, ionotropic, N-methyl D-aspartate 1	0.77	0.79	0.29	−2.27
ESTs, Highly similar to inorganic pyrophosphatase	−0.63	−1.12	−1.23	−2.27
Activation-induced down-regulation of retinoid receptor RXR alpha	−1.02	−0.90	−1.34	−2.11
68 kDa type I phosphatidy linositol-4-phosphate 5-kinaseal phamRNA	−0.78	1.10	−0.72	−2.10
ESTs, Weaklysimilar to cell division proteinkinase5	0.07	1.10	−0.72	−2.07
ESTs, Highly similar to GTP-binding protein SARA	0.09	0.06	−0.71	−2.06
G1/S-specific cyclin D3 (CCND3)	1.14	1.46	−0.07	−2.05

Protein phosphatase 2 (PPP2R3A).Gene expression profiles showed that 14 genes were down-regulated in stage I and 24 were down-regulated in stage III (Table 3); e.g Endothelial transcription factor GATA2 (GATA), Janus kinase 3 (JAK3), Androgen receptor, and Nuclear factor I/C NFI, and signal transduction regulation gene such as Mitogen-activated protein kinase P38 beta (MAP kinase P38 beta).

Figure 4 is Scatter plot for comparing the expression profiles of according to cancer stage in clear cell RCC and control patients. Expression profiles of clear cell RCC and control patients are shown as scatter plot of 1,152 genes from the microarray. Regression analysis of Z scores from two independent samples of clear cell RCC and control were performed and Z scores of individual genes were plotted. To obtain a molecular portrait of relationships metabolism associated with clear cell RCC, we used a hierarchical clustering algorithm to group genes on the basis of similar expression patterns[12], and the data are presented in a matrix format (Figs. 2, 3). Each row of Figure 2 and Figure 3 represents all hybridization results for a single DNA element of the array, and each column represents the expression levels for all genes in a single hybridization sample. The expression level of each gene was visualized in color, relative to its median expression level across all samples. Red represented expression greater than the mean, green represents expression less than the mean, and color intensity denotes the degree of deviation from the mean. Gray represented median expression level. Distinct samples representing similar gene patterns from control cells were aligned in adjacent rows. The cells included in this map were samples from right

Fig. 2. Genes expression in Clear-cell Type of RCC (Stage I). Scatter plot for comparison of expression profile between controls and clear cell RCC patients. Expression profiles of controls and clear cell RCC patients are shown as bivariated scatter plot of 1,152 genes from the microarray. The values are corrected intensities relative to control, representing levels of expression for the cDNA elements of the microarrays.

clear cell RCC and control patients. Coordinately expressed genes were grouped into clusters, which we named on the basis of the cellular process in which component genes participated. The clustergram revealed that clusters of genes related to progression were up- and down-regulated in clear cell RCC patients, as compared to controls (Figs. 2, 3).

Pathologic stage is the most powerful predictor of prognosis in renal cell carcinoma. In tumours without metastasis, prognostic differences in T1/2 tumours versus T3/4 tumours are well known[13]. However it is unknown the differences of gene expression patterns according to cancer stage in RCC. In this study, we carried to discover some novel evidences for changes of gene expression in stage I and stage III of RCC.

To better understand the genetics and biology of clear cell RCC, we profiled the expression of 1,152 genes in clear cell RCC tissue samples along with corresponding patient-matched normal kidney tissue samples. The gene expression profiles were analyzed, visualized using singular value decomposition analysis, and categorized depending on their functions, as follows.

In this study, most notably, genes involved in cell growth were up-regulated in stage I more than stage III. The eight genes were differentially expressed in the cell growth group are up-regulated in clear cell type of RCC, such as Insulin-like growth factor binding protein 3 (IGFB-3), insulin-like growth factor-binding protein 4 (IGFB-4), Protein tyrosine phosphatase, receptortype, mupolypeptide (PTPRA), ribosomal S6 kinase 1 (RSK1), vascular endothelial growth factor (VEGF), ProteinkinaseC (PKC), Arachidonate12-lipoxygenase (ALOX12 and Protein phosphatase 2 (PPP2R3A)).

The insulin-like growth factors (IGF), their recep-

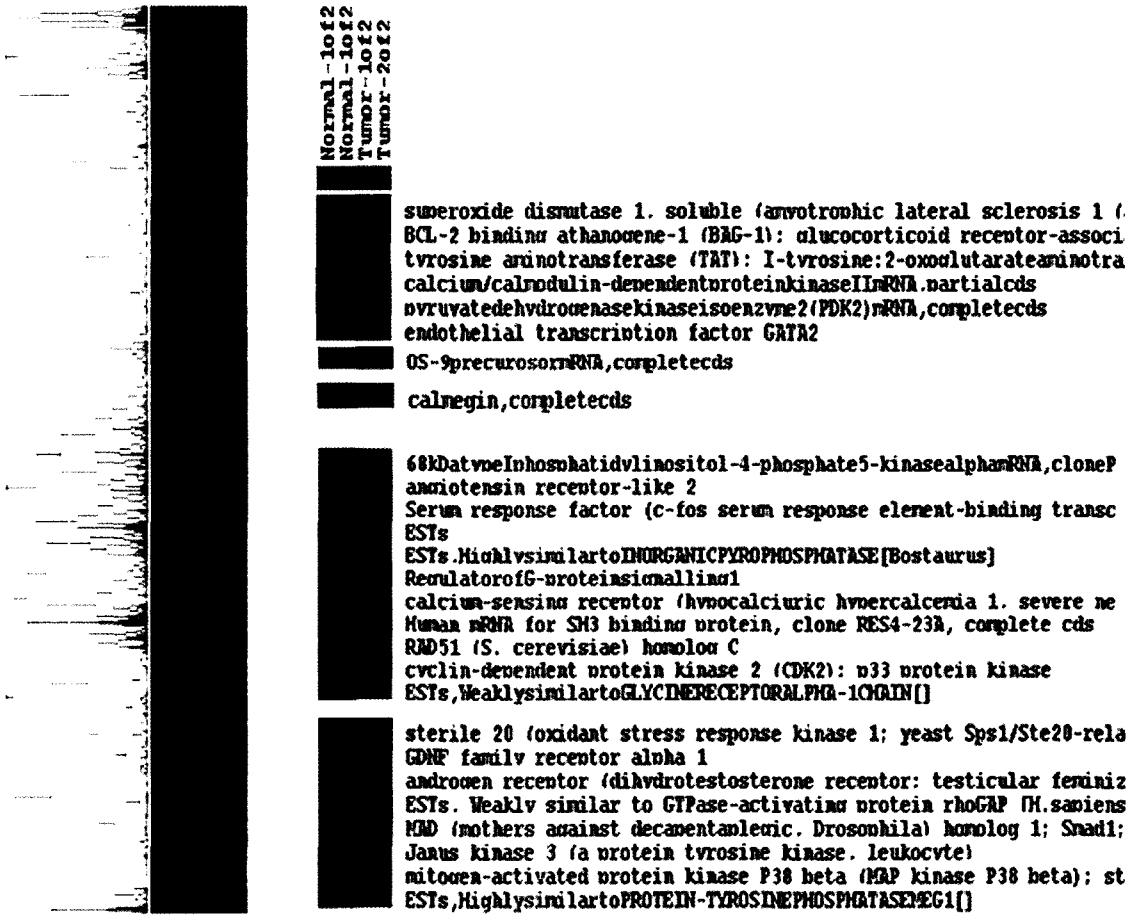

Fig. 3. Genes Expression in Clear-cell Type of RCC (Stage III). Clustergram of gene expression in clear cell RCC (Stage I). Microarray data from controls and clear cell RCC (stage I) patients were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate programs available as shareware from Michael Eisen's lab. Each gene is represented by a single row of colored boxes; each experimental sample is represented by a single column. The entire clustered image is shown on the left. These clusters contain uncharacterized genes and genes not involved in these processes.

tors, and their binding proteins play key roles in regulating cell proliferation. IGF regulates the cellular growth of normal and malignant epithelial cells, and stimulates cellular proliferation with a role in malignant transformation or potent mitogens[14,15,16]. IGFB family which alters cell adhesion expression greatly is very likely associated with carcinogenesis, tumor invasion[17,18]. Also, PTPRA encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. Another cell proliferation related genes, such as RSK, VEGF, PKC, PPP2R3A, and ALOX12 were shown in our study. Sassone-Corsi *et al.* (1999) reported that RSK phosphorylates histone H3 and mediates chromatin remodeling[19]. This gene encodes a member of the RSK family of serine/threonine kinases. The activity of this protein has been implicated in controlling cell growth and differentiation. We and others showed that VEGF activates the small GTPase Rac1 that is important for VEGFR2-mediated signaling linked to formation of lamellipodia associated with focal complexes, cell migration, and proliferation[20] as well as activation of NAD (P)H oxidase, a major source of reactive oxygen species (ROS) in ECs[21,22]. Protein kinase C encoded by PKC has been reported to be involved in many different cellular functions, such as B cell activation, apoptosis induction, endothelial cell proliferation, and intestinal sugar absorption. ALOX12 which was highly up-regulated in stage I was believed to play important roles in tumor promotion, progression. 12-lipoxygenase and its metabolites have

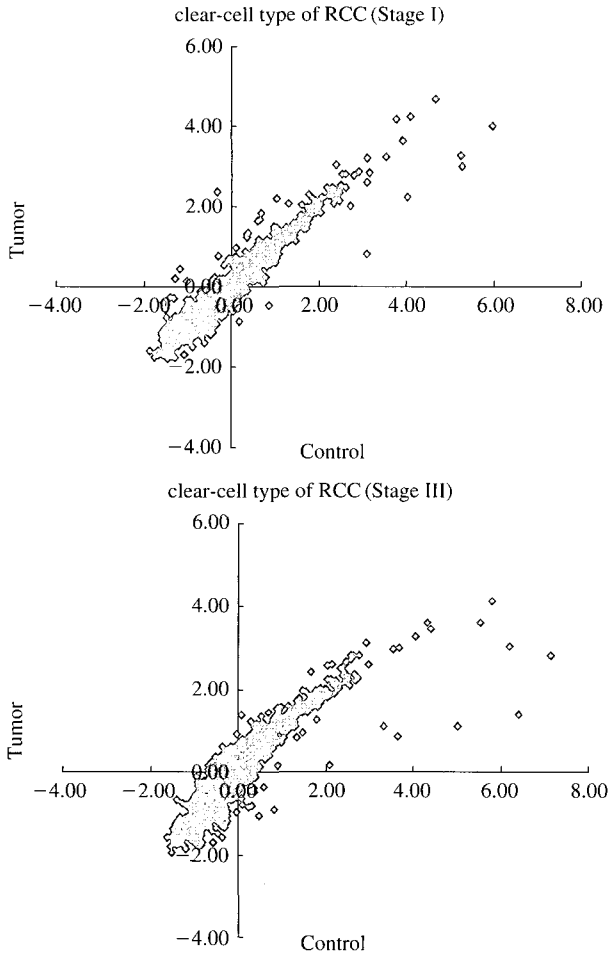

Fig. 4. Scatter Plot: Clustergram of Gene Expression in Clear Cell RCC (Stage III). Microarray data from controls and clear-cell RCC patients were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate programs available as shareware from Michael Eisen's lab. Each gene is represented by a single row of colored boxes; each experimental sample is represented by a single column. The entire clustered image is shown on the left. These clusters contain uncharacterized genes and genes not involved in these processes.

been shown to induce the growth of tumour in animal models and increase angiogenesis in breast cancer and prostate cancer[23]. The product of PPP2R3A belongs to the phosphatase 2 regulatory subunit B family. It is one of the four major Ser/Thr phosphatases, and it is implicated in the negative control of cell growth and division.

The remaining over-expressed genes whose expression were more attenuated in stage I than in stage III were mainly associated with cell cycle, signal transduction, and cell adhesion. Cell cycle regulatory genes such as Cyclin K (CPR4) and Clk1mRNA were shown to be up-regulated in stage I specially. The product encoded by CPR4 may play a dual role in regulating CDK and RNA polymerase II activities, and Clk1mRNA which is denoted "CLK" (for CDC-like kinase), represents a prototype for a new family of human protein kinases bearing significant homology to the yeast cdc2/CDC28 kinases that regulate the cell cycle[24].

The three genes involved in signal transduction, GTP-binding protein Ran/TC4 (RAN), Guanine nucleotide binding protein betasubunit-likeprotein12.3 (GNAI2) and Guanine nucleotide binding protein (G protein). RAN (ras-related nuclear protein) is a small GTP binding protein belonging to the RAS superfamily that is essential for the translocation of RNA and proteins through the nuclear pore complex. The RAN protein is also involved in control of DNA synthesis and cell cycle progression. The protein encoded by GNAI2 is a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression. Ohneda K *et al.* (2002). suggested that somatic cell mutations in the cAMP-dependent signal transduction pathway occurring during myocardial development might be responsible for some forms of idiopathic ventricular tachycardia[25].

Another more up-regulated gene in stage I than in stage III, Fibronectin 1 is an adhesive protein that binds to the external face of the plasma membrane and enables cells to interact with the extracellular matrix[26]. The cell-binding region of fibronectin 1 binds and releases integrin, a complex of proteins that span the plasma membrane[4]. These interestingly up regulated genes in stage I seem to be associated with proliferation and carcinogenesis of normal cell in initiation stage.

Whereas, genes involved in signal transduction related genes encoding transcription factor, apoptosis relate protein, and antioxidant enzyme were down-regulated in which both stage I and stage III. Also, these down regulation were more significant in stage III than in stage I.

In this study, down-regulated genes in RCC were transcription factor genes such as Endothelial transcription factor GATA2 (GATA), Janus kinase 3 (JAK3), Androgen receptor, and Nuclear factor I/C NFI, and signal transduction regulation gene such as Mitogen-activated protein kinase P38 beta (MAP kinase P38 beta). GATA-1 and GATA-2 have been identified as key molecules in gene regulation during erythroid cell differentiation[27]. JAK3 encodes Janus kinase 3, a tyrosine kinase that belongs to the Janus family. JAK3 functions in signal transduction and

interacts with members of the STAT (signal transduction and activators of transcription) family[28]. It is predominantly expressed in immune cells and transduces a signal in response to its activation via tyrosine phosphorylation by interleukin receptors. MAP kinase targets specific transcription factors, and thus mediates immediate-early gene expression in response to various cell stimuli[29].

Another down-regulated genes were apoptotic genes such as Apoptosis-associated tyrosine kinase (AATYK) and Bruton agammaglobulinemia tyrosine kinase (BTK), and antioxidant gene such as Superoxide dismutase 1 (SOD1). AATYK expressed during apoptosis may induce growth arrest and/or apoptosis of myeloid precursor cells. BTK, a non-receptor-associated tyrosine kinase of the Tec family, appears to participate in many myeloid cell functions[30]. SOD1 which defenses against oxidants as an antioxidant enzyme were down regulation in both stage I and III[9].

The new technology allows automated imaging analysis and is well suited for the large-scale study of gene expression patterns in vitro and in vivo. The large-scale analysis of the gene expression levels can provide insights into the underlying molecular mechanism of RCC and possibly lead to the finding of molecular tumor markers that can potentially be used for more accurate diagnosis, prognosis and possibly can serve as drug targets for effective therapies. Many genes were found to be differentially expressed in RCC compared with normal kidney.

In conclusion, this study has identified 67 gene expression alterations in clear cell RCC. Most notably, genes involved in cell growth were up-regulated in stage I more than stage III whereas genes involved in signal transduction were down-regulated in which both stage I and stage III. The identified alteraions of gene expression will likely give in sight in to clear-cell type of RCC and tumor progression. Also, we demonstrated that RCC is one of the feasible model to assess the nephrotoxicity of environmental mutagen and carcinogen with the standing point of structure and function in the kidney; and ^{33}P-labeled cDNA microarray is highly likely to be an efficient technology to evaluate the gene regulation related to progress in clear cell RCC model.

Methods

Patients

Renal cell carcinoma were four clear cell RCC tissue samples (three of them were stage I, one stage III) along with four corresponding patient-matched normal kidney tissue samples were obtained from patients undergoing partial or radical nephrectomy at the Cleveland clinic Foundation Korea University Medical Center. All subjects completed a questionnaire, which include item on, sex, age, stage, type ectc. Table 1 details clinical characteristics of clear cell RCC patients.

Human cDNA Microarray

A human cDNA microarray was primarily derived from a commercially available master set of approximately 15,000 human verified sequences (Research Genetics, Inc., Huntsville, AL). The 15,000-human cDNA clone set was sorted for a list of genes (1,152 elements) representing families such as differentiation, development, proliferation, transformation, cell-cycle progression, immune response, transcription and translation factors, oncogenes, and molecules involved in cell growth and maintenance. PCR-amplified cDNAs were spotted on nylon membranes. The general methodology of arraying is based on the procedures of DeRisi *et al.* (1996).

cDNA Radiolabeling

Total RNAs prepared from renal tissues of patients with or without RCC were used to synthesize ^{33}P-labeled cDNAs by reverse transcription. Briefly, 3-10 g of RNA were labeled in a reverse transcription reaction containing 5 X first-strand PCR buffer, 1 g of 24-mer poly dT primer, 4 *l* of 20 mM each dNTP excluding dCTP, 4 *l* of 0.1 M DTT, 40 U of RNase inhibitor, 6 *l* of 3000 Ci/mmol-^{33}P dCTP to a final volume of 40 *l*. The mixture was heated at 65 C for 5 min, followed by incubation at 42 C for 3 min. Two *l* (specific activity: 200,000 U/ml) of Superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) was then added and the samples were incubated for 30 min at 42 C, followed by the addition of 2 *l* of Superscript II reverse transcriptase and another 30 min of incubation. Five *l* of 0.5 M EDTA was added to chelate divalent cations. After the addition of 10 *l* of 0.1 M NaOH, the samples were incubated at 65 C for 30 min to hydrolyze the remaining RNA. Following the addition of 25 *l* of 1 M Tris (pH 8.0), the samples were purified using Bio-Rad 6 purification columns (Hercules, CA). This resulted in 5×10^6 to 3×10^7 cpm per reaction[10] (Vawter *et al.*, 2001).

Hybridization & Scanning

cDNA microarrays were pre-hybridized in hybridization buffer containing 4.0 ml Microhyb (Research Genetics), 10 *l* of 10 mg/ml human Cot 1 DNA (Life Technologies), and 10 *l* of 8 mg/ml poly dA (Pharmacia, Peapack, NJ). Both Cot 1 and poly dA were denatured at 95 C for 5 min prior to use. After 4 h of

prehybridization at 42 C, approximately 10^7 cpm/ml of heat-denatured (95 C, 5 min) probes were added and incubation continued for 17 h at 42 C. Hybridized arrays were washed three times in 2 × SSC and 0.1% SDS for 15 min at room temperature. The microarrays were exposed to phosphoimager screens for 1-5 days, and the screens were then scanned in a FLA-8000 (Fuji Photo Film Co., Japan) at 50-m resolution[10].

Data Analysis

Microarray images were trimmed and rotated for further analysis using L-Processor (Fuji Photo Film Co., Japan). Gene expression of each microarray was captured by the intensity of each spot produced by radioactive isotopes. Pixels per spot were counted by Arrayguage (Fuji Photo Film Co., Japan) and exported to Microsoft Excel (Microsoft, Seattle, WA). The data were normalized with Z transformation to obtain Z scores by subtracting each average of gene intensity and dividing with each standard deviation. Z scores provide each of 2,304 spotted (two sets of 1,152 genes) genes with the distance from the average intensity and were expressed in units of standard deviation. Thus, each Z score provides flexibility to compare different sets of microarray experiments by adjusting differences in hybridization intensities. Gene expression difference as compared with untreated control cells was calculated by comparing Z score differences (Z differences) among the same genes. This facilitates comparing each gene that had been up- or down- regulated as compared with the control cells. Z differences were calculated first by subtracting Z scores of the controls from each Z score of the samples. These differences were normalized again to distribute their position by subtracting the average Z difference and dividing with the standard deviation of the Z differences. These distributions represent the Z ratio value and provide the efficiency for comparing each microarray experiment[10]. Scatter plots of intensity values were produced by Spotfire (Spotfire, Inc., Cambridge, MA)[11]. Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisen's laboratory (http://rana.lbl.gov). Clustering of changes in gene expression was determined by using a public domain cluster based on pair-wise complete-linkage cluster analysis[12].

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