Expression of Stress-Response and Cell Proliferation Genes in Renal Cell Carcinoma Induced by Oxidative Stress

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Ferric nitrilotriacetate induces oxidative damage in renal proximal tubules that ultimately leads to a high incidence of renal cell carcinoma (RCC) in rats. In search of genes specifically involved in oxystress-induced carcinogenesis, we have applied a modified fluorescent differential display technique to the tumors and an established cell line as well as their non-neoplastic counterparts. We screened approximately 84,000 products. Reverse Northern blotting confirmed differential expression of 20 transcripts, which showed either significant increase, decrease or lack of expression in the RCCs. Five cDNA clones encoded novel products of unknown function. Fifteen cDNA clones were identified by homology search, which included annexin II, Y-box binding protein, ribosomal proteins, heat shock proteins, DNA polymerase, nonmuscle caldesmon (increased); protein tyrosine phosphatase (decreased); selenoprotein P, stromal cell-derived factor 1, intestinal trefoil protein, nicotinamide adenine dinucleotide, reduced form (NADH) dehydrogenase, and insulin-like growth factor binding protein 7 (deleted). Most of the identified genes were associated with stress-response or cellular proliferation. These results suggest that multiple, interactive genetic pathways are involved in carcinogenesis induced by oxidative stress. (*Am J Pathol* 2000, 156:2149–2157)

A role of oxidative stress in carcinogenesis has been widely accepted.^{1,2} However, the mechanisms involved are still largely unknown. To elucidate the detailed genetic processes, we have been working on a rat model in which involvement of reactive oxygen species (ROS) are highly likely; an iron chelate, ferric nitrilotriacetate (Fe-NTA), induces renal proximal tubular degeneration, a consequence of oxidative injury after Fenton-like reaction, that ultimately leads to a high incidence of renal cell carcinoma (RCC).^{3–6} We have thus far demonstrated in the target organ of this model increased levels of lipid peroxidation products (thiobarbituric acid-reactive sub-

stances, various aldehydes including malondialdehyde and 4-hydroxy-2-nonenal),^{7,8} aldehyde-modified proteins,^{9,10} and a variety of oxidatively modified DNA bases,¹¹ as well as thymine-tyrosine DNA-protein cross-links.¹² A load of oxidative stress on the renal tubular cells is further thought to continue for the whole life in the rats that received repeated injecting of Fe-NTA, chiefly because of heavy iron deposition.^{5,6} These data strongly suggest the involvement of ROS in the initiation, promotion and progression processes of carcinogenesis. In a previous report, we found by a genetic approach frequent loss of heterozygosity (LOH) on chromosome 5 and 8 in Fe-NTA-induced rat RCCs. This led to a finding that p15^{INK4B} and p16^{INK4A} tumor suppressor genes are two of the major targets, which were either deleted or methylated in the 5' CpG island region in approximately half of the cases.¹³

In the present study, to identify and isolate differentially expressed genes during oxystress-induced renal carcinogenesis, we have developed and used modified fluorescent differential display (FDD) technique. We identified 15 genes that revealed either significant increase, decrease, or lack of expression, and also found five novel transcripts. Most of the identified genes were associated either with stress-response or cellular proliferation.

Materials and Methods

Tissue Samples and Cell Lines

RCCs were induced in F1 hybrid rats between Wistar strain (Shizuoka Laboratory Animal Center, Shizuoka, Japan) and Long-Evans strain (originally outbred from Ben May Laboratory for Cancer Research, University of Chicago, Chicago, IL) by administering Fe-NTA as previously described.^{13–15} RCCs or nontumorous renal tissues were carefully dissected with razor blades and kept fro-

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zen at -80°C until use. FRCC001 cell line was established from a Fe-NTA-induced renal cell carcinoma (F1 hybrid rat, No. 10-21-4, grade-2, granular cell subtype, solid structure, INF- β with lung metastasis)¹⁵ using a procedure previously described,¹⁶ and were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and antibiotics/antimycotics (GIBCO; penicillin G 100 U/ml; streptomycin sulfate 100 $\mu\text{g}/\text{ml}$; amphotericin B 0.25 $\mu\text{g}/\text{ml}$). This cell line formed typical epithelial monolayers on plastic, revealed anchorage-independent growth after confluence, and proliferated well in the subcutis of nude mice with no evidence of metastasis.

RNA Isolation and Northern Blotting

Total RNA was isolated from each frozen tissue or cells by means of a modified acid guanidinium/phenol/chloroform method (Isogen, Nippon Gene, Tokyo). Poly(A)-rich RNA was isolated by oligo(dT)-latex beads (Nippon Roche, Tokyo). For Northern blot analysis, poly(A)-rich RNA sample (2 μg) was separated on 1% agarose gel containing formaldehyde and transferred onto nylon membrane as previously reported.¹⁷ We used as probes the isolated cDNA clones by FDD, which were labeled with [α -³²P]dCTP by Megaprime DNA labeling system (Amersham Pharmacia Biotech, Tokyo). Autoradiography was performed using an imaging plate (Fuji Film, Tokyo) and the visualization and quantitation were done with BAS2000 (Fuji Film). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a standard for quantitation.

Differential Display

Total RNA was treated with RNase-free DNase using a Message clean kit (GenHunter Corp., Brookline, MA). Random oligonucleotide primers (20–22 mer) synthesized were used as arbitrary primers. Anchor primers were synthesized as follows: 5'-CCCGGATCC (dT)₁₅N-3' (where N is C, G, or A; this primer was designed to include *Bam*HI site) and were labeled with rhodamine at the 5'-end of the oligonucleotides. A 500-ng aliquot of total RNA was reverse transcribed in 1 $\times$ reverse transcriptase buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl) with 5 mmol/L MgCl₂, 1 mmol/L deoxynucleotide phosphates (dNTP), 20 U RNase inhibitor, and 50 pmol of each anchor primer. Each sample was incubated for 10 minutes at 70°C and then cooled on ice. This was followed by addition of 1.5 mU avian myeloblastosis virus reverse transcriptase (Takara Bio, Shiga, Japan) in a total volume of 20 μl , which was incubated at 30°C for 10 minutes and at 55°C for 30 minutes. Reverse transcriptase was inactivated by incubation at 95°C for 5 minutes. For each PCR reaction, 2 μl of cDNA was dissolved in a volume of 20 μl in PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂) in the presence of 0.1 mmol/L dNTP, 10 pmol of arbitrary primer, 5 pmol of

anchor primer, and 0.5 U *Taq* polymerase (Takara Bio). PCR conditions were 94°C for 5 minutes, 37°C for 5 minutes, 72°C for 5 minutes, which was followed by 34 cycles of 94°C for 30 seconds, 37°C for 2 minutes, and 72°C for 1 minute. A final extension reaction was carried out at 72°C for 5 minutes. Five microliters of each PCR product was analyzed by electrophoresis in 7 mol/L urea/4% polyacrylamide gel at 40 W constant power for 2 to 3 hours. Gels were visualized with a fluorescence image analyzer (FMBIO-100, Takara Bio). The fragments showing differential expression were excised from the gel with razor blade, extracted by heating in water, and then reamplified by PCR using the corresponding primer pair.

Cloning and cDNA Sequencing of Fragments Obtained by Differential Display

An aliquot of reamplified cDNA was checked for a single band by urea/polyacrylamide gel electrophoresis. In case of multiple bands, the procedures were repeated until single band was obtained. Then the rest of the product was digested with *Bam*HI (Takara Bio) to remove rhodamine labeling as well as to obtain cohesive end, and was loaded to 1% agarose gel. The band was carefully excised and extracted from the gel by the use of QIAEX II gel extraction kit (QIAGEN, Tokyo). The product was finally cloned into the pBluescript II SK(+) vector (Stratagene, La Jolla, CA) after treating the vector first with *Hinc*II digestion (Takara Bio), then with terminal deoxynucleotidyl transferase (Takara Bio) for adding dT at the blunt end and finally with *Bam*HI. The plasmid DNAs were sequenced with the AmpliTaq Cycle Sequence kit (Perkin Elmer, Foster City, CA) according to the manufacturer's protocol using an ABI PRISM 377 DNA sequencer. The homology search was performed with Genomenet World Wide Web server (<http://www.genome.ad.jp>).

Results

Differential Display and Reverse Northern Blotting

To isolate genes differentially expressed in oxystress-induced rat RCCs, we have used five samples as a set: cortical portion of normal kidney (1-year-old F1), cortical non-neoplastic portion of Fe-NTA-treated kidney (1-year-old), two independent RCCs (a grade 2 RCC with lung metastasis and a grade 1 RCC with no metastasis), and a RCC cell line (FRCC001) established from Fe-NTA-induced RCC of grade 2. Total RNA was isolated from each sample and was reverse transcribed to cDNA. Using three kinds of anchor primers in combination with 280 arbitrary primers, an average of approximately 100 cDNA transcripts was amplified and displayed in each lane of the gel. Thus, in total, approximately 84,000 clones were compared on display for the set of samples.

Our strategy was to select only clones that showed different expression pattern between the former two sam-

Figure 1. Fluorescent differential display of Fe-NTA-induced RCCs and non-neoplastic renal tissues. Total RNA was isolated from normal cortical kidney (lane 1), non-neoplastic cortical kidney after Fe-NTA treatment (lane 2), Fe-NTA-induced RCCs (grade 2 RCC, lane 3; grade 1 RCC, lane 5), FRCC001 cell line established from a grade 2 RCC of lane 3 (lane 4), reverse transcribed and followed by PCR in the presence of rhodamine-labeled anchor primer and each arbitrary primer. The PCR fragments were displayed on 7 mol/L urea-4% polyacrylamide gels and visualized as detailed in Materials and Methods and in Results. Fragments of clone 29 (A), clone 61 (B), clone 91 (C), clone 126 (D), clone 230 (E), and clone 256 (F) are indicated by arrows. Transcript clones are summarized in Table 1.

ples (normal control and non-neoplastic after Fe-NTA administration) and the latter three samples (two RCC tissues and one RCC cell line) to avoid false positive clones. We found a total of 290 clones to be differentially expressed. Examples of representative FDD runs are shown in Figure 1. Each band was carefully excised from the gel by a razor blade, purified, and reamplified. Among these, 142 clones were successfully subcloned and sequenced. Using each cDNA clone as a probe, 72 selected clones (more than fivefold increase or decrease by FDD) were subjected to reverse Northern blotting for which a different set of samples (normal renal cortex of a 1-year-old male F1 rat, renal cortex of a 12-week-old male Wistar rat after 6 weeks of repeated Fe-NTA administration, two pairs of RCCs and their nontumorous counterparts: a grade 1 RCC with no metastasis and a grade 3 RCC with lung metastasis and peritoneal invasion) were prepared (Figure 2). Finally, 20 cDNA clones were confirmed to be differentially expressed (Figure 1, A–F, and Figure 2, A–F); the remaining cDNA clones either showed no signal or difference in expression by Northern blots or exhibited no difference between RCCs and its non-neoplastic counterparts. These were interpreted as false positive and excluded from the present study.

Unidentified Genes Differentially Expressed in the Fe-NTA-Induced RCCs

Sequence analyses revealed clones 28, 29, 256, 418, and 435 as novel gene fragments (Figure 3). Clone 28 was overexpressed, whereas the others were deleted in expression in the Fe-NTA-induced RCCs. Homology search through GenBank and EMBL databases using BLAST programs revealed no significant homology with any published genes in the five clones. Each clone showed single band by Northern blots; the sizes were 2.6 kb in clone 28, 2.8 kb in clone 29, 1.6 kb in clone 256, 2.0 kb in clone 418, and 2.4 kb in clone 435. All of the fragments were flanked by DNA sequence of the corresponding oligo(dT) primer and arbitrary primer used for FDD and further revealed polyadenylation signal sequence (AAUAAA) upstream of the putative polyA tail.

Identified Genes Differentially Expressed in the Fe-NTA-Induced RCCs

In the present FDD study, we have identified 15 genes as differentially expressed. Increased in the Fe-NTA-in-

Figure 2. Northern blot analysis of identified and novel transcripts in neoplastic and non-neoplastic rat renal tissues. Two μ g of poly (A)-rich RNA was isolated from rat tissue (**lane 1**, normal cortical kidney; **lane 2**, non-neoplastic cortical kidney after 6 weeks of repeated Fe-NTA treatment; **lanes 3** and **5**, non-tumorous part of the cortical kidney; **lanes 4** and **6**, Fe-NTA-induced RCCs of grades 1 and 3, respectively. **Lanes 3** and **4**, and **lanes 5** and **6** are paired samples) and subjected to Northern blot analysis as detailed in materials and methods. Subcloned cDNA fragments (**A**, clone 29; **B**, clone 61; **C**, clone 91; **D**, clone 126; **E**, clone 230; **F**, clone 256) summarized in Table 1 were used as probes, respectively. Size of each band was determined by relative migration of 18S and 28S ribosomal RNA. GAPDH was used as loading controls. Transcript clones are summarized in Table 1.

duced RCCs were homologues of Y-box binding protein, heat shock protein (HSP) 86, DNA polymerase catalytic subunit α and glycoproteins of 96 kd (gp96); ribosomal proteins (RPs) S13, S15 and L19; non-muscle caldesmon and annexin II (calpactin I heavy chain) as shown in Table 1. We estimated the increase of expression in the RCCs as 7.8- to 14.3-fold (1.3- to 2.3-fold) for the homologue of Y-box binding protein, 1.7- to 1.8-fold (0.93- to 1.4-fold) for the homologue of HSP 86, 2.8- to 4.0-fold (0.8- to 1.5-fold) for RP S15, 3.1- to 3.3-fold (1.4- to 2.7-fold) for RP S13, 1.7- to 2.4-fold (1.1- to 1.3-fold) for RP L19, and 2.3- to 2.6-fold (1.6- to 1.8-fold) for the homologue of gp96 as compared with a control sample of normal age-adjusted kidney. (Data for kidney after 6 weeks of repeated Fe-NTA administration are shown in parentheses.) Expression of the homologue of DNA polymerase catalytic subunit α , nonmuscle caldesmon (Figure 2C) and annexin II in the samples of non-neoplastic kidneys was under the detection limit of Northern blot.

Expression of the homologue of protein tyrosine phosphatase (PTP) β were decreased in the RCCs. We estimated the expression level in the RCCs to be 9 to 18% as compared with a control sample of normal age-adjusted kidney, whereas kidney samples after 6 weeks of repeated Fe-NTA administration showed 69 to 117% (data not shown). Expression of selenoprotein P, intestinal trefoil protein, nicotinamide adenine dinucleotide, reduced form (NADH) dehydrogenase, homologue of human mac25, and homologue of mouse stromal-derived factor 1 (SDF-1) were under the detection limit in the RCCs, whereas they were abundantly expressed in the nontumorous counterparts, as summarized in Table 1.

Discussion

The present study was conducted to find genes that reveal marked alteration in expression during ROS-induced rat renal carcinogenesis, especially in promotion and/or progression stages. We have identified 15 genes (Table 1) and found five novel transcripts (Figure 3).

Y-box binding protein (YB-1) is a member of DNA-binding protein family with a unique cold shock domain, which is highly conserved from prokaryotes to eukaryotes.¹⁸ YB-1 regulates gene expression through binding to Y-box sequence, 5'-CTGATTGG-3', which is contained in the *cis*-regulatory elements of several genes, including thymidine kinase, cyclin-dependent kinase 1, proliferating cell nuclear antigen (PCNA), DNA polymerase, epidermal growth factor receptor, multidrug resistance 1 (MDR1),^{19,20} and OxyR, a thiol-containing transcriptional activator²¹ whose redox status controls the expression of downstream genes.²² YB-1 is an integral component of redox signaling pathway. Recently it was reported that cisplatin resistance was proportionally associated with the expression of YB-1,²³ that YB-1 binds preferentially to cisplatin-modified DNA²⁴ and further that increased amounts of nuclear YB-1 was associated with a poor prognosis of ovarian cancer.²⁵ Therefore, it is possible that cells with high expression of YB-1 were selected during carcinogenesis in consideration of the fact that cancer cells, especially adenocarcinoma, are exposed to persistent oxidative stress.²⁶

Ribosomes catalyze protein synthesis with a complex structure including both RNA and protein components. There is increasing evidence that the ribosomal RNA

Clone 28

GTCAAGAGAG AGTACGGAGT GTCATGAGG CCCGGAAGGA CCGAGCACCC 50
 CCACCACGAT TCAGACCTGC TGGCGCTGCA CCACGACCTC CACCAAAGCC 100
 CATGTAAAGA GGTGATTTCA TGAAGACAGA AAGAAAAATA CCATGGAAAA 150
ATAAACTGA AGTTATACTT TAAAAAAAAA AAAAA

Clone 29

GCTCTACGAC TGAGCTGCAC TAGACCGTTA TCTTCCTGTG ATAGTTTATT 50
 TTTATTCTCC TTTATTCTGG TTTGAGACAA GGTCTCTCTA GCTTTCCTGT 100
 CTGGGAAATA ACCACACCGC CAACGATGTC CTTGACCTTG AACTCGGCTC 150
 CTCCTGCCTC TCTACTTCTC AAATGCTGAG AGGACAGGCT TGAGCTCCTC 200
 GCCTGGAGTC CCTGCAGTAT TTCAAGTGTT ATGATCGTGA **AAATAAACAT** 250
 CTGCCTTGTT CACCGTTAAA AAAAAAAAAA AA

Clone 256

CGGCTTTTGT CTCTGCTCCT TCCCATCATG TCCTACCTGG CCTTCCATCT 50
 TAGCTCAGGA CTCACCTTCT TGAAGCGAAA CTTATCCACC TTTCTACTTC 100
 TCCTGGGGAC AGGAATCCCT ATACCCTTGA GTGCTCCACC TTCAGAACAA 150
 CCCATTAATC TAAGACTATT GGGGACCGGG ATCGGACTGT CAGACTGGGC 200
 AAAGCTAGAG CCCTGCCCTG AGCAGATTCC TGACCTTTTC AAAGAAAGAA 250
 CATGTCCCCG GAAGACTGTT GCCTCATTGT CTAAGGAGGA TATCTTGCGG 300
 TTTAATGATT CACCTTATGT CCTTGGGCAC TTCCCAGTAC TCCCCAGCC 350
 CTAAGCTTCT ATTCCTGCTT CAGAGCTTTA AATGCTGACA TTCCACTCAA 400
TAAACAGACC TTGACAAGGA CAAAAAAAAA AAAAA

Clone 418

ATACGGTCCT CTCTGCTCCT TCCATCATGT CCTACCTGGG CCTTCCATCT 50
 TAGCTCAAGG ACTCACTTTC TTGAAGCGAA ACTTATCCAC CTTTCTACTT 100
 CTCCTGGGGC AGGAATCCCT ATACCCTTGA GTGCTCCACC TTCAGAACAA 150
 CCCATTAATC TAAGACTATT GGGGACCGGG ATCGGACTGT CAGACTGGGC 200
 AAAGCTAGAG CCCTGCCCTG AGCAGATTCC TGACCTTTTC AAAGAAAGAA 250
 CATGTCCCCG GAAGACTGTT GCCTCATTGT CTAAGGAGGA TATCTTGCGG 300
 TTTAATGATT CACCTTATGT CCTTGGGCAC TTCCCAGTAC TCCCCAGCC 350
 CTAAGCTTCT ATTCCTGCTT CAGAGCTTTA AATGCTGTAC ATTCCACTCA 400
ATAAACAGAC CTTGACAAGG AAAAAAAAAA AAAAA

Clone 435

CGTCCAATGT CAGTCTTGTC TAGATAGTGA GAGTCTTGTC TCAAAAATAA 50
ACAAAACCAA AACTAAACA GAGCTTAACT CTGTTGGAAT TGTCCAAAGC 100
 GATGGATTCA GAGGCATAAT TCAGGTTCTA GGATGGACAG ATTCATTTCT 150
 GTCCAGATAA ATTCGCCACC TTGGGCAATG AGCCAGTCC TGTGGATTGG 200
CAGATGGAGA CGGGAAAATT GAAAAAAAAA AAAAA

Figure 3. Nucleotide sequence of five differentially expressed novel transcripts. Five clones reveals no homology to the GenBank/EMBL data base. Flanking sequences of primer sets are underlined. The polyadenylation signal sequence is in bold letters.

molecules play a central role in its catalytic activities; many of the ribosomal proteins have been relatively poorly conserved, so it has been suggested that the

ribosomal proteins mainly enhance the function of the ribosomal RNAs. Overexpression of RP S13 is observed in actively growing cells such as human colorectal car-

Table 1. Genes Differentially Expressed in Fe-NTA-Induced Renal Cell Carcinoma of Rats

Clone No.	Non-T	RCC	Homologue	Homology	Function	Chromosomal Location		
						Mouse	Human	Rat
28	—	↑ ↑ ↑	Unknown	ND	ND	ND	ND	ND
29	↑ ↑	—	Unknown	ND	ND	ND	ND	ND
34	→	—	Selenoprotein P	Rat 1.4e-67	S	Chr. 15, 6.7 cM	5q31	ND
40	↑	↑ ↑	Y-box binding protein (YB-1)	Mouse 9.3e-46	S, P	ND	1p34	nr
61	→	↑ ↑	Heat shock protein 86	Mouse 5.1e-48	S	Chr. 12, 48 cM	ND	nr
82	→	↑	Ribosomal protein S15	Rat 1.4e-128	P	ND	ND	ND
83	—	↑	DNA polymerase catalytic subunit α	Mouse 2.0e-79	P	Chr. X, 34 cM	Xp21.1-p21.3	nr
91	—	↑ ↑	Nonmuscle caldesmon	Rat 3.3e-58	P	Chr. 6, 11.5 cM	7q33	ND
109	↓	↓ ↓	Protein tyrosine phosphatase β	Human 2.2e-78	P, communication	ND	12q15-q21	nr
116	↑	↑ ↑	Ribosomal protein S13	Rat 4.1e-106	P	ND	ND	ND
126	↑	↑ ↑ ↑	Ribosomal protein L19	Rat 1.4e-72	P	ND	ND	ND
128	→	—	Stromal cell-derived factor 1 (SDF-1)	Mouse 1.5e-70	P, angiogenesis	Chr. 6, 53 cM	10q11.2	4q42.1
208, 246, 421	↑	↑ ↑ ↑	Annexin II (calpactin I)	Rat 4.2e-258	S, P	Chr. 9, 37 cM	15q21-q22	ND
230	↓	—	Intestinal trefoil protein	Rat 8.9e-100	S, adhesion	Chr. 17, 17 cM	21q22.3	ND
250	↓	—	NADH dehydrogenase	Rat 5.9e-49	electron transfer	MT	MT	MT
256	→	—	Unknown	ND	ND	ND	ND	ND
418	↓	—	Unknown	ND	ND	ND	ND	ND
432	→	—	Mac25/IGF binding protein-7	Human 5.9e-84	P	ND	4q12	nr
435	→	—	Unknown	ND	ND	ND	ND	ND
444	→	↑ ↑	Glycoproteins of 96 kDa (gp 96)	Mouse 1.8e-101	S	Chr. 10, 49 cM	12q24.2-qter	nr

↑, increased expression; ↓, decreased expression; →, no change; —, under detection limit, compared with age-adjusted normal control kidney (Northern blots); Non-T, non-tumorous part of the kidney after Fe-NTA treatment; RCC, Fe-NTA-induced renal cell carcinoma.

Non-T and RCC were obtained from the same rat. S, stress response-associated; P, proliferation-associated. See Discussion for detail.

Chr., chromosome; ND, not determined; nr, not reported; MT, mitochondrial. Chromosomal information was obtained from <http://www.ncbi.nih.gov/genome/guide/>.

cinoma,²⁷ and overexpression of RP L19 was reported in human breast tumors that overexpress erbB-2.²⁸ There are many reports on the overexpression of other RPs in human cancer: L31 in colorectal tumors,²⁹ P0 in hepatocellular carcinoma and colon carcinoma,³⁰ and L7a and L37 in prostate cancer.³¹ As far as we know, there are no data available that deal with the association of RP S15 and tumors. Moreover, it was reported that overproduction of mycobacterial RP S13 induces catalase/peroxidase activity, presumably as a stress response which, in this situation, may cause hypersensitivity to isoniazid in *Mycobacterium smegmatis*.³² Our results support the idea that increased expression of certain ribosomal proteins is associated with oxidative stress and is a common feature of cancer.

Glycoprotein of 96 kd (gp96) and heat shock protein (HSP) 86 belong to the HSP family. Exposure to stress such as heat shock, oxygen deprivation, and calcium or glucose limitation results in the cellular induction of HSPs. These two proteins are involved in stress response, working as a molecular chaperon in the cytoplasm. For example, irradiation and interleukin-6 administration induced gp96 in human cervical and breast cancers, respectively.^{33,34} These two proteins are coexistent with a broad range of antigen peptides derived from that particular cell and are named as tumor rejection antigen for gp 96³⁵ or tumor-specific transplantation antigen for HSP86,³⁶ and autologous tumor-derived gp96 was used successfully for immunotherapy of tumors.³⁵ HSP86 has been constitutively induced in murine embryonal carcinoma, but is

decreased on differentiation,³⁷ suggesting induction of these proteins as a common molecular event in cancers.

Annexin II (also known as calpactin I, p36, or lipocortin II), a Ca²⁺-binding protein, is a substrate for an oncogene growth factor-associated protein tyrosine kinase. Increased expression of annexin II is observed in pancreatic adenocarcinoma of Syrian hamsters,³⁸ human hepatocellular carcinoma,³⁹ human brain tumor,⁴⁰ and cell lines of hereditary RCC from Eker rats.⁴¹ Recent evidence indicates a role for annexin II in DNA synthesis by interacting with DNA polymerase α ,⁴² cell proliferation,⁴³ and differentiation of F9 teratocarcinoma cells.⁴⁴ Moreover, it has been reported that expression of annexin II was induced in several sets of transformed cells irrespective of the nature of oncogene products, including v-H-ras-, v-mos-, and SV40-transformed cells.⁴⁵

Nonmuscle caldesmon, an actin-binding and phosphorylation-activated protein, is closely associated with mitosis; caldesmon stays dissociated from microfilaments from prometaphase until at least the early stages of cytokinesis, suggesting that continued dissociation of caldesmon may be required for mitosis-specific organization of microfilaments, including disassembly of stress fibers and formation and activation of contractile rings for cell division.⁴⁶ In this context, induction of DNA polymerase catalytic subunit α and down-regulation of protein-tyrosine phosphatases (PTPases) were consistent in the metabolism of RCCs in the present study.

PTPases regulate tyrosine phosphorylation, which plays a key role in cellular proliferation, differentiation,

and oncogenesis.^{47,48} PTPases can be structurally classified into transmembrane and non-transmembrane isozymes.⁴⁸ One of the functions of PTPases is to reverse the effect of protein tyrosine kinases, many of which are oncogenes, suggesting that they may act as tumor suppressors.⁴⁹ Indeed, recently it was reported that expression of PTPase were not observed in 55% of grade 4 glioblastomas, whereas all grade 1 gliomas showed expression⁵⁰ and were dramatically decreased in lung adenocarcinomas.⁵¹ Furthermore, PTPases were found to play a role in cell-to-extracellular matrix communication as well as cell-to-cell communication.⁵² Our results suggest that decrease in PTPase expression might work for promotion or progression of the initiated cells.

Selenoprotein P is a glycoprotein containing selenocysteine that has been purified from rat and human plasma.⁵³ Selenium is an essential trace element in mammals, and its remarkable biological effect in eukaryotes may be related to a unique function of various selenoproteins such as glutathione peroxidase and thioredoxin reductase.⁵⁴ Selenoprotein P has been postulated to serve as an extracellular oxidant defense.⁵³ So far there is no report on the association of selenoprotein P and cancer. Deleted expression of selenoproteins in RCCs might lead to more oxidative stress than that of adjacent non-neoplastic tissue, which in turn may contribute to higher oxidative stress and, therefore, genomic instability in cancer.

Trefoil peptides are stable, secreted molecules containing a conserved 6-cysteine motif and are known to be expressed in the gastrointestinal tract and kidney.⁵⁵ Induction of human spasmolytic polypeptide and porcine spasmolytic polypeptide in the trefoil protein family has been reported in association with peptic ulcers and mucosal injury in inflammatory bowel disease.⁵⁵ In gastric cell lines, the trefoils were shown to act in a manner suggestive of immediate-early genes capable of auto- and cross-induction through *cis*-acting regulatory regions: trefoil-mediated transcriptional regulation required activation of the Ras/MEK/MAP kinase signal transduction pathway. Intestinal trefoil factor (ITF) stimulation of gastric cell lines led to phosphorylation of epidermal growth factor receptor.⁵⁶ Further, it was shown recently that transfection of ITF-3 induced apoptosis in human colon adenocarcinoma cells by perturbing the complexes between E-cadherin, β -catenin, and associated proteins.⁵⁷ Although function of this protein in the kidney is still unknown, the same mechanism may work for RCCs. However, we cannot rule out the possibility of "cell population effect," because ITFs were reported to be localized to the collecting ducts of the kidney,⁵⁸ and the majority of Fe-NTA-induced rat RCCs are thought to arise from proximal tubular epithelial cells.

The mac25 gene is a newly discovered member of the insulin-like growth factor-binding protein (IGFBP) family and renamed as IGFBP-7.⁵⁹ The IGFBP family regulates the interaction between insulin-like growth factor (IGF)-1 and -2, and their receptors. IGF-1 and -2 play important roles in normal growth and development and have been implicated as growth regulators and potent mitogens in some cancer. It has been reported that decreased ex-

pression of mac25/IGFBP-7 during progression of breast carcinomas was associated with allelic loss at human chromosome 4q12-13, where mac25/IGFBP-7 is located.⁶⁰ This report and our data suggest that this gene may have tumor suppressor or associated function in renal carcinogenesis, and loss of expression may lead to tumor cell growth.

A chemokine, stromal cell-derived factor 1 (SDF-1), is an important regulator of leukocyte and hematopoietic precursor migration and pre-B cell proliferation.⁶¹ There are few reports about function and expression of SDF-1 in solid organ and carcinoma. In a recent report, the chemokine receptor CXCR4, which is G-protein-coupled receptor for the CXC chemokine SDF-1, is essential for vascularization of the gastrointestinal tract,⁶² suggesting SDF-1 and CXCR4 are involved in a new signaling system of organ vascularization. Our results suggest that no expression of SDF-1 mRNA may lead to abnormal angiogenesis in RCCs.

NADH dehydrogenase plays a fundamental role in cellular respiration in both eukaryotes and prokaryotes. This protein is an integral membrane protein whose activity is crucial to the buildup and maintenance of a transmembrane proton gradient, sustained by the reduction of dioxygen to water and by coupling with translocations across the mitochondrial membrane. The genome is present in the mitochondrial DNA. There are reports that expression of NADH dehydrogenase or cytochrome c oxidase is up-regulated in several kinds of cancers.^{63,64} Our result was the opposite. Of note is the fact that human RCCs show a high incidence of mitochondrial DNA deletion.⁶⁵ The frequency with which this phenomenon occurs in RCCs, but not in other types of cancers, suggests that this may be an important phenotype associated with renal cell neoplastic transformation. Further study is necessary to elucidate the significance of our results.

The purpose of the present study was to find genes differentially expressed in oxystress-induced cancer based on a hypothesis that either alteration in transcriptional control or genomic deletion may contribute directly to carcinogenesis. We performed a careful selection and identified 15 different clones by screening approximately 84,000 transcripts. It was interesting to find that most of these clones were associated with either stress response or cellular proliferation, but that multiple genetic pathways are apparently involved, as shown in Table 1. One transcription factor, YB-1, was included. Most of the clones could be classified into "information" and "communication" categories by a proposed genome classification.⁶⁶ We believe that these transcriptional controls of the identified genes or genomic deletion are working in the promotion and progression processes of carcinogenesis. Further study is now in progress to differentiate between transcriptional shutoff and genomic deletion in the genes with no expression in the RCCs, to find links among the identified genes, and to find earlier changes in combination with morphological approach. Our data also warrant investigation of human cancers for the expression of genes reported in the present study, to test a

hypothesis that oxystress-induced cancers share a common panel of gene expression.

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