Jpn. J. Cancer Res. **93**, 1201–1206, November 2002

Analysis of *Fas* Gene Mutations on Laser Capture Microdissected Specimens from Renal Cell Carcinoma

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Renal cell carcinoma (RCC) expresses Fas antigen on the cell surface, and thus could be sensitive to apoptosis induced by the binding of Fas ligand. *Fas* gene mutations might be involved in the development of RCC. *Fas* gene mutations were examined in genomic DNA extracted from RCC lesions. With use of laser capture methods, one RCC and one non-neoplastic lesion per case were microdissected from 15 patients with RCC. Polymerase chain reaction-amplified products were directly sequenced. Loss of heterozygosity (LOH) was examined at four sites of known polymorphism. Mutations of the *Fas* gene were detected in 3 RCC lesions from 3 (20%) of 15 cases. All mutations were point mutations, 2 missense and one silent, in exons 7 and 9. Non-neoplastic tissues never showed *Fas* gene mutations. Nine of 15 cases (60.0%) were heterozygous for one or more sites of the known biallelic polymorphisms, i.e., at nucleotides −1377, −670, 416, and 836. Two of these 9 cases showed LOH at promoter region −670. Mouse T-cell lymphoma cells transfected with missense mutated genes were resistant to apoptosis induced by anti-Fas antibody, indicating these to be loss-of-function mutations. The results of the present study suggest that *Fas* gene mutations play a role in the pathogenesis of RCC.

Key words: Renal cell carcinoma — Fas — Apoptosis — Mutation — Laser capture microdissection

Fas antigen is a 45-kDa transmembrane protein of the tumor necrosis factor (TNF) receptor superfamily that can induce programmed cell death (apoptosis) through cross-linking with Fas ligand (FasL).[1, 2] *Fas* is located on chromosome 10q24.1 and comprises 9 exons and 8 introns. The *Fas* gene encodes 325 amino acids, which are divided into extracellular, transmembrane, and intracytoplasmic domains. The 80-amino acid portion in the intracytoplasmic domain is essential for apoptotic signal transduction, and is thus designated as the death-signaling domain. Mutations of the *Fas* gene in the death domain lead to loss of its apoptotic function (loss-of-function mutation), resulting in accumulation of cells, and thus might contribute to the pathogenesis of human malignancies. Indeed, *Fas* gene mutations have been reported in both the lymphoid lineage and epithelial malignancies: approximately 10% of cases with multiple myeloma[3] and sporadic non-Hodgkin's lymphoma,[4] 7.7% with lung cancer,[5] and 28% with urinary bladder cancer.[6]

Renal cell carcinoma (RCC) is the commonest malignancy of kidney, accounting for 85% of renal carcinoma in adults. Nephrectomy is the treatment of choice, because RCC is usually resistant to chemotherapy. Defects in the apoptotic pathway in RCC might be one of the causes for its resistance to chemotherapy.[7] Indeed Nonomura *et al.* reported that RCC expressed Fas antigen on the cell surface, but about 30% of the RCC cells survived under apoptotic stimuli by agonistic anti-Fas antibody.[8] From these findings, it is postulated that mutations of the *Fas* gene might be involved in carcinogenesis and/or chemoresistance of RCC cells.

Because RCC contains neoplastic and non-neoplastic tissues, often with massive necrotic changes, DNAs extracted from the paraffin-embedded samples by microdissection were used to examine the non-neoplastic and neoplastic tissues. Laser capture microdissection under direct microscopic visualization enable rapid one-step procurement of selected human cell populations from histologic sections. This method made microdissection of selected cells much easier, so that extensive study on the lesions become possible. In the present study, mutations of *Fas* gene were examined in 15 cases of RCC.

MATERIALS AND METHODS

Patients Fifteen patients, who received nephrectomy for RCC, were selected for the current study: they were admitted to Osaka University Hospital during the period of 1999 to 2000. None of the 15 patients had received preoperative chemotherapy or radiation therapy. There were 9 males and 6 females with an age range of 36 to 79 (median, 67) years. Based on the TNM system classification of the International Union Against Cancer (UICC),[9] pathological stage was determined as follows: 11 cases (73.3%) in stage T1 and 4 (26.7%) in T2. Tumor size

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ranged from 2.8 to 9.2 (mean, 4.97±2.47) cm. None of the patients had distant metastases to other organs. Histologic specimens were fixed in 10% formalin, and routinely processed for paraffin-embedding. Histologic sections cut at 4 μm thickness were stained with hematoxylin and eosin, and were reviewed by three of the authors (H. T., Y. T., K. A.) for diagnosis. Tumor grading was based on the nuclear grading system of Fuhrman and colleagues.[10] Tumor grading was determined as follows: 2 (13.3%) in grade 1, 11 (73.3%) in grade 2, and 2 (13.3%) in grade 3.

Microdissection and genomic DNA extraction Microdissection of each lesion was performed using a PixCell laser capture microscope (Arcturus Engineering, Santa Clara, CA) according to the previously described methods with some modifications (Fig. 1).[11] The dissected pieces were allowed to adhere to the transparent cap and collected in 0.6 ml Eppendorf tubes. The procured cells were subsequently resuspended in 20–50 μl of extraction buffer containing 10 m*M* Tris (pH 8.0), 2 m*M* EDTA, 0.2% Tween 20, and 200 μg/ml proteinase K, and were incubated overnight at 37°C. The mixture was heated at 100°C for 10 min to inactivate the proteinase K, and 3–5% of the solution was used as a template for each PCR. One cancerous and one benign lesion were microdissected per one case.

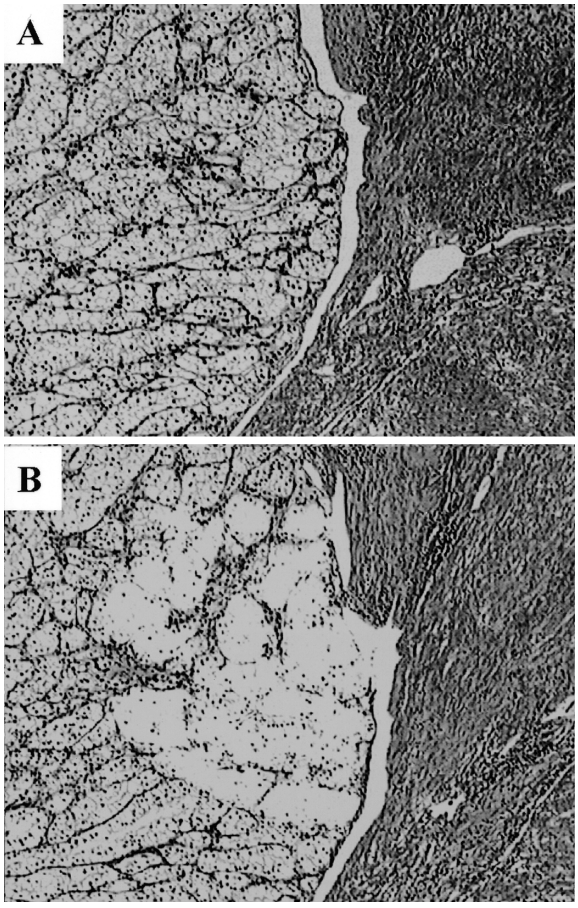

Fig. 1. RCC lesion (A) in a representative case is successfully microdissected (B) (Arcturus Engineering, hematoxylin and eosin staining, ×100).

Detection of mutations The death domain is necessary for the transduction of the apoptotic signal,[12–14] and therefore we examined mutations in exons 7, 8 and 332 bp of exon 9. DNA was subjected to 1st round PCR of 10 cycles with the oligonucleotide primers followed by 2nd round PCR of 35 cycles with the use of 0.1% of the 1st round PCR products as the template. The cycles consisted of denaturation for 15 s at 95°C, annealing for 30 s at variable temperatures, and extension for 30 s at 72°C (Table I), in an Applied Biosystems 9700 Thermocycler (Foster City, CA). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Santa Clara, CA), and were sequenced by the dideoxy chain termination method using a DNA sequencing kit (Applied Biosystems). The samples were analyzed by the Genetic Analyzer (ABI PRISM 310, Applied Biosystems). PCR products with suspected mutations were cloned in pCR 2.1-TOPO (Invitrogen, San Diego, CA), then sequenced to confirm whether the mutation exists or not. All clones were mutation-positive.

Construction of mutant Fas receptor The mutants carrying a point mutation were constructed by PCR. Primers Fas-F (atgctgggcatctggaccct, nucleotides from 195–214 of the human Fas receptor cDNA), Fas-R (ctaattgcatatactcagaa, nucleotide 1243–1224), 784-F (gaagagaaaggaagtacagagaacatgc, 764–791), 784-R complementary to 784-F, 1053-F (gcatcaacttcatggagagaaagaagcg 1037–1064), 1053-R complementary to 1053-F were synthesized. Primers 784-F, -R and 1053-F, -R contain mutated nucleotides that convert Lys-181 (AAA) to Arg-181 (AGA) and Lys-271 (AAG) to Glu-271 (GAG), respectively, of the Fas receptor. During primary PCR, the Fas receptor cDNA was amplified with two pairs of primers, either Fas-F, 784-R and 784-F, Fas-R or Fas-F, 1053-R and 1053-F, Fas-R. The amplified products were isolated by agarose gel electrophoresis, and mixed at a ratio of 1:1, then secondary PCR was performed using Fas-F and Fas-R primers. The products were cloned in pCR 2.1-TOPO. Mutant constructions were verified by DNA sequencing.

Plasmid construction, transfection and cell-killing assay The mouse T-cell lymphoma cell line WR19L (ATCC TIB52) was grown in RPMI 1640 medium containing 10% fetal calf serum (FCS). The 1.1 kb *Eco*RI-*Xba*I fragment containing the full-length human *Fas* cDNA with and without point mutations was transferred into the mammalian expression plasmid pEF-BOS-EX.[15] The WR19L cells were transfected with the plasmid by electroporation as described previously.[12] After selection

with 0.9 mg/ml of G-418, transformants expressing the mutated Fas were identified by fluorescence-activated cell sorting (FACS) analysis using mouse anti-human Fas monoclonal antibody (CH-11, MBL, Nagoya) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Ig) antibody (Capel, Aurora, OH). The cell-killing assay was carried out in 96-well microtiter plates as described previously. Briefly, 2.5×10^4 transformed cells (100 μl) were incubated at 37°C for 16 h with various concentrations (3 ng/ml–2 μg/ml) of anti-Fas antibody. Dead and viable WR19L cell transformants were distinguished by staining with trypan blue.

RESULTS

***Fas* gene mutations and allelic status** Mutations of the *Fas* gene were detected in 3 lesions from 3 (20%) of 15 cases. All mutations were point mutations, 2 missense and

Table I. Amplification Primers for Mutation Analysis of the *Fas* Gene

Primer (Exon)	First PCR			Second PCR		
	Sequence (5′→3′)	Size of PCR product (bp)	Annealing temperature (°C)	Sequence (5′→3′)	Size of PCR product (bp)	Annealing temperature (°C)
PA-F (Promoter, −1377)	5′-gccctataccatcctccttat-3′	172	55	5′-gccctataccatcctccttat-3′	145	55
PA-R	5′-ctgtcactgcacttaccacc-3′			5′-gtaggtgttgataggcttgtct-3′		
PB-F (Promoter, −670)	5′-cctcttgaaaataaaaact-3′	242	56	5′-cctcttgaaaataaaaact-3′	228	56
PB-R	5′-tcactcagagaaagacttgcgg-3′			5′-gacttgcggggcatttgac-3′		
3-F (Exon 3)	5′-acttcccaccctgttacctg-3′	310	55	5′-acttcccaccctgttacctg-3′	250	55
3-R	5′-acttcccaccctgttacctg-3′			5′-tgtgtgtcaacatagcaccac-3′		
7-F (Exon 7)	5′-tcttagtgtgaaagtatgttctc-3′	223	46	5′-ctacaaggctgagacctgagtt-3′	203	55
7-R	5′-caaatcactaatttctctatttt-3′			5′-aggaagtaacaaaaagccaaatc-3′		
8-F (Exon 8)	5′-attaaggaaaaattagaagttcacat-3′	217	50	5′-attaaggaaaaattagaagttcacat-3′	181	50
8-R	5′-atcccataatatgtcactgaaa-3′			5′-atcccataatatgtcactgaaa-3′		
9-F1 (Exon 9)	5′-ggttttcactaatgggaatttca-3′	192	50	5′-attttcagactattttctattttctat-3′	156	50
9-R1	5′-cattgtcattcttgatctcatct-3′			5′-cattgtcattcttgatctcatct-3′		
9-F2 (Exon 9)	5′-cactaagtcaagttaaaggct-3′	170	50	5′-cactaagtcaagttaaaggct-3′	155	50
9-R2	5′-gatctttaatcaatgtgtcatac-3′			5′-gtgtcatacgcttctttcttt-3′		
9-F3 (Exon 9)	5′-gaaagttcaactgcttcgtaattg-3′	230	50	5′-cttcgtaattggcatcaacttcat-3′	217	50
9-R3	5′-ctaattgcatatactcagaa-3′			5′-ctaattgcatatactcaggaa-3′		

PA, promoter region A containing the polymorphism at nucleotide −1377; PB, promoter region B containing the polymorphism at nucleotide −670.

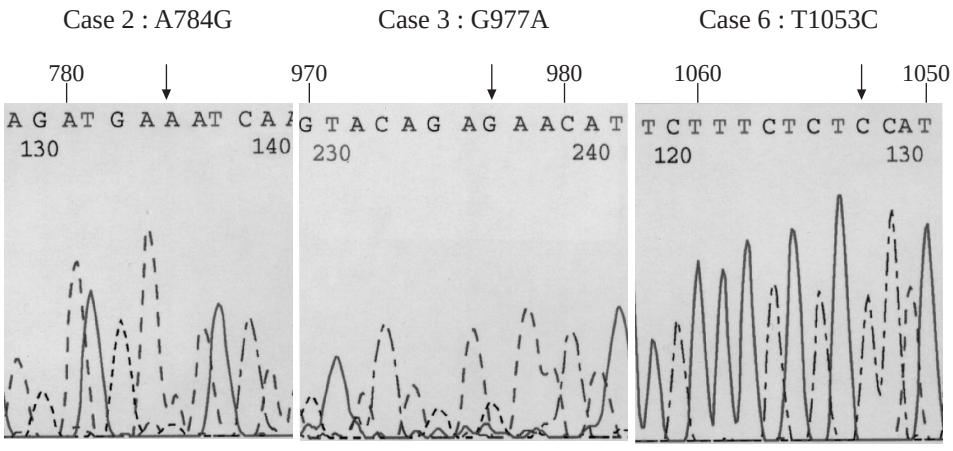

Fig. 2. Sequencing analysis of PCR products revealed point mutations: two missense mutations at codon 181 [Arg (AGA) to Lys (AAA)] and codon 271 [Lys (AAG) to Glu (GAG)] in case 2 and 6; silent mutation at codon 246 [Glu (GAA) to Glu (GAG)] in case 3. Each nucleotide substitution is indicated by an arrow.

Table II. Mutations and LOH of the *Fas* Gene in Renal Cell Carcinoma

Case	Histology	Pathologic stage	Histologic grade	Diameter of tumor (cm)	Mutation				LOH analysis			
					Sites	Nucleotide changes	Codon	Amino acid	−1377	−670	Exon 3	Exon 7
2	Clear cell carcinoma	pT1a	2	3	Exon 7	A784G	181	Lys/Arg	NI	HET	NI	NI
3	Clear cell carcinoma	pT1a	2	3.6	Exon 9	G977A	246	Glu/Glu	NI	LOH	NI	NI
5	Clear cell carcinoma	pT1b	1	5.7					NI	LOH	NI	NI
6	Clear cell carcinoma	pT1a	3	3.5	Exon 9	A1053G	271	Lys/Glu	NI	NI	NI	NI

NI, not informative; HET, retention of heterozygosity; LOH, loss of heterozygosity.

one silent mutations detected in exon 7 and exon 9, which encodes the death domain region of the Fas receptor (Fig. 2, Table II). Regarding to the mutational patterns, two cases were A to G and one case was G to A transition. Non-neoplastic tissues adjoining RCC showed no mutations of the *Fas* gene. When *Fas* gene mutations were evaluated in relation to histological grade and stage, 2 (18.2%) of 11 lesions with grade 2 and 1 (50%) of 2 lesions with grade 3 showed mutations, but grade 1 lesions never showed mutations. All lesions with *Fas* mutations were T1.

The apoptotic signal transduction of mutant Fas receptor Two missense mutations in exons 7 and 9 caused substitutions of non-conserved amino acids. To examine whether these mutations abolish the apoptotic signal transduction of Fas, these mutant *Fas* genes were constructed by PCR, introduced into a mammalian expression vector, and expressed in WR19L cells. FACS analysis of stable transformants using anti-Fas monoclonal antibody indicated that more than 80% of G-418-resistant transformant clones expressed human Fas on the cell surface. The expression levels of Fas in the positive clones were similar among different mutant proteins (data not shown). The ability of the mutated Fas to transduce the apoptotic signal was then examined by treatment of the transformants with agonistic anti-Fas antibody. To exclude clonal variations of Fas expression, two independent clones were chosen for each mutant. The transformant clones expressing the wild-type Fas were killed within 16 h by 40 ng/ml–2 μg/ml anti-Fas antibody. On the other hand, clones expressing Fas with the missense mutation in exon 9 were resistant to apoptosis induced by the anti-Fas antibody even at 2 μg/ml (Fig. 3). A half of the cells transfected with mutant *Fas* gene at codon 181 were killed by 2 μg/ml anti-Fas antibody. These findings indicate that the two missense mutations found in RCC were loss-of-function mutations.

DISCUSSION

It is postulated that apoptosis induced via the Fas/FasL system could prevent the development of RCC, and therefore escape from apoptosis through *Fas* gene mutations might be involved in renal carcinogenesis. Indeed, the present study showed a higher frequency of *Fas* gene mutations in cases with RCC (20%). Mutations of the *Fas* gene were detected in the neoplastic lesions, but never in the non-neoplastic tissues. All of the mutations in the current series were transitions, suggesting that some "endogenous" mutagens act in the pathogenesis of RCC. Only the death domain (exons 7–9) was examined in the current cases, because the majority of the previous studies on *Fas* gene mutations in human malignancies showed that the death domain was mostly involved in cases with Fas mutations.[3, 5, 6, 16] Mutations might occur in other regions of the *Fas* gene, and thus an extended study might reveal a

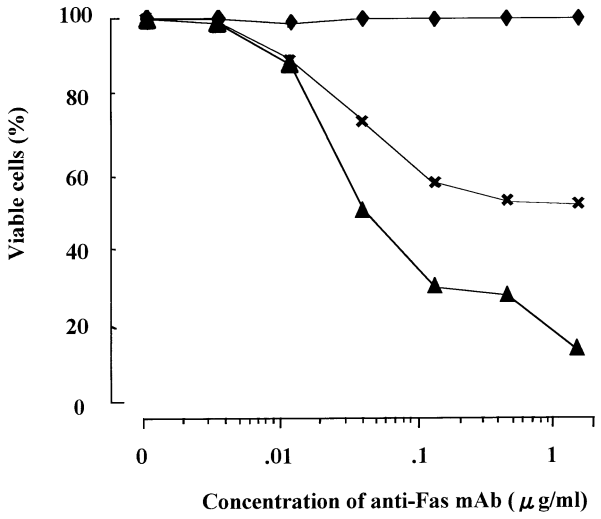

Fig. 3. Cytolytic effect of anti-Fas antibody on WR19L transformant clones. The mouse WR19L cell line expressing recombinant human Fas protein with missense mutations found in case 2 (×), case 6 (◆) or without (wild-type) (▲) point mutations was incubated with various concentrations of anti-Fas antibody at 37°C for 16 h. Clones expressing Fas with the missense mutation in exon 9 were resistant to apoptosis. A half of the cells transfected with mutant *Fas* gene at codon 181 were killed by 2 μg/ml anti-Fas antibody.

higher frequency of *Fas* gene mutations in cases with RCC.

Two of the three mutations were detected in exons 7 and 9. To examine whether these mutations in the *Fas* gene result in loss-of-function of the Fas receptor, transfection experiments with the mutant *Fas* gene were performed. Clones transfected with the missense mutant *Fas* gene proved to be resistant to apoptosis induced by the anti-Fas antibody, indicating that the missense mutations found in RCC were loss-of-function mutations. As a result, cells with mutant *Fas* genes accumulate, and this might provide a basis for the development of RCC. Nuclear magnetic resonance (NMR) spectroscopy revealed that the death domain was localized in residues 202–319 and the core protein in residues 210–305.[17] The region consisting of the fifteen carboxy-terminal residues of Fas (residues 305–319) was reported to play a negative regulatory role for apoptosis.[13] Indeed, cells transfected with the *Fas* gene with missense mutation (case 6) in the core region of the death domain were resistant to apoptosis in the current series.

Two (13.3%) of 15 cases with RCC showed missense mutations which could induce resistance of RCC to apoptosis. The remaining 13 cases had wild-type or nonsense-mutated *Fas* gene. A previous study showed that the RCC cell lines with a functionally intact Fas signaling cascade were resistant to apoptosis induced through anticancer drugs which upregulate Fas receptor and ligand.[18] Therefore further studies are required to clarify whether alterations in the downstream part of the same pathway of Fas-mediated apoptosis, such as FADD/MORT1,[19] caspase 8[19, 20] and FLICE-inhibitory proteins,[21] cause resistance to apoptosis in cases with the wild-type *Fas* gene. The present study suggests that the *Fas* gene mutations in part play a role in renal cell carcinogenesis.

(Received June 11, 2002/Revised September 4, 2002/Accepted September 17, 2002)

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