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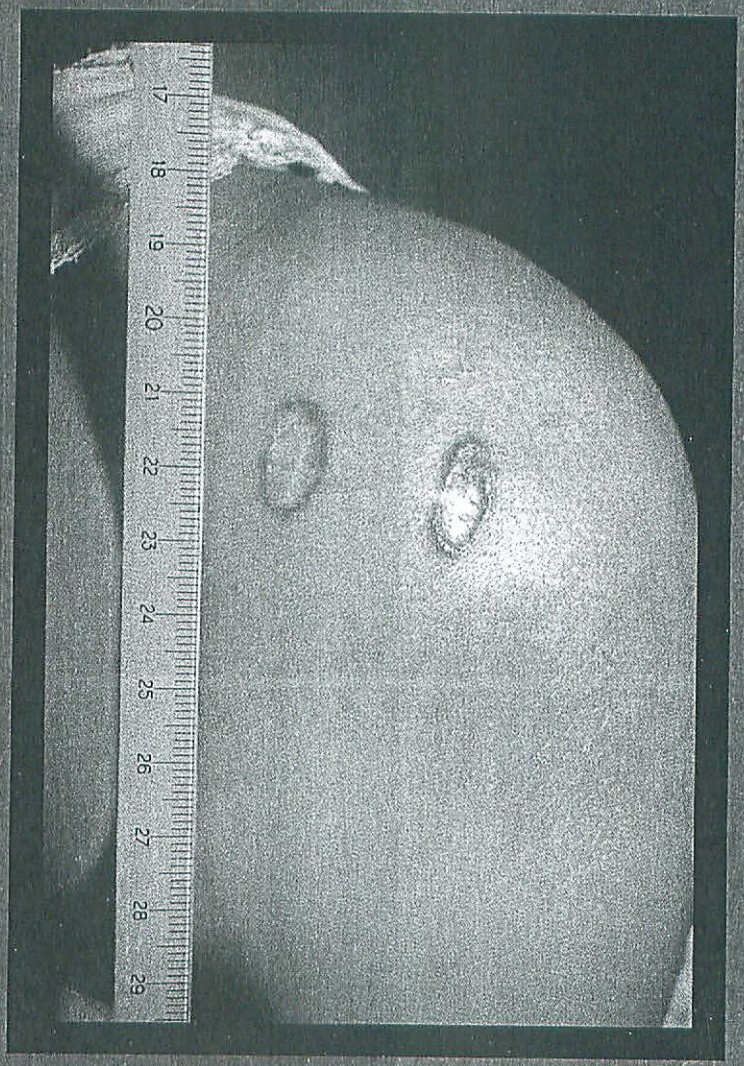

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PTEN/MMAC1/TEP1 MUTATIONS IN HUMAN PRIMARY RENAL-CELL CARCINOMAS AND RENAL CARCINOMA CELL LINES

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Extensive allelotyping studies have implicated several tumor-suppressor loci on chromosomes 3p, 5q, 6q, 8p, 9pq, 10q, 11q, 14q, 17p, 18q and 19p in human kidney tumorigenesis. The *PTEN* (also called *MMAC1* and *TEP1*) gene, a candidate tumor suppressor located at chromosome 10q23.3, is mutated in a variety of sporadic malignancies as well as in patients with Cowden disease. To investigate the potential role of the *PTEN* gene in renal tumorigenesis, we searched for abnormalities of the gene in 68 primary renal-cell carcinomas (RCCs) as well as in 17 renal carcinoma–derived cell lines, using DNA-SSCP, sequencing and microsatellite analysis. Five of 68 (7.5%) primary RCCs exhibited intragenic mutations (3 missense, 1 deletion and 1 splice-site), and 1 of 17 (5.9%) cell lines had an insertion mutation. Loss of heterozygosity of the *PTEN* gene occurred in 25% of primary RCCs, including the 3 cases with intragenic mutation and the 1 *PTEN*-mutated cell line. Clinical and histopathological examinations revealed that 4 of the 5 primary tumors with *PTEN* mutation were high-grade, advanced clear-cell RCCs with distant metastases or renal vein tumor invasions, resulting in poor prognostic courses. The other was a low-stage papillary/chromophilic RCC. Our data suggest that *PTEN* mutation is observed in a subset of RCCs and that, especially in clear-cell RCCs, it occurs as a late-stage event and may contribute to the invasive and/or metastatic tumor phenotype.

Key words: *PTEN/MMAC1/TEP1 gene; tumor suppressor; mutation; carcinoma; renal cell; cell line*

Renal cell carcinoma (RCC) is the most common malignant neoplasm in the adult kidney. Cytogenetic and molecular genetic studies have demonstrated that several cancer-associated genes, including oncogenes and tumor-suppressor genes, are involved in the development and progression of human neoplasms.[1] Regarding the molecular pathogenesis of RCC, chromosome 3p loss has been observed in most clear-cell RCCs, and the von Hippel-Lindau (VHL) disease tumor-suppressor gene, located at 3p25, has been shown to be mutated and inactivated frequently in this tumor subtype.[2,3]

In addition to the *VHL* gene, extensive allelotyping studies have implicated several other tumor-suppressor loci on chromosomes 5q, 6q, 8p, 9pq, 10q, 11q, 14q, 17p, 18q and 19p in human kidney tumorigenesis.[4–6] A putative tumor-suppressor gene, *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) also called *MMAC1* (mutated in multiple advanced cancers-1) and *TEP1* (TGF-β–regulated and epithelial cell–enriched phosphatase), located at chromosome 10q23.3, has been identified.[7–9] Somatic mutation of the *PTEN* gene has been found in a variety of sporadic tumors, including gliomas, melanomas and prostate, breast and endometrial carcinomas.[7,8,10–13] Moreover, germ-line mutation of the gene was identified in Cowden/Bannayan-Riley-Ruvalcaba syndrome, an autosomal-dominantly inherited multiple hamartoma syndrome with a high risk of breast and thyroid carcinomas.[14,15] The *PTEN* gene product is a dual-specificity phosphatase with sequence homology to the cytoskeletal protein tensin; it suppresses the growth, apoptosis and migration of cells through negatively regulating the phosphatidylinositol 3-kinase/Akt signaling pathway.[16–19]

Although somatic *PTEN* mutation in renal carcinomas has been reported,[8,20,21] the detailed mutational status of the gene in sporadic RCC has not been well characterized. To further investigate the potential role of *PTEN* in human renal tumorigenesis, we searched for abnormalities of the gene in primary sporadic RCCs as well as in RCC-derived cell lines.

MATERIAL AND METHODS

Tissue samples

Sixty-eight primary sporadic RCCs and matched normal kidney tissues were obtained from patients during surgery at Yokohama City University Hospital and its affiliated hospitals. All specimens were frozen rapidly with liquid nitrogen and stored at –80°C until nucleic acid extraction. Tumors were confirmed to be sporadic according to medical records. Tumor stage was determined according to the TNM classification. A system of 3 nuclear grades (G1, low-grade; G2, intermediate-grade; G3, high-grade) was used, according to the *General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma*.[22]

Cell lines

Human renal carcinoma–derived cell lines included SGE-RC, VH-Renal, VMRC-RCW, VMRC-PCZ, SN12C, KC12, MTS-RC, KWA-RC, OUR10, OUR20, YCR-1, A498, CAKI1, SMKT-R2, SMKT-R3, ACHN and NSK-RC. Cell lines were maintained in Ham's F12 or DMEM with 10% FBS.

DNA-SSCP and sequencing analysis

High m.w. genomic DNA was prepared by a proteinase K/phenol chloroform extraction method.[23] We used PCR primer sets for DNA-SSCP analysis for all 9 exons and exon–intron boundaries of the *PTEN* gene, as described previously.[8] The PCR conditions in each primer set were essentially as described previously[8] with [α-^{35}S]dATP added to the reaction. Following 40 cycles of PCR amplification, products were denatured, electrophoresed on MDE gel (FMC Bioproducts, Rockland, ME), dried and exposed to X-ray films at room temperature overnight. When aberrant SSCP patterns were detected, PCR-SSCP was repeated with both tumor and normal corresponding kidney samples, to confirm the results. PCR products were purified using the Wizard PCR-Prep kit (Promega, Madison, WI) and directly sequenced, or sequencing was performed after the product was cloned into the pCR2.1 vector, using a TA cloning kit (Invitrogen, La Jolla, CA). Sequencing was

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performed using a Thermo Sequenase radiolabeled terminator cycle sequence kit (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. Following the sequence reaction, these products were electrophoresed on 6% polyacrylamide gels. Gels were dried and exposed to radiographic films for 1 day.

RNA extraction, RT-PCR and cDNA sequencing

Total RNA was isolated from primary RCCs and corresponding normal tissues by a guanidine thiocyanate extraction method.[24] Total RNA was treated with DNase I to avoid contamination of the DNA from PTEN pseudogene.[25] First-strand cDNA was then synthesized using a reverse transcription system (Promega) according to the manufacturer's protocol. For RT-PCR amplification of the *PTEN* fragment from exons 1 to 4, PCR primers (forward 5′-ATG ACA GCC ATC ATC AAA GAG-3′, reverse 5′-AGG ATA TTG TGC AAC TCT GCA-3′) were used with the following conditions: denaturation at 95°C for 10 min; then 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C for 40 cycles; then 5 min at 72°C. Following amplification, PCR products were evaluated on 1.5% ethidium bromide–stained agarose gels, purified using the QIA quick Gel Extraction (Qiagen, Chatsworth, CA), cloned into the pCR2.1 vector and sequenced using the same protocol described above.

LOH detection by PCR-microsatellite analysis

Two microsatellite markers, D10S215 and D10S541, both flanking the *PTEN* gene, were used for determining the allelic imbalance of the gene.[26] Another polymorphic dinucleotide-repeat marker, PTENCA, which contains the 5′ end of the gene, was also used for loss of heterozygosity (LOH) analysis.[27] PCR conditions were essentially as described previously with [α-^{32}P]dATP added to the reaction.[26,27] Following amplification for 20 cycles, products were electrophoresed through denaturing 6% polyacrylamide gels, dried and exposed to X-ray films at –80°C overnight. Each allelic intensity was measured with a Hewlett-Packard (Palo Alto, CA) Scan Jet digital scanner with NIH image software (version 1.55) for determining LOH status.

RESULTS

We screened 68 primary sporadic RCCs and matched normal kidney samples as well as 17 renal carcinoma–derived cell lines for *PTEN* mutations throughout all exons and exon–intron boundaries using DNA-SSCP and sequencing analysis. Sixty-eight primary RCCs consisted of 54 clear-cell and 7 papillary/chromophilic carcinomas, 6 sarcomatoid variants and 1 chromophobic carcinoma.

Five primary RCCs, tumors 27, 68, 86, 263 and 333, and 1 cell line, OUR20, showed aberrant mobility shifts on DNA-SSCP analysis for exons 2, 5, 3, 3, 8 and 8, respectively (Fig. 1). Sequencing studies revealed that tumor 27 had a T-to-C transition at nucleotide (nt.) 158, and it made an amino acid change (valine to histidine) at codon 53. In tumor 68, a C-to-G transversion was found at nt. 303, and it made an amino acid change (isoleucine to methionine) at codon 101. In tumor 263, a T-to-C transition was found at nt. 202, and it made an amino acid change (tyrosine to histidine) at codon 68. In tumor 333, a 1 bp deletion at nt. 968 (968 delA) was identified. In OUR20, a 1 bp insertion at nt. 1005 (1005 insA) was found (Fig. 2). The presence of a single mutant allele in both SSCP and direct sequencing of PCR products in OUR20 indicated that the wild-type PTEN allele is lost in this cell line.

In tumor 86, a G-to-C transversion 5 bp from the splice donor site of intron 3 was found, and we suspected that it made a splice variant. It is typical for nucleotide changes involving splice-junction sequences to produce a variety of splice variants together with a normally spliced message.[28] To look for splice aberrations in this tumor, we reverse-transcribed mRNAs, amplified the cDNA and sequenced the fragments. RT-PCR and sequencing analysis revealed that tumor 86 exhibited at least 2 aberrant-sized transcripts; one was an abundant longer message with abnormal 52 bp sequences between exons 3 and 4, and the other was a shorter, faint message due to the absence of all of exon 3 (Fig. 3). In addition to these aberrant messages, we detected a normally spliced PTEN transcript by RT-PCR. Unfortunately, we could not determine whether this normal message was derived from tumor cells with a splice-site mutation since we analyzed mRNAs from fresh tumor specimens usually containing some amount of non-cancerous cells.

Histopathological and clinical data revealed that 4 of the 5 primary tumors displaying *PTEN* mutation were high-grade, advanced clear-cell RCCs with distant metastasis (cases 68 and 86) or renal vein tumor invasion (cases 263 and 333) at the time of nephrectomy. All 4 patients subsequently died of multiple metastatic lesions within 3, 3, 48 and 22 months, respectively (Table I). In contrast, the fifth *PTEN*-mutated tumor (case 27) was a papillary/chromophilic RCC with T1bN0M0G1 staging and good prognosis (Table I).

Exons 7 and 8 of the *PTEN* gene contain an A(6) repeat, and it has been shown that these mononucleotide repeats are targets for mutations in replication error-positive colorectal carcinomas.[29] However, we did not find any change in these 2 A(6) repeats in our tumors or cell lines.

During mutational analysis of the *PTEN* gene, we identified 2 sequence polymorphisms, both of which had been reported by

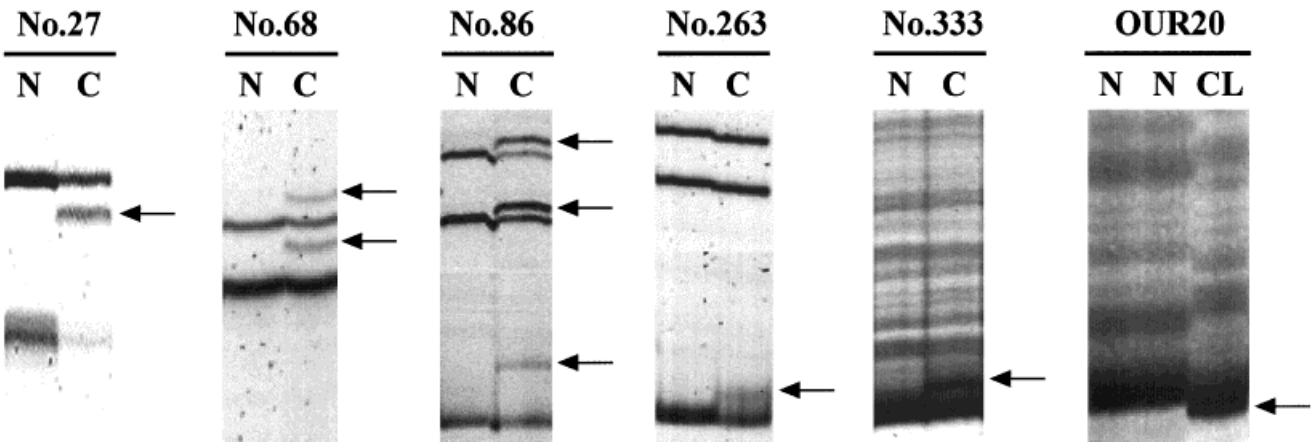

Figure 1 – DNA-SSCP analysis of the *PTEN* gene. Primary RCCs 27, 68, 86, 263 and 333 and the OUR20 cell line showed aberrant mobility shifts in exons 2, 5, 3, 3, 8 and 8, respectively. Aberrant bands (arrows) were observed only in renal carcinoma (C) or the OUR20 cell line (CL) but not in normal corresponding or control kidney samples (N).

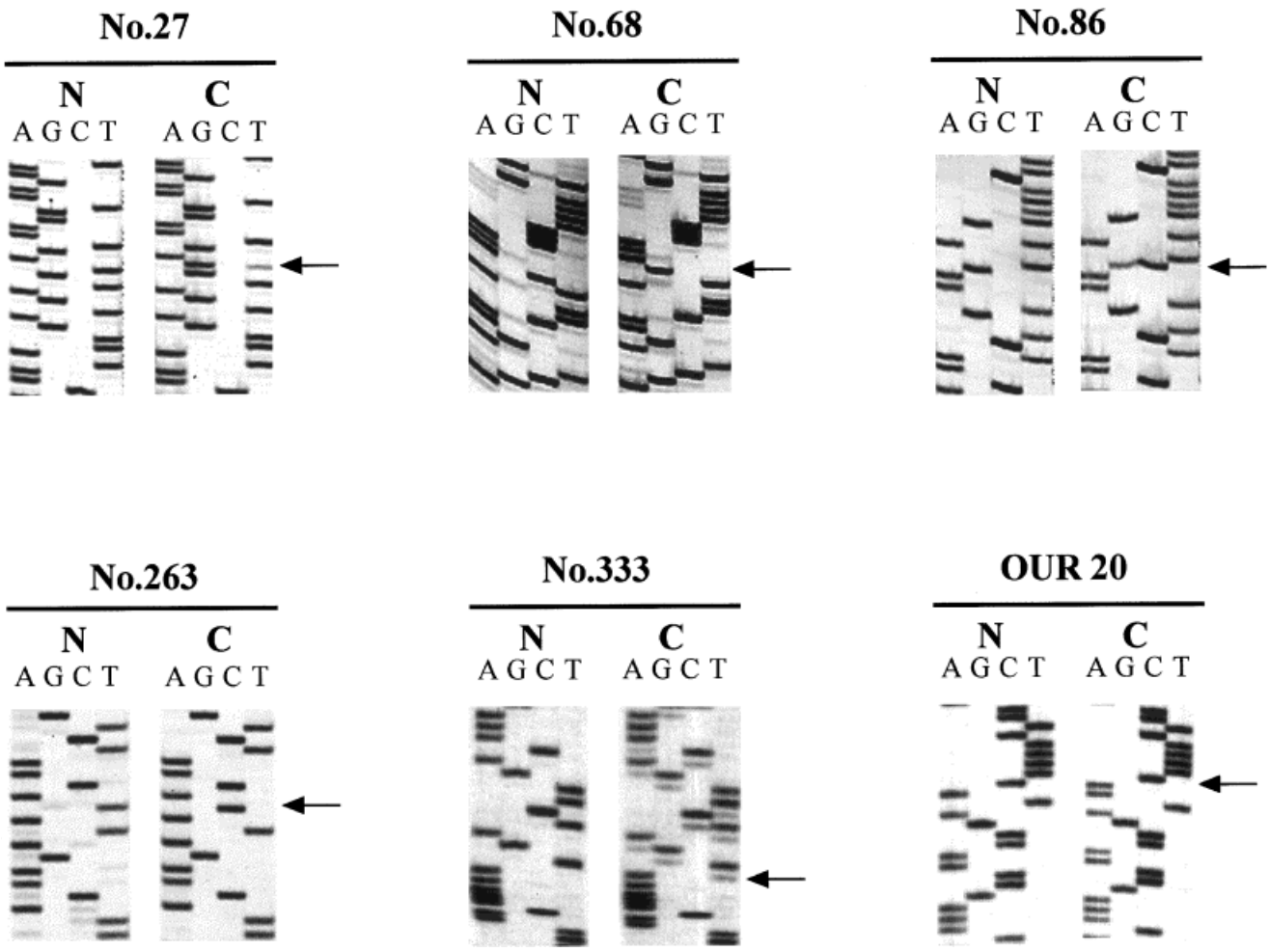

Figure 2 – Sequence analysis of the *PTEN* gene, demonstrating that tumor 27 had a T-to-C transition at nt. 158, tumor 68 had a C-to-G transversion at nt. 303, tumor 86 had a G-to-C transversion 5 bp from the splice donor site of intron 3 [nt. position 209(+5)], tumor 263 had a T-to-C transition at nt. 202, tumor 333 had an A deletion at nt. 968 and renal-carcinoma cell line OUR20 showed an A insertion at nt. 1005. N, corresponding normal kidney; C, renal-carcinoma sample.

others.[27,30] One was a C/G variant 9 bp from the splice donor site of the 5′ side of exon 1 with allelic frequencies of 0.97 (C) and 0.03 (G). The other was a T/G variant 32 bp from the splice donor site of intron 8 with allelic frequencies of 0.43 (T) and 0.57 (G) in our panel of Japanese patients.

We next examined primary RCCs for LOH status at the *PTEN* locus using 3 highly polymorphic dinucleotide-repeat markers, D10S215, D10S541 and PTENCA. LOH at the *PTEN* locus was found in 14 (25%) of 57 informative cases. Three of the 5 primary RCCs with intragenic mutation (tumors 86, 263 and 333) also showed LOH at this locus (Fig. 4), which means that the *PTEN* gene was completely inactivated in a "2-hit" manner in these tumors. We did not detect any aberrant patterns exhibiting microsatellite instability (MSI) for these 3 dinucleotide-repeat markers in the 68 fresh tumors analyzed.

DISCUSSION

In the current study, we examined the mutational status of the *PTEN1/MMAC1/TEP1* gene in primary RCCs as well as in renal-carcinoma cell lines and found somatic *PTEN* mutations in 5 of 68 (7.5%) primary tumor specimens and 1 of 17 (5.9%) cell lines. The *PTEN* gene was completely inactivated in 3 of the 5 primary tumors and in 1 cell line by "2-hit" mechanisms.[31] Histopathological and clinical data revealed that 4 of the 5 primary tumors with *PTEN* mutation were high-grade, advanced clear-cell RCCs with poor prognosis.

In sporadic gliomas, *PTEN* mutation was observed exclusively in high-grade, high-stage tumors.[11] Loss of *PTEN* expression was associated with tumor progression and poor prognosis in these malignancies.[32] Moreover, functional analysis demonstrated that wild-type *PTEN* can inhibit cell spreading and motility by suppressing both the focal adhesion kinase and the mitogen-activated protein kinase pathways, while mutated *PTEN* that had lost these functions enhanced the spreading and migration of tumor cells.[16,33] Our data suggested that *PTEN* inactivation also occurs as a late-stage event in the tumorigenesis of clear-cell RCCs and may contribute to the invasive and/or metastatic tumor phenotype.

In contrast, the fifth *PTEN*-mutated tumor (case 27) was a relatively early-stage, low-grade papillary/chromophilic carcinoma. The patient is alive without tumor recurrence more than 11 years after surgery. This subtype of RCC is morphologically and genetically distinct from clear-cell RCCs.[34–36] Indeed, we have previously analyzed tumor samples in the present series for somatic *VHL* mutations. Three clear-cell RCCs with *PTEN* mutations (tumors 86, 263 and 333) also showed somatic *VHL* mutations, while tumor 27 had no *VHL* mutation[3] (data not shown). These

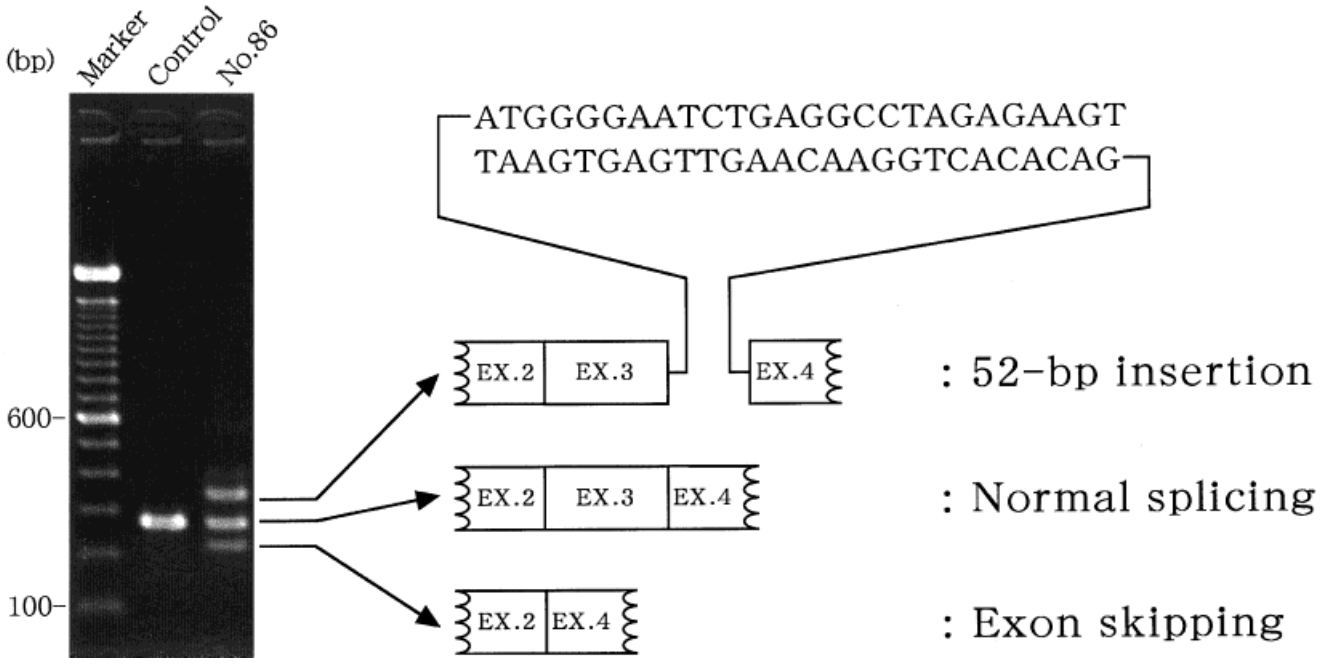

FIGURE 3 – Results of RT-PCR analysis of *PTEN* transcripts and schematic illustration of aberrant splicing identified in primary RCC. RT-PCR of *PTEN* exons 1–4 in renal tumor sample 86 (No. 86) showed 2 aberrant-sized transcripts together with a normal-sized transcript identical to that in a normal corresponding kidney sample (Control). Sequencing analysis revealed that the longer transcript contained 52 bp aberrant insertions between exons 3 and 4, the shorter band lost all of exon 3 and a normal-sized transcript had normally spliced cDNA sequences. Marker, 100 bp DNA ladder (GIBCO-BRL, Gaithersburg, MD) was used as a size marker.

TABLE I – SUMMARY OF *PTEN* MUTATIONS IN PRIMARY RENAL-CELL CARCINOMAS AND RENAL-CARCINOMA CELL LINES

Tumor/ cell line	Mutation	Exon/intron	Consequence	LOH[1]	Histopathology	TNM[2]	Renal vein invasion	Grade[3]	Outcome (months)[4]
27	158T→C	Exon 2	Val^{53} Gly	NI	Papillary/ chromophilic	T1bN0M0	(−)	G1	Alive, NED (133)
68	303C→G	Exon 5	Ile^{101} Met	No	Clear/compact	T3aN2M1	(−)	G3	Dead of disease (3)
86	209 (+5)G→C	Intron 3	Splice mutation	Yes	Clear/compact	T3aN0M1	(−)	G3	Dead of disease (3)
263	202T→C	Exon 3	Tyr^{68} His	Yes	Clear/compact	T3bN0M0	(+)	G3	Dead of disease (48)
333	968 delA	Exon 8	Frameshift	Yes	Clear/compact	T3bN0M0	(+)	G3	Dead of disease (22)
OUR20	1005 insA	Exon 8	Frameshift	Yes	—	—	—	—	—

[1]NI, not informative.–[2]TNM classification.–[3]Nuclear grading system: G1, low grade; G2, intermediate grade; G3, high grade.–[4]Survival months were calculated from the date of surgery. NED, no evidence of disease.

mutational data suggested that clear-cell and papillary/chromophilic RCCs have different tumorigenic pathways and that *PTEN* may play distinct roles in these 2 subtypes of RCC.

PTEN was frequently mutated in endometrial carcinomas with MSI, suggesting that this gene might be one of the mutational targets for a mismatch repair defect.[13,37] MSI has been observed in only a small fraction of sporadic RCCs.[6,38] Indeed, most of the tumor specimens in the present series were the subject of previous studies, including *VHL* mutations and MSI status, and we have found infrequent MSI in these RCC tumors.[38] In the current analysis, again, we did not find any aberration of the 2 A(6) repeats in the *PTEN* coding, which have been shown to be targets in replication error (RER)-positive colorectal cancers,[29] nor did we find MSI in 3 dinucleotide-repeat markers in our 68 RCC samples. The MSI/RER^+ phenotype, therefore, appears to be rare, and there is no observable correlation between *PTEN* mutation and MSI in sporadic RCCs.

Somatic *PTEN* mutation was originally found in 1 of 6 primary RCCs by Steck *et al.*.[8] Then, Sakurada *et al.*[39] and Cairns *et al.*[40] reported no *PTEN* mutations out of 24 and 15 RCC samples analyzed, respectively. Alimov *et al.*[20] found somatic *PTEN* inactivation in 3 of 54 (5.6%) primary RCCs and 1 of 9 (11%) RCC cell lines. In this study, we identified somatic *PTEN* mutation in primary RCCs and renal-carcinoma cell lines at a relatively low incidence (7.5% in primary tumor samples and 5.9% in RCC cell lines). Based on these findings, we conclude that *PTEN* alteration is likely to be involved in a subset of RCCs; nevertheless, our data suggest that inactivation of this tumor-suppressor gene occurred as a late-stage event and may be associated with invasive and metastatic tumor phenotypes in some clear-cell RCCs. Further detailed analysis including mutational detection and functional study will be needed to evaluate the importance of the *PTEN* gene in human kidney tumorigenesis.

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