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Aberrations of the *p14^{ARF}* and *p16^{INK4a}* Genes in Renal Cell Carcinomas

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The *INK4a*/*ARF* locus on chromosome 9p21, which encodes two distinct genes, *p14^{ARF}* and *p16^{INK4a}*, is frequently altered in human neoplasms. To investigate the potential roles of *p14^{ARF}* and *p16^{INK4a}* genes in human renal cell carcinomas (RCCs), we analyzed 6 human RCC cell lines and 91 primary RCCs for homozygous deletion, promoter hypermethylation and expression of the *p14^{ARF}* and *p16^{INK4a}* gene products using differential PCR, methylation-specific PCR, and immunohistochemistry, respectively. Five cell lines showed homozygous co-deletion of both genes and one demonstrated promoter hypermethylation of the *p16^{INK4a}* gene only. Eight of 91 RCCs showed aberrations of *p14^{ARF}* or *p16^{INK4a}* status and six of these featured gross extension into the renal vein. The results suggest that *p14^{ARF}* and *p16^{INK4a}* aberrations may play roles in the relatively late stage of renal tumorigenesis associated with tumor progression.

Key words: *p14^{ARF}* — *p16^{INK4a}* — Methylation — Homozygous deletion — Renal cell carcinoma

Renal cell carcinoma (RCC), the most common malignancy of the adult kidney, occurs in sporadic and hereditary forms. Recent studies characterizing genetic aberrations have implicated a number of chromosomal loci in cancer development and progression, with inactivation of tumor suppressor genes as one of the most important steps. Chromosome 3p deletions constitute the most common genetic event in primary RCCs and aberration of the *VHL* gene at 3p25 is known to occur early in the genesis of clear cell lesions.[1, 2] However, chromosome 3p is not cytogenetically aberrant in papillary RCCs and adult rather than in childhood tumors, even among individuals who inherit a mutated *VHL* gene. The RCC is also characterized by an accumulation of complex chromosomal alterations during tumor progression with loss of heterozygosity (LOH) relatively frequently observed for chromosomes 6q, 8p, 9p, 13q and 17p.[3, 4]

Loss of the short arm of chromosome 9, in particular 9p21–22, is common in many human neoplasms, for example melanomas,[5] bladder tumors,[6] and leukemia.[7] Furthermore, the *p16^{INK4a}* gene isolated from 9p21 has been found to be homozygously deleted in many types of tumor cell lines, including examples derived from RCCs.[8] In human neoplasms, loss of *p16^{INK4a}* expression is mainly due to homozygous deletion or to hypermethylation of CpG islands in the promoter region, while mutational inactivation is rare.[9] In primary RCCs, LOH on chromosome 9p has been detected in 20–30% of cases,[10, 11] but inactivation of *p16^{INK4a}* by homozygous deletion or point mutation is rare.[12–14] Recently, the *p14^{ARF}* gene was isolated as a second alternative reading frame gene of *p16^{INK4a}* of the *INK4a* locus and the human *p14^{ARF}* promoter has been cloned and shown to contain a CpG island.[15] The *INK4a* locus features two unique first exons, designated 1α and 1β, which are spliced into common exons 2 and 3.[16] Exons 1α, 2 and 3 encode *p16^{INK4a}* which induces cell cycle arrest by inhibiting phosphorylation of the RB protein.[17] The product of exons 1β, 2 and 3 is termed p14^{ARF}, which acts by binding directly to MDM2, resulting in the stabilization of both p53 and MDM2.[18, 19] Despite the potential importance of *p14^{ARF}* and *p16^{INK4a}* in tumorigenesis, there have been few studies of inactivation by homozygous deletions or altered methylation in primary RCCs.

To investigate whether the *INK4a*/*ARF* locus on chromosome 9 is involved in renal cell tumorigenesis, we screened 91 primary RCCs and 6 cell lines for homozygous deletion, promoter hypermethylation of the *p14^{ARF}* and *p16^{INK4a}* genes and expression of the gene products.

MATERIALS AND METHODS

Cell lines Six human RCC cell lines (SKRC-12, -14, -17, -29, -52, and -59) were cultured in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum.[20] Genomic DNA was extracted from each cell line as described previously.[21] Adherent cells were harvested by trypsinization, collected by centrifugation for 5 min at 1500 rpm and pellets of cells were fixed in 10% buffered formalin and embedded in paraffin.

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Tumor samples and DNA extraction Ninety-one primary tumor specimens were obtained from patients who had undergone nephrectomy between 1995 and 2000 in the Department of Urology, Nara Medical University Hospital and Nara Prefectural Hospital. Tumors were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 3 μm. The sections were stained with hematoxylin and eosin for histological diagnosis. Pathological evaluation and grading were according to the classification by the UICC and the AJCC.[22, 23]

Under a light microscopy, tumor lesions were marked and scraped off. Genomic DNA was extracted from each section as described previously.[24]

Differential PCR assay for *p14^{ARF}* and *p16^{INK4a}* To assess homozygous deletions in the *p14^{ARF}* gene, we carried out differential PCR with primers for exon 1β, using the *GAPDH* gene as an internal control. The primer sequences and PCR conditions were as described previously[25] and the lengths of PCR products for *p14^{ARF}* and *GAPDH* were 149 bp and 160 bp, respectively.

Differential PCR for homozygous deletions of *p16^{INK4a}* (exon 1) was carried out using the *β-actin* gene as an internal control. The primer sequences and PCR conditions were as previously described.[25, 26] The lengths of PCR products for *p16^{INK4a}* and *β-actin* were 204 bp and 187 bp, respectively. They were loaded onto 8% acrylamide gels and signal intensity was measured with Kodak Digital Science ID Image Analysis Software Version 3.0 (Kodak, NY). Values less than 0.2 for the target gene/internal control ratio were considered to represent homozygous deletion.[25–28]

Methylation-specific PCR Promoter hypermethylation of the *p14^{ARF}* and *p16^{INK4a}* genes was determined by methylation-specific PCR (MSP),[29] which distinguishes unmethylated from methylated alleles based on sequence changes produced after bisulfite conversion of unmethylated (but not methylated) cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA. Sodium bisulfite modification was performed using a "CpGenome" DNA Modification Kit (Intergen, Oxford, UK) according to the manufacturer's protocol with minor modifications.[25, 30]

Primer sequences for the methylated and unmethylated PCR of *p14^{ARF}* were as previously reported by Esteller *et al.*,[31] and those for *p16^{INK4a}* were reported by Herman *et al.*[29] PCR was carried out in a 10 μl mixture containing 1× PCR buffer (10 m*M* Tris pH 8.3, 50 m*M* KCl), 2 m*M* $MgCl_2$, 0.25 m*M* deoxyribonucleotide triphosphates

Table I. Summary of Data for the *p14^{ARF}*, *p16^{INK4a}* Genetic Status of Renal Cell Carcinoma Cell Lines

	p14del	p14met	p14IHC	p16del	p16met	p16IHC
SKRC12	–	–	+	–	+	–
SKRC14	+	–	–	+	–	–
SKRC17	+	–	–	+	–	–
SKRC29	+	–	–	+	–	–
SKRC52	+	–	–	+	–	–
SKRC59	+	–	–	+	–	–

del, homozygous deletion; met, hypermethylation; IHC, immunohistochemistry.

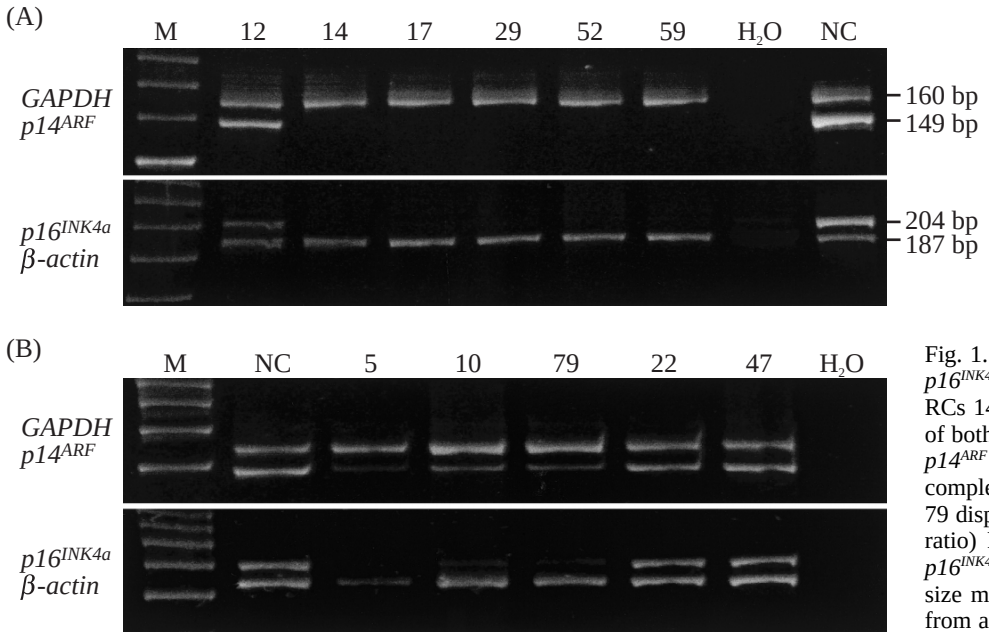

Fig. 1. Differential PCR for *p14^{ARF}* and *p16^{INK4a}* homozygous deletion. (A) SKRCs 14, 17, 29, 52 and 59 show deletion of both *p14^{ARF}* and *p16^{INK4a}*, the bands for *p14^{ARF}* and *p16^{INK4a}* PCR products being completely lacking. (B) RCCs 5, 10 and 79 display loss or very low levels (<20% ratio) PCR products of both *p14^{ARF}* and *p16^{INK4a}* genes. M, 25 bp ladder of DNA size markers; NC, normal control (DNA from a normal blood sample).

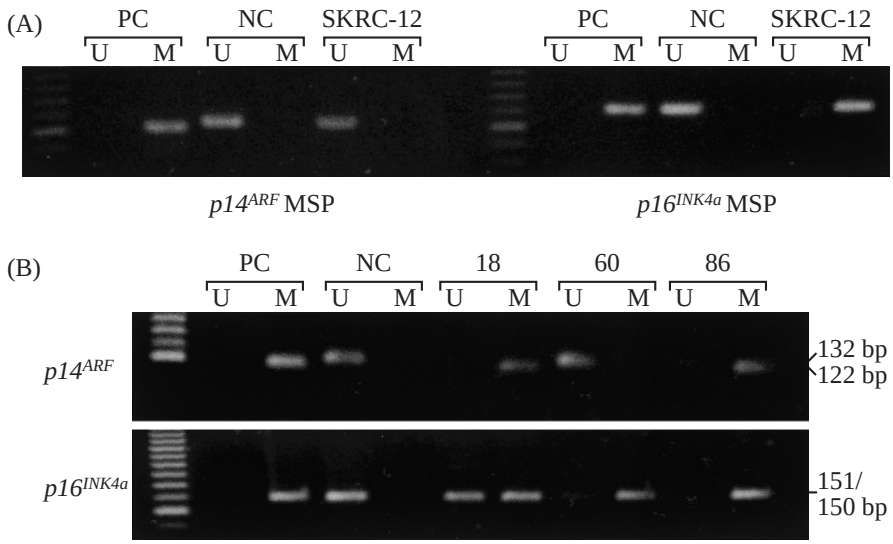

Fig. 2. Methylation-specific PCR analysis of $p14^{ARF}$ and $p16^{INK4a}$. PCR products amplified by unmethylated (U) and methylated (M) specific primers. (A) SK-RC 12 shows hypermethylation of the $p16^{INK4a}$ gene promoter. There is no hypermethylation of $p14^{ARF}$. (B) RCC18 and 86 show hypermethylation of both $p14^{ARF}$ and $p16^{INK4a}$ gene promoters. RCC60 demonstrates $p16^{INK4a}$ without $p14^{ARF}$ hypermethylation. MSP, methylation-specific PCR; PC, positive control for the methylated DNA; NC, normal control (DNA from a normal blood sample).

Table II. Clinicopathological Features of the Primary RCC Cases

	Clear	Chromophobe	Cystic	Granular	Papillary	Spindle	Total
T1	46	3	7	4	2	1	63
T2	6	0	0	0	0	0	6
T3	13	3	0	0	2	2	20
T4	0	0	0	0	1	1	2
Total	65	6	7	4	5	4	91
V(−)[a)]	56	3	7	4	2	1	73
V(+)[a)]	9	3	0	0	3	3	18
Total	65	6	7	4	5	4	91

a) Gross extension into the renal vein.

(dNTPs) (each), 0.5 μM primers (each), 0.4 units of FastStart *Taq* DNA polymerase (Roche, Mannheim, Germany) and approximately 40 ng of bisulfite-modified DNA. Amplification was performed according to the previous PCR conditions.[25, 29, 31)] Amplified products were electrophoresed on 2% agarose gels, and were visualized by ethidium bromide staining.[25)]

$p14^{ARF}$ and $p16^{INK4a}$ immunohistochemistry After deparaffinization, sections were heated for 5 min at 120°C in an autoclave in 10 m*M* sodium citrate buffer (pH 6.0). For $p14^{ARF}$ immunohistochemistry, the sections were incubated overnight at 4°C with rabbit polyclonal antibody 14^{ARF} (SC-8348, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:400 dilution. For $p16^{INK4a}$ immunohistochemistry, sections were incubated for 1 h at room temperature with a mouse monoclonal antibody (SC1661, Santa Cruz Biotechnology, Inc.) at 1:500 dilution. The reactions were visualized using a Histofine SAB-PO Kit (Nichirei Corp., Tokyo) and diaminobenzidine, and sections were counterstained with hematoxylin. The percentage of neoplastic cells with nuclear immunoreactivity was recorded as negative (−) when only occasional (<10%) tumor cells were stained and positive (+) when over 10% of the tumor cells were stained.[32)]

RESULTS

Cell lines Table I summarizes data for the homozygous deletion and methylation status of the 6 human RCC cell lines. Homozygous co-deletion of the $p14^{ARF}$ and $p16^{INK4a}$ locus was detected in 5 of the 6 (83.3%) cell lines (Fig. 1), and one (16.7%) showed promoter hypermethylation of the $p16^{INK4a}$ gene. There was no case with hypermethylation of the $p14^{ARF}$ gene promoter (Fig. 2).

The one of the 6 cell lines without deletion or hypermethylation of $p14^{ARF}$ showed $p14^{ARF}$ immunoreactivity, while all 6 exhibited loss of $p16^{INK4a}$ expression.

Primary renal cell carcinomas Table II shows the clinicopathologic characteristics of the 91 cases (64 males, 27 females; mean age, 62.5 years; range, 27–88 years).

Table III. Summary of Data for the *p14ARF*, *p16INK4a* Genetic Status of the Eight Primary RCCs with Alteration

Case	p14del	p14met	p14IHC	p16del	p16met	p16IHC	Histology	Grade	Stage	V[a)]
RCC5	+	−	−	+	−	−	clear	2	T3b	+
RCC10	+	−	−	+	−	−	clear	2	T3b	+
RCC79	+	−	−	+	−	−	cystic	2	T3	+
RCC18	−	+	+	−	+	+	spindle	3	T4	+
RCC86	−	+	−	−	+	−	papillary	3	T4	+
RCC60	−	−	+	−	+	−	clear	2	T2	−
RCC94	−	−	−	−	−	−	clear	2	T3b	+
RCC49	−	−	+	−	−	−	clear	2	T1	−

a) Gross extension into the renal vein.

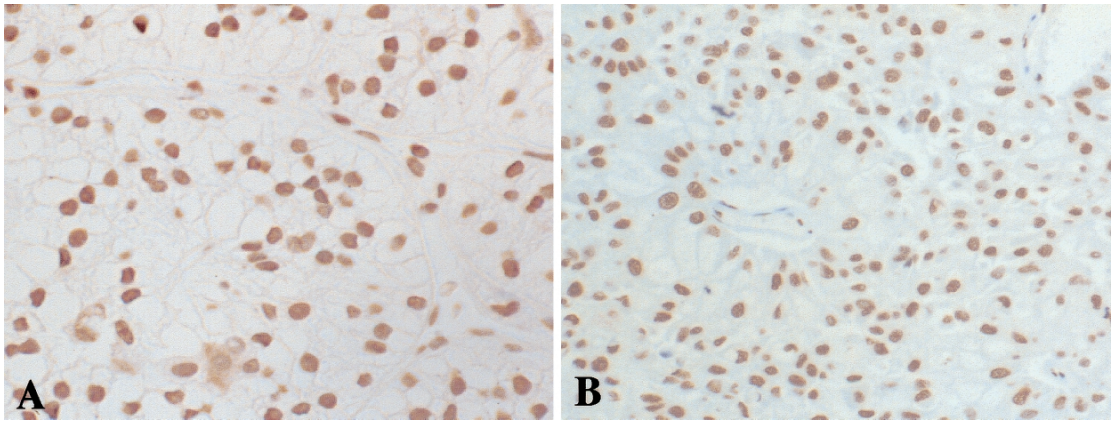

Fig. 3. Immunohistochemical staining of $p14^{ARF}$ and $p16^{INK4a}$. $p14^{ARF}$ (A) and $p16^{INK4a}$ (B) immunohistochemistry showing nuclear staining in the majority of tumor cells in renal cell carcinomas (A, RCC13; B, RCC22).

Homozygous deletion of both *p14ARF* and *p16INK4a* was detected in three of 91 (3.3%) samples (Fig. 1), and another three samples (3.3%) showed hypermethylation of *p14ARF* and/or *p16INK4a* gene promoters (Fig. 2).

Nuclear immunoreactivity for $p14^{ARF}$ and $p16^{INK4a}$ was observed in almost all tumor cells. Five of 91 (5.5%) samples showed loss of both $p14^{ARF}$ and $p16^{INK4a}$ expression and two samples showed loss of $p16^{INK4a}$ expression only (Table III). Three samples with homozygous deletion showed defective immunoreactivity, although one sample with promoter hypermethylation showed expression of $p14^{ARF}$ and $p16^{INK4a}$ (Fig. 3). Aberrations of *p14ARF* or *p16INK4a* were detected in eight of 91 (8.8%) samples by one or more of the three methods. There was no significant correlation with histological findings, tumor size or grading. However, six of the 8 cases featured gross extension into the renal vein (Table III).

DISCUSSION

The chromosome 9p21 region harbors two genes: *p14ARF* and *p16INK4a*, whose products have growth-suppressive activity. Two primary mechanisms have been postulated for inactivation of potential tumor suppressor genes on 9p21: homozygous deletion and promoter hypermethylation, with intragenic mutations occurring in only a small proportion of tumors and cell lines.[33)] The present results showed aberration of *p14ARF* and *p16INK4a* due to homozygous deletion and promoter hypermethylation to be more frequent in RCC cell lines than in primary RCCs, suggesting that the alterations might provide some advantage associated with acquisition of immortality and selective cell growth.

Homozygous deletion of the *p16INK4a* gene has been demonstrated frequently in cell lines derived from a variety of different tumors,[34, 35)] and therefore, *p16INK4a* is widely regarded as a major target with 9p21 deletion. A previous study showed inactivation of *p16INK4a* by homozygous deletion to be relatively rare in RCCs,[13)] and the type alteration of *p14ARF* and *p16INK4a* was detected in only three of 91 (3.3%) primary RCCs in our study. To assess the sensitivity of differential PCR for *p14ARF* and *p16INK4a* deletion, we previously carried out a titration experiment with various ratios of normal DNA and DNA from A172

glioma cells with homozygous co-deletion of the $p14^{ARF}$ and $p16^{INK4a}$ genes.[25] The results indicated that the ratios of $p14^{ARF}$/*GAPDH* and $p16^{INK4a}$/*β-actin* meaning hemizygous deletion are ~0.42 and ~0.35, respectively. In this study, 11 (12%) primary tumors showed decreased density (0.35–0.5) of the $p14^{ARF}$ and $p16^{INK4a}$ genes (data not shown), suggesting that these might show LOH of 9p21 at the *INK4a*/*ARF* locus, although many tumors have positive immunoreactivity to both genes.

$p14^{ARF}$ plays a major role in the *p53* pathway by binding to MDM2, and $p16^{INK4a}$ affects the *RB* pathway by inhibiting phosphorylation of the RB protein. Involvement of $p14^{ARF}$ and $p16^{INK4a}$ in malignant transformation is supported by studies on mice lacking *INK4a*/*ARF*[36] or *ARF* alone.[37] In mice, malignant transformation is induced by inactivation of either the *p53* pathway or the *RB* pathway, and the $p14^{ARF}$ and $p16^{INK4a}$ genes are frequently co-deleted in several human neoplasms,[38, 39] with inactivation of both having a cooperative negative effect on tumor progression.[40–42] Co-deletion of $p14^{ARF}$ and $p16^{INK4a}$ was here found in five RCC cell lines and three primary RCCs. Because of the dual encoding capacity of the $p14^{ARF}$ and $p16^{INK4a}$ locus, a deletion occurring in $p16^{INK4a}$ exons 2 or 3 could also disrupt $p14^{ARF}$. The unique genomic structure and compact organization of these genes which have common reading frames may be essential for maintaining a balanced *Rb* and *p53* pathway function.

Hypermethylation of $p14^{ARF}$ is often observed in colon cancer cell lines, with hypermethylation of $p16^{INK4a}$.[15] Recently, Esteller *et al.* showed promoter hypermethylation incidences of 23% for $p16^{INK4a}$ and 13% for $p14^{ARF}$ in primary RCCs.[43] In our study, the respective figures were 3.3% and 2.2%, respectively. Two showed promoter hypermethylation of both $p14^{ARF}$ and $p16^{INK4a}$ genes, and one sample showed hypermethylation of the $p16^{INK4a}$ gene only. We consider that this discrepancy is due to race or sampling (for example T1 samples accounted for 72% of all our tumors). Considering the results for expression of $p14^{ARF}$ and $p16^{INK4a}$ obtained by immunohistochemical staining, we conclude that inactivation of $p14^{ARF}$ and $p16^{INK4a}$ genes by promoter hypermethylation is a rare event in primary Japanese RCCs.

The present study in fact revealed a close correlation between immunohistochemical results and alteration by homozygous deletion/hypermethylation of $p14^{ARF}$ and $p16^{INK4a}$ genes. All 5 cell lines and 3 primary RCCs with homozygous co-deletion showed loss of both $p14^{ARF}$ and $p16^{INK4a}$ expression. While one of 3 primary RCCs with hypermethylation was immunoreactive for both $p14^{ARF}$ and $p16^{INK4a}$, it showed extensive areas with loss of expression. In this case, both unmethylated and methylated signals for $p16^{INK4a}$ were seen by MSP. This may be explained by incomplete gene silencing due to histological heterogeneity. In contrast, the unmethylated signal for $p14^{ARF}$ was very faint in the tumor, possibly due to insufficient density and/or partial extent of DNA methylation. One primary RCC showed loss of $p14^{ARF}$ expression without alteration, and two primary RCCs showed loss of $p16^{INK4a}$ expression without $p16^{INK4a}$ alteration. This may have been due to disruption of translational mechanisms in the inactivation of $p14^{ARF}$ and $p16^{INK4a}$ or mutations in exons 2 or 3 of the $p16^{INK4a}$ gene.

Previous studies showed LOH at 9p to be associated with stage, grade and recurrence,[10, 11] suggesting a contribution to tumor progression. Recent studies showed that loss of chromosome 9p13 is associated with progression in papillary RCCs, so that a tumor suppressor gene on chromosome 9p13 may play a role.[44, 45] In our study, eight cases (8.8%) demonstrated $p14^{ARF}$ and $p16^{INK4a}$ aberration. Although there was no significant correlation between aberration and histological types, tumor size, or grading, six of these cases (75%) showed gross extension into the renal vein (Table III). Therefore, aberrations such as deletion, methylation and loss of expression of $p14^{ARF}$ and $p16^{INK4a}$ may be associated with tumor extension into the renal vein. Venous invasion is characterized by local destruction of the endothelium by tumors. In a cohort study of RCC, patients with venous invasion were found to have a worse prognosis.

In summary, aberrations of $p14^{ARF}$ and $p16^{INK4a}$ such as homozygous deletions and hypermethylation were detected in a small subset of primary RCCs. Although a relatively rare event, such alteration appeared to be associated with tumor extension into the renal vein. Our results thus suggest the $p14^{ARF}$ and $p16^{INK4a}$ genes to be important with regard to 9p deletion, having a possible role in primary RCC progression.

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