Homozygous Deletions of the INK4a/ARF Locus in Renal Cell Cancer

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Abstract. *Background: Genetic alterations of $p14^{ARF}$ contribute to dysfunction of p53 pathways by disruption of MDM2-mediated inhibition of p53. $P14^{ARF}$ was investigated by focusing on the homozygous deletion (HD) in the INK4a/ARF locus and hypermethylation of the $p14^{ARF}$ promoter in renal cell cancer (RCC). Materials and Methods: Using 6 RCC cell lines, RT-PCR and Western blotting was performed for $p14^{ARF}$. DNA from 34 RCCs was analyzed for HD in the INK4a/ARF locus, promoter hypermethylation and p53 gene mutation. Results: HD was confirmed in 4 out of 6 cell lines and in 8 out of 34 (23.5%) RCC specimens, which correlated with the presence of metastasis, high tumor grade and had a tendency to more advanced stage (I vs. II–IV). No hypermethylation of the $p14^{ARF}$ promoter or p53 mutation was detected among the RCC specimens. Conclusion: These results indicate that the deletion in the INK4a/ARF locus might contribute to tumor progression in RCC at least partly by functional inactivation of wild-type p53.*

Renal cell cancer (RCC) is highly resistant to apoptosis-triggering agents such as radiation and anticancer drugs, resulting in the poor prognosis of patients with locally advanced or metastatic tumors due to limited treatment options. One key molecule that regulates apoptosis is p53. P53 is expressed at a low level in the normal state, but once cells suffer DNA damage, it accumulates, and consequently determines whether the cell goes in the direction of cell cycle arrest and DNA repair (1) or in the direction of apoptosis (2, 3). Mutations of the *p53* gene have been reported in more than half of human cancers, disrupting p53's normal response (4, 5). In RCC, however, p53 pathways are inactivated in spite of rare *p53* gene abnormalities, as demonstrated by many reports (6-8).

Loss of heterozygosity (LOH) on 9p21-22 has been frequently observed in RCC. Moreover, it presented with much higher rate in metastatic tumors (9). LOH of 9p21 in RCC was considered a putative major factor of tumorigenesis (10). Thus, essential tumor suppressor gene(s) might be encoded in these loci. The INK4a-ARF locus on human chromosome 9p21 encodes two different transcripts, $p14^{ARF}$ and $p16^{INK4a}$, derived from alternative splicing of upstream exons (Figure 1) (11). $P16^{INK4a}$ is a cyclin-dependent kinase (CDK) inhibitor that acts upstream of the retinoblastoma (Rb) protein to promote cell cycle arrest. $P14^{ARF}$ is one of the major p53-stabilizing proteins, preventing p53 from MDM2-mediated ubiquitination, transport into the cytoplasm and degradation (12). Recently, evidence has been provided for the idea that alteration of $p14^{ARF}$ might cause dysfunction of wild-type p53, resulting in cell cycle progression and inhibition of the apoptotic pathway (13, 14). Homozygous deletions (HD) of $p14^{ARF}$ were often seen in lymphomas (15), gliomas (16), lung cancers (17) and cutaneous melanoma (18). In liposarcoma, reduced expression of $p14^{ARF}$ due to hypermethylation of the $p14^{ARF}$ promoter was associated with a cell component reported to correlate with worse prognosis (19). The incidence of $p14^{ARF}$ promoter methylation was significantly higher in patients with invasive than superficial bladder cancer (20). However, genetic and epigenetic alterations of this locus in RCC are poorly understood. Kinoshita *et al.* revealed a low prevalence of p16 deletions in RCC cell lines and tumor samples (9). Kawada *et al.* reported homozygous co-deletions of both genes in only 3.3% of tumor samples, but in 5 out of 6 cell lines with the absence of $p14^{ARF}$ promoter methylation (21). Another publication reported a wide range of $p14^{ARF}$

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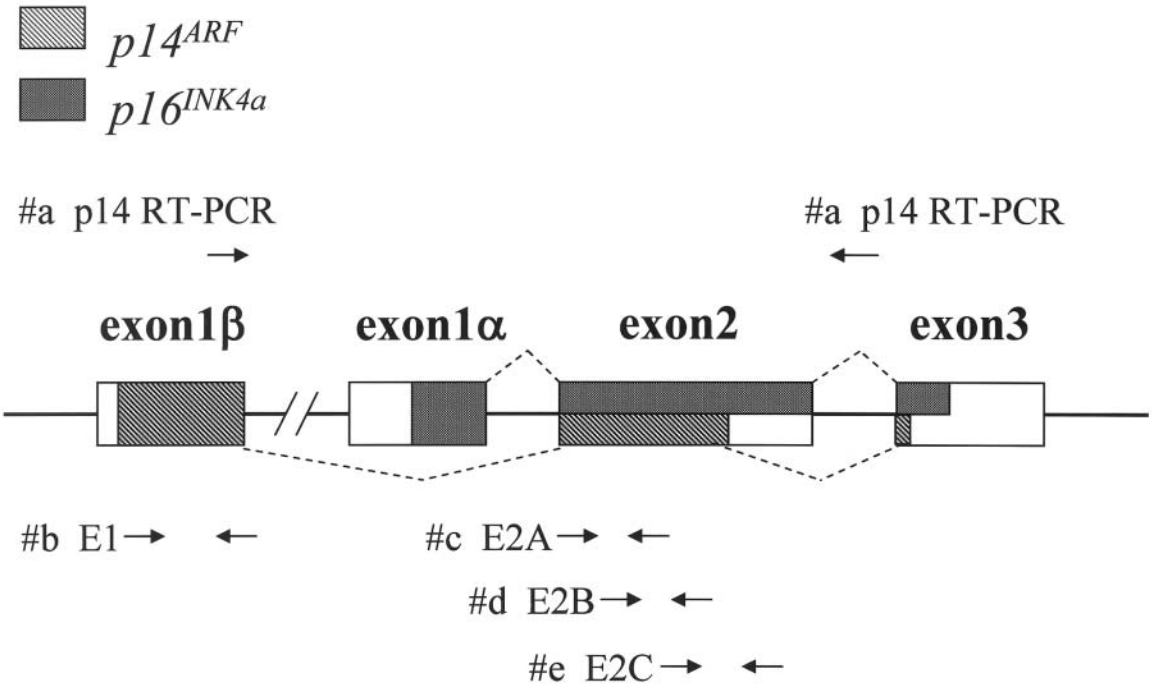

Figure 1. *Schematic diagram of the human INK4a/ARF locus and location of primers.*

promoter methylation in RCC, depending on the hystological type (from 12% in a Clear cell type to 40% in unclassified RCC) (22). Moreover, no HD of $p14^{ARF}$ or $p16^{INK4a}$ was observed in a small series of RCC, whereas aberrant $p14^{ARF}$ promoter hypermethylation was found in patients from radio-contaminated areas in the aftermath of the Chernobyl accident (23).

The discrepancy of previous reports prompted us to investigate the INK4a/ARF locus status, focusing on HD in the locus, $p14^{ARF}$ expression and hypermethylation of the $p14^{ARF}$ promoter region in 6 RCC cell lines and 34 primary RCC specimens.

Materials and Methods

Tumor samples and cell lines. RCC specimens obtained from 34 patients (27 males and 7 females; 36 to 81 years old, mean age: 61 years) were examined. Informed consent was obtained from all patients. The maximal and average follow-up was 60 and 32.7 months, respectively.

Pathological staging was determined according to the TNM classification of malignant tumors. Pathological grades were assigned according to a system developed by the Japanese Urological Association based on the degree of atypia of tumor cells.

Six established human RCC cell lines, A498, ACHN, Caki-1, Caki-2, KH39 and KRC/Y, were described earlier (6). Caki-1 and KH39 were transfected by electroporation with 10 μg of pBabe-puro expression vector carrying human $p14^{ARF}$. The transfected cells were selected for resistance to puromycin.

RT-PCR for $p14^{ARF}$ mRNA. RNA isolated from RCC cell lines was subjected for RT-PCR using first-strand cDNA synthesis kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and AmpliTaq™ (Perkin Elmer, Branchburg, NJ, USA), as described previously. To ensure appropriate first-strand cDNA synthesis *beta-actin* gene RT-PCR was performed. Primers (Table I) were designed to amplify the full-length $p14^{ARF}$ open reading frame.

DNA extraction, homozygous deletion analysis. High molecular weight DNA was isolated from RCC cell lines and tumor tissue samples. H&E-staining revealed that the samples used for DNA extraction contained over 80% tumor cells. Genomic DNA of the RCC cell lines was used as the template for PCR amplification of INK4a/ARF locus (Figure 1, Table I). *GAPDH* PCR was performed to certify the quality of the DNA. The location of the primers is listed in Figure 1. The PCR was performed using AmpliTaq™ (Perkin Elmer), as described previously.

Allelic dosage analysis for INK4a/ARF locus exon 2 was performed using the TaqMan ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Chiba, Japan). Real-time PCR was first performed for the *gamma-globin* (PE Applied Biosystems) gene as an internal control. Consecutively, equal volumes of DNA aliquots from RCC samples were used for INK4a-ARF locus exon 2 amplification with the primer set of E2B and Taq-man probe FAM-5'-ttcctggacacgctggtggtg-3'-TAMRA. The relative copy number was calculated, referring to the standard curve using different dilutions of normal genomic DNA. Relative amount (RA) was determined as [the relative copy number of target]/[the relative copy number of internal control]. The positive control RA was determined using DNA derived from peripheral blood lymphocytes of a healthy volunteer. All analyses of tumor

Table I. *Summary of PCR conditions, primers and amplicons.*

#, Exon and primer name	Forward primer sequence	Reverse primer sequence	Annealing temperature	Amplicon size
#a RT-PCR *p*14 RT-PCR	aaggatccatggtgcgcaggttcttgg	gttgtggcgggggcagttgt	61˚C	611
#b Exon 1β E1	gcctgcggggcggagat	tggtcttctaggaagcggct	58˚C	315
#c Exon 2 E2A	ctggctctgaccattctgt	agcaccaccagcgtgtcc	58˚C	171
#d Exon 2 E2B	gaccccgccactctcacc	aggtaccgtgcgacatcgc	58˚C DNA PCR 60˚C Real-time PCR	170
#e Exon 2 E2C	gatgcctggggccgtct	cagggtacaaattctcagat	55˚C	169

samples were performed in triplicate. The RCC samples presenting <20% of the positive control RA were considered to possibly harbor homozygous deletions (24).

Methylation-specific PCR (MSP). The methylation status of $p14^{ARF}$ promoter was determined by MSP. The first step was bisulfate modification using a CpGenome DNA Modification Kit (Intergen Company, Temecula, CA, USA) according to the manufacturer's instructions. The second step was PCR amplification with the following specific primers designed to distinguish methylated from unmethylated DNA (25). Approximately 30 ng of bisulfate-modified DNA was applied. HCT15 (colon cancer cell line) and Kato-III (gastric cancer cell line) DNA were used as methylated DNA (26) and DNA from normal lymphocytes as unmethylated DNA.

PCR-SSCP. The p53 gene was screened for genetic alterations by PCR-SSCP. All procedures and primers sets were described previously (6).

Immunostaining. Monoclonal anti-human $p14^{ARF}$ antibody (Neo Marker, Fremont, CA, USA) was used for immunostaining of the frozen tumor tissues and for Western blotting, as described previously (6). As a positive control, cut sections of HeLa cells embedded in an optimum cold temperature compound (Miles Laboratories) and HeLa cell lysate were used.

Results

$P14^{ARF}$ mRNA and protein expression in RCC cell lines. RT-PCR revealed that only ACHN (wild-type p53) and KRC/Y (mutant p53) showed *$p14^{ARF}$* mRNA expression (Figure 2A). Western blotting for detection of the $p14^{ARF}$ protein was subsequently performed. Fifty micrograms of protein were applied per lane and no specific band was detected in any cell line, although the specific band was detected in HeLa cells lysate with the application of as little as 20 μg of

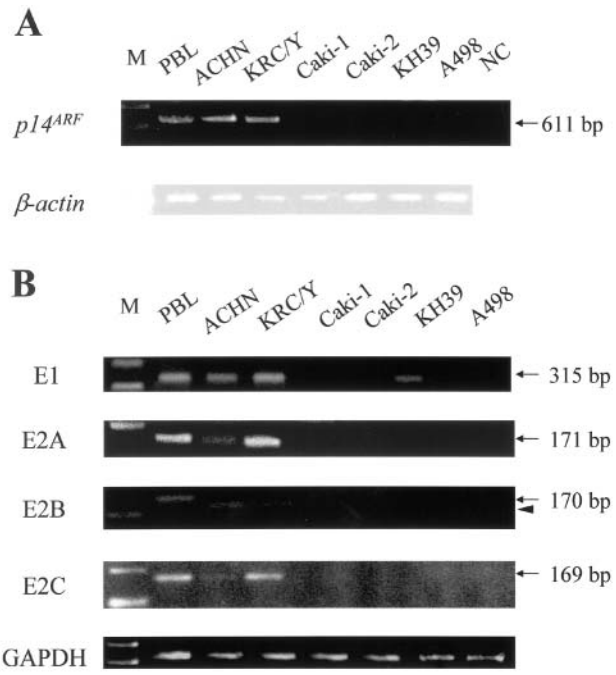

Figure 2. *A) RT-PCR for $p14^{ARF}$ mRNA. B) DNA PCR for INK4a/ARF locus. M: 100-base-pair molecular marker, PBL: peripheral blood lymphocytes of a healthy volunteer.*

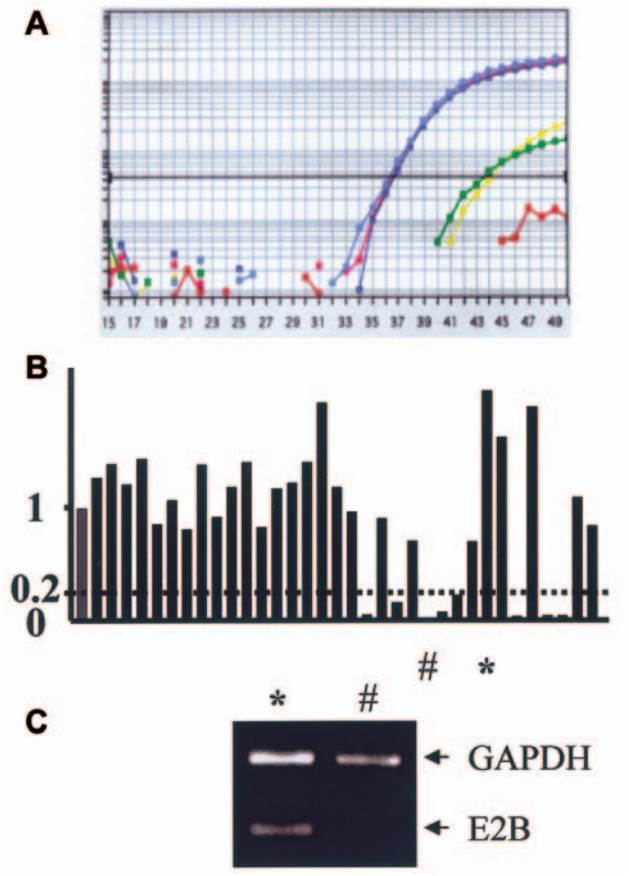

Figure 3. *A) Real-time PCR result of two representative RCC patients, each done in triplicate. Note that the amplification curves for DNA samples from a patient with homozygous deletion (HD) (green, yellow and red curves) are shifted to the right, and the number of cycles needed to reach the threshold level (about 44 cycles) is larger than DNA samples from a patient without HD (about 37 cycles) (the other curves). B) Relative amount (RA) of INK4a/ARF locus exon 2 in RCC specimens. Closed bars represent log ratio compared with the RA of control DNA (hatched bar). The samples presenting <20% of the control RA were considered to possibly harbor homozygous deletions. C) Multiple PCR of the two patients presented in (A). Left: patient without HD (marked with * in (B)). Right: patient with HD (marked with # in (B)). Using primer sets of GAPDH and E2B, 40 cycles of amplification were performed.*

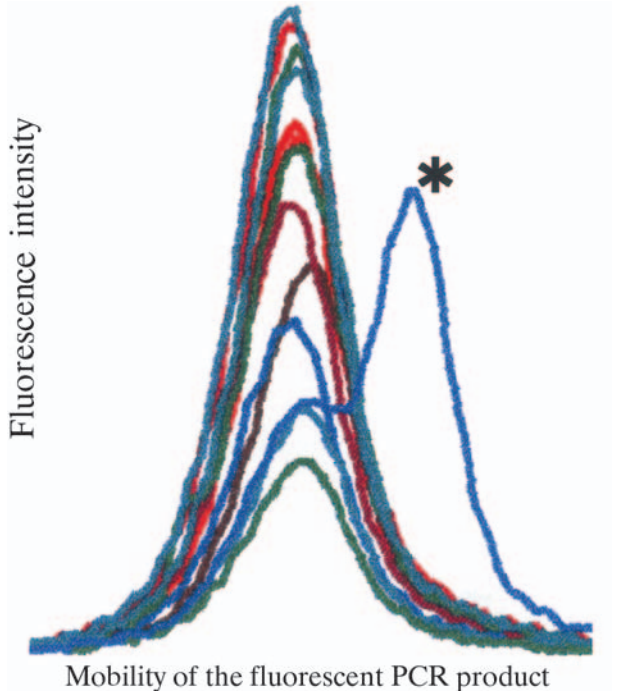

Figure 4. *Representative PCR-SSCP results of the p53 gene in RCC specimens. No mobility shift was detected in any of the specimens. * Control DNA carrying p53 mutation.*

total protein per lane (data not shown).

Frequent deletions of INK4a-ARF locus in RCC. In the ACHN and KRC/Y cells, a specific DNA PCR product was observed using each primer's set, except for E2B, in which the amplicon was slightly shorter than in control PBL DNA, which may indicate a small deletion (Figure 2B, E2B, arrowhead). E1 also produced a specific amplicon in KH39. However, all other PCR products were undetectable (Figure 2B).

DNA extracted from RCC specimens was subjected to real-time PCR. In 8 out of 34 specimens (23.5%) the amplification curve was shifted to the right (Figure 3A). Calculation of the relative amount compared with the copy number of the internal control confirmed the presence of HD of $p14^{ARF}$ in these samples (Figure 3B and C).

Absence of hypermethylation in the $p14^{ARF}$ promoter and p53 mutations in RCC. None of the samples showed promoter hypermethylation even though the positive and negative methylation controls were included. In the PCR-SSCP analysis, no aberrant or shifted peaks in comparison with the peaks in the normal control were detected in any individual examined (Figure 4). The absence of *p53* mutations in exons 4 to 8 was confirmed by direct sequencing of the PCR products. These results suggested that no genetic alteration of *p53* had occurred in those tumors since the majority of *p53* mutations were located in the DNA-binding domain (exons 5 through 8) (27).

Clinical implications of HD in INK4a/ARF locus. The presence of metastasis ($p=0.0371$, chi-square 4.344) and grade-3 tumor status ($p=0.0086$, chi-square 6.9) was significantly correlated with the HD of INK4a/ARF locus (Table II). In addition, patients with the HD appeared to be diagnosed at more advanced stages (stage I *vs.* II-IV), although this demonstrated a borderline significance ($p=0.0595$) (Table II). Patients with HD had shorter

Table II. *Association between homozygous deletion (HD) of the INK4a/ARF locus and clinicopathological variables of the RCC patients.*

	HD (+)	HD (–)	Total
Tumor stage 1	1 (7.1%)	13 (92.9%)	14 (100%)
Tumor stage≥2*	7 (35%)	13 (65%)	20 (100%)
Metastasis (–)	3 (13.0%)	20 (87.0%)	23 (100%)
Metastasis (+)**	5 (45.5%)	6 (54.5%)	11 (100%)
Tumor grade 1	4 (21.1%)	15 (78.9%)	19 (100%)
Tumor grade 2	2 (15.4%)	11 (84.6%)	13 (100%)
Tumor grade 3***	2 (100%)	0	2 (100%)
Total	8 (23.5%)	26 (76.5%)	34 (100%)

P-value: chi-square test; **p*=0.0595; ***p*=0.0371; ****p*=0.0086.

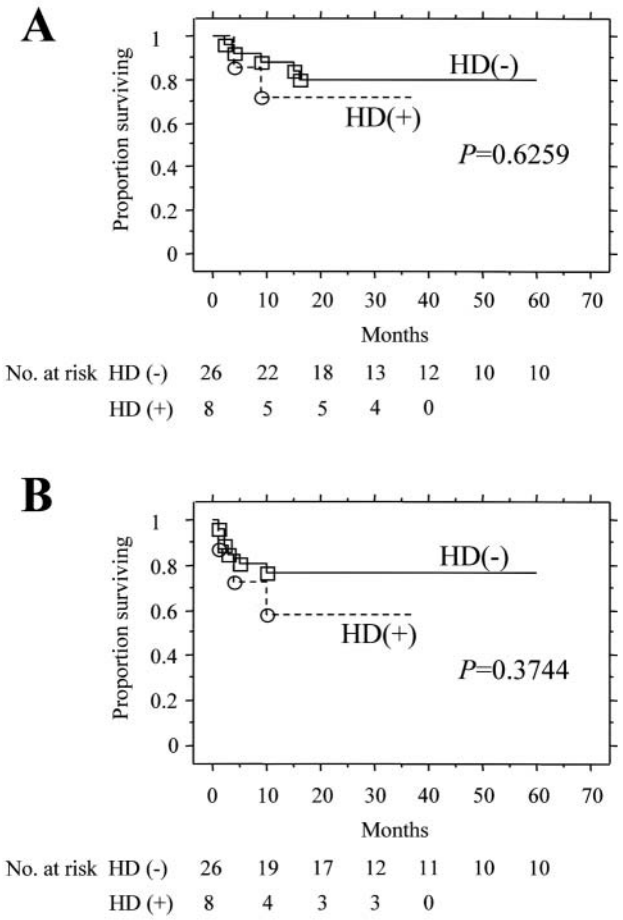

Figure 5. *A) Disease-specific survival of the RCC patients. B) Progression-free survival of the RCC patients. ○: patients with homozygous deletion (HD), □: patients without HD. The significance of the difference between the curves was calculated by the log-rank test.*

disease-specific and particularly progress-free survival by Kaplan-Meyer analysis. However, those were not significantly different by the log-rank test, presumably due to the limited number of patients (Figure 5).

Discussion

In RCC, p53 pathways are inactive in spite of wild-type *p53* (6-8). The amount of p53 protein is regulated *via* an autoregulatory feedback loop in cooperation with MDM2 and $p14^{ARF}$ (28). Activation of *p53* by cellular stress induces the transcription of the *MDM2* gene. MDM2 binds to p53, then blocks the activity of p53 and promotes its degradation. $P14^{ARF}$ protein can stabilize p53 by binding to and promoting the degradation of MDM2, providing a molecular mechanism for p53 function in cell cycle control and tumor suppression. Thus, loss of $p14^{ARF}$ and/or gene amplification or overexpression of *MDM2* could disable p53 pathways even though *p53* is not genetically altered (15). Studies of a variety of tumor cell lines have demonstrated that genetic alterations of *p53* and $p14^{ARF}$ are inversely correlated, suggesting that *p53* inactivation and $p14^{ARF}$ loss are functionally equivalent (29, 30). $P14^{ARF}$ is frequently inactivated by HD. Since another gene, $p16^{INK4a}$, is encoded in the same locus (11), HD can result in simultaneous disruption of $p16^{INK4a}$, which is a CDK inhibitor acting on cell cycle arrest. Thus, a single genetic hit can disrupt two main pathways – cell cycle control and p53, which both have anti-carcinogenic effect.

Our PCR study indicated that 4 out of 6 RCC cell lines and 8 out of 34 RCC specimens (23.5%) had HD in INK4a/ARF locus (Figures 2, 3). On the other hand, no hypermethylation of the $p14^{ARF}$ promoter was found in the RCC specimens. In colorectal and gastric cancer, $p14^{ARF}$ promoter hypermethylation was detected frequently (26). However, in RCC, the HD of INK4a/ARF locus might be a dominant genetic deficiency leading to disruption of $p14^{ARF}$ function alongside with $p16^{INK4a}$. As for MDM2, amplification of the *MDM2* gene was not observed in the above-mentioned 6 RCC cell lines (personal unpublished data).

Although ACHN and KRC/Y showed $p14^{ARF}$ mRNA expression, no $p14^{ARF}$ was detected by Western blotting in these 2 cell lines without HD. It is interesting to note that genomic PCR with E2B primers resulted in a shorter amplicon compared with control PBL DNA, which may indicate small deletion (Figure 2B, E2B, arrowhead). Moreover, none of the RCC specimens, including samples without HD, showed *in situ* immunoreactivity for $p14^{ARF}$. Other genetic alterations, such as point mutations or small deletions, might exist, leading to loss of or insufficient protein expression. Alternatively, $p14^{ARF}$, which is potentially harmful for cell proliferation, might be quickly degraded.

No *p53* mutation was detected in the RCC specimens examined. Based on the analysis of p53 mutation database

(7) and our own data, RCC has a particular character of high resistance to treatment with a very low frequency of *p53* gene abnormalities. However, due to the fact that p53 has a plethora of downstream molecules regulating various intracellular processes involving cell cycle arrest, DNA repair and apoptosis, and, thus, is called the "guardian of genome", we believe that RCC possesses some mechanisms which disrupt the p53 pathway. Although further analysis is required, loss of $p14^{ARF}$ might be a main contributor to the functional inactivation of wild-type p53, resulting in tumor progression and resistance to apoptotic stimuli in RCC.

Gene therapy-based treatment replacing the $p14^{ARF}$ gene may revitalize p53 function. In mesothelioma cell lines, overexpression of $p14^{ARF}$ using adenoviral vector transfer induced cell cycle arrest and/or apoptosis (31). Furthermore, greater effects were achieved in cell lines with wild-type p53 than in p53-null cells. We generated $p14^{ARF}$-stable transfectants and mock-transfected cells in wild-type p53 RCC cell lines. The stable transfection resulted in change of morphology (spindle-like shape) and retarded growth compared to the parental and mock-transfected cells. Furthermore, the transfectants presented obvious senescence and subsequent cell death. For this reason, no additional functional experiments were feasible with these transfectants. Further research may clarify the exact role of $p14^{ARF}$ in the restoration of the apoptotic machinery in RCC cells.

HD of the INK4a/ARF locus was significantly correlated with tumor grade ($p=0.0086$) and the presence of metastasis ($p=0.0371$) (Table II), and tumors containing such HD tended to be diagnosed at a more advanced stage. Patients with HD had shorter disease-specific and particularly progress-free survival by Kaplan-Meyer analysis. However, those were not significantly different by the log-rank test (Figure 5). A larger number of patients and additional follow-up study may clarify whether this gene alteration can be a certain predictor of worse prognosis.

In conclusion, we demonstrated HD of the INK4a/ARF locus in RCC. Although further studying is needed, this gene alteration might contribute to the functional inactivation of wild-type p53, resulting in tumor progression and poor prognosis.

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