J Gastroenterol 2004; 39:355–361
DOI 10.1007/s00535-003-1302-9

Journal of Gastroenterology

Alteration of the *p14ARF* gene and p53 status in human hepatocellular carcinomas

Teruaki Ito[1], Naoshi Nishida[1], Yoshihiro Fukuda[2], Takafumi Nishimura[1], Toshiki Komeda[1], and Kazuwa Nakao[1]

[1]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[2]Kyoto University College of Medical Technology, Kyoto, Japan

Background. The INK4a/ARF locus encodes p16^{INK4a} and p14^{ARF}, both of which are crucial for two tumor suppressor pathways, retinoblastoma (RB)/p16^{INK4a} and p53/ARF. Inactivation of RB/p16^{INK4a} was frequently reported, but alterations of the *p14ARF* gene in hepatocellular carcinoma (HCC) in the Japanese population have been insufficiently analyzed. ***Methods.*** To determine the role of p53/ARF alteration in hepatocarcinogenesis, we examined 44 HCCs for mRNA expression, deletion, mutation, and promoter hypermethylation of the *p14ARF* gene; alterations of p53 were also analyzed in the same series of HCCs. ***Results.*** Homozygous deletion, spanning from exon 1β to exon 2, was found in 1 HCC mutations within exon 2 were found in 2 HCCs, but no promoter hypermethylation was detected. All 3 HCCs with *p14ARF* alteration were well differentiated. Twelve of the 44 HCCs (27.2%) showed immunohistochemical evidence of p53 alteration; however, only 1 of the tumors with p53 alteration was well differentiated. TaqMan polymarase chain reaction (PCR) indicated that the expression of p14^{ARF} in HCCs was higher than in that in all but three of the corresponding nontumorous tissues ($P < 0.0001$), and increased expression of p14^{ARF} seemed to be associated with poorly differentiated phenotype. Absence of p14^{ARF} expression was seen in only one HCC, with homozygous deletion of the *p14ARF* gene. ***Conclusions.*** Compared with p53 alteration, *p14ARF* alteration does not occur frequently, but may play a role in a subset of Japanese HCCs in the early stage of hepatocarcinogenesis. On the other hand, overexpression of p14^{ARF} was frequently observed in HCC, especially in poorly differentiated tumors, probably reflecting oncogenic stimuli in these tumors.

Key words: p14^{ARF}, p53, INK4a/ARF locus, hepatocellular carcinoma

Received April 14, 2003 / Accepted: July 25, 2003
Reprint requests to: N. Nishida

Introduction

The INK4a/ARF locus on chromosome 9p21 has a unique genetic organization, which codes two proteins with different functions, the cyclin-dependent kinase inhibitor (CDKI) p16^{INK4a} and p14^{ARF}. While p16^{INK4a} prevents S-phase entry by inhibiting CDK4/6-mediated phosphorylation of retinoblastoma (RB), p14^{ARF} is a key trigger of p53 stabilization in response to oncogenic signaling.[1–3]

The *p16INK4a* gene is known to be inactivated by mutations, homozygous deletions, or promoter methylation in many tumors of diverse origin,[4–7] and its frequent elimination as a result of methylation of the promoter region in hepatocellular carcinoma (HCC) has been reported recently.[8,9]

As for p14^{ARF}, deletion inactivation of the INK4a/ARF locus has been reported in several human cancers.[10–12] The promoter hypermethylation of the CpG islands in the *p14ARF* gene, as well as in the *p16INK4a* gene, has been reported in primary colorectal,[13] gastric,[14] and esophageal cancers.[15] Moreover, alterations of p14^{ARF} expression levels have been observed in lung cancer,[16] breast carcinomas,[17] colorectal tumors,[18] and hematological malignancies.[19] Although inactivation of p16^{INK4a} has been documented as a frequent event in human HCCs, the role of p14^{ARF} alteration in human hepatocarcinogenesis is still poorly understood.

We previously reported that alteration of the *p53* gene was detected in 32% of human HCCs,[20] but the frequency and mechanism of inactivation of p14^{ARF}, the key trigger of p53 stabilization, have not yet been identified. The focus of the study presented here was, therefore, on alterations of p14^{ARF} in 44 primary HCCs in Japan, in terms of mRNA expression, homozygous deletion, mutation, and promoter hypermethylation, in order to determine the involvement of p14^{ARF} inactivation in human HCCs. In addition, we investigated p53 alterations in the same series of HCCs. In this report, we

describe alterations of p14ARF in HCCs in the Japanese population with special attention to p53 inactivation. We also discuss the role of disruption of the p53/ARF pathway during human hepatocarcinogenesis.

Methods

Samples

HCC samples and their corresponding non-tumorous tissues were obtained from 44 patients (40 men and four women), ranging in age between 41 and 79 years, during surgery or autopsy at Kyoto University Hospital. The samples were immediately stored at −80°C. Eight patients were positive for hepatitis B surface antigen (HBsAg), 27 for hepatitis C virus antibody (HCVAb), and 2 for both HBsAg and HCVAb, while 7 patients were negative for both. The grade of differentiation of HCC was determined at the Clinical Pathology Department of the hospital. Sixteen of the HCCs were well differentiated, 19 were moderately differentiated, and 9 were poorly differentiated. Tumor tissues were carefully separated from non-tumorous tissues, and high-molecular-weight DNA and RNA were extracted from tumorous and non-tumorous tissues according to standard protocols.[21] Of the 44 HCCs, 28 were subjected to quantitative RNA expression analyses. Informed consent was obtained from all patients.

Methylation analysis of the p14ARF *gene promoter*

The methylation status of the CpG islands in the *p14ARF* gene promoter was analyzed by means of methylation-specific polymerase chain reaction (MS-PCR). DNA samples from fresh frozen tissues were chemically modified by sodium bisulfite, and amplified by using primers specific for methylated and unmethylated sequences of the *p14ARF* promoter. The details of the modification reaction, PCR conditions, and sequences of the primers used have been described previously.[13] DNA from KATO-III (JCRB0611; Health Science Research Resources Bank, Osaka, Japan) a gastric cancer cell line which is reported to contain the methylated promoter of the *p14ARF* gene,[14] was used as a positive control for the methylated sequence, and DNA from normal peripheral lymphocytes was used as a negative control.

Homozygous deletion analysis of the p14ARF *gene*

We used comparative multiplex PCR to detect homozygous deletions in exon 1β and exon 2 of the *p14ARF* gene, as previously described.[9,15] Two primer sets of the *p14ARF* exon 2 and the β-actin, as well as exon 1β and the β-actin, were amplified simultaneously in a single reaction for each comparative multiplex PCR. The PCR products were electrophoresed on 10% polyacrylamide gels, and the intensities of the bands were densitometrically quantified (Densitograph AE-6905C; ATTO, Tokyo, Japan). The tumor was considered to contain a homozygous deletion if the signal was less than 10% of that of the control. PCR and gel analyses were performed at least three times for each sample.

PCR-single strand conformation polymorphism (PCR-SSCP) analysis and sequencing of the p14ARF *gene*

PCR-SSCP analyses for exon 1β and exon 2 of the *p14ARF* gene were performed, by using primer pairs designed to cover the entire coding region and under the conditions described by Kita et al.[7] Samples showing abnormal mobility in the PCR-SSCP analysis were further analyzed by direct sequencing with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 310 genetic analyzer (Applied Biosystems).

Relative quantitative real-time reverse transcription (RT)-PCR of p14ARF mRNA

cDNA was generated from total RNA by using SuperScript reverse transcriptase (Gibco RBL, Rockville, MD, USA) according to the manufacturer's instructions. The sequences of the PCR primer pair and the TaqMan probe used were as follows: forward, 5′-TTCGTGGTTCACATCCCGCGGC-3′; reverse, 5′-CCCATCATCATGACCTGGTC-3′; TaqMan probe, 5′-FAM-CAGCAGCCGCTTCCTAGA-TAMRA-3′. In order to avoid false-positive PCR results from the genomic DNA, the reverse primer for RT-PCR was designed to contain the junction of two exons. All semi-quantitative PCRs were performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The PCR reaction mixture contained 12.5 µl of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM of the primers, 100 nM of the TaqMan probe, 1 µl of the cDNA sample, and water. The thermal cycling conditions comprised the initial steps at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The standard curve was constructed with serial dilutions of cDNA from the HLF cell line (JCRB 0450; Health Science Research Resources Bank, Osaka, Japan) for analysis of p14ARF mRNA expression, which clearly expressed the mRNA of the target genes. In order to compare findings under the same conditions, data for the target genes were normalized to the expression of an internal housekeeping gene, the

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, by means of TaqMan GAPDH control reagents (Applied Biosystems). All the reactions for standard samples and samples of HCC cases were performed in duplicate, and the data were averaged from the values of duplicate reactions.

Immunohistochemistry for p53

The primary antibody used in this study was as follows: monoclonal mouse anti-human p53 protein, clone DO-7 (1:50 dilution; Dako, Copenhagen, Denmark). Briefly, 4-μm slices of tissue sections were reacted with the primary antibody overnight at 4°C. Negative controls were reacted with normal mouse immunoglobulin under similar conditions. The tissue sections were then incubated at room temperature for 30 min with biotinylated anti-mouse IgG (1:200 dilution; Vector Laboratories, Burlingame, CA, USA), and incubated for 30 min together with the avidin-biotin peroxidase complex reagent with the aid of a Vectastain ABC kit (Vector Laboratories). Diaminobenzidine (0.05%) was used as the final chromogen, and hematoxylin was used as the nuclear counterstain.

Statistical analyses

The Wilcoxon signed rank test or Mann-Whitney *U*-test was used for comparisons of expression levels of $p14^{ARF}$ among HCCs of various differentiation grades and non-tumorous tissues. For analysis of the relationship between grade of differentiation and alteration of *p14ARF* or p53, Fisher's exact test was applied. $P < 0.05$ was considered to be statistically significant.

Results

Analysis of the $p14^{ARF}$ *gene in HCC*

MS-PCR analysis showed that DNA from the gastric cancer cell line KATO-III was methylated at the *p14ARF* CpG island, while DNA from normal peripheral lymphocytes was unmethylated at the same site, as previously described.[14] On the other hand, no hypermethylation of the *p14ARF* promoter was observed in either the 44 HCCs or their corresponding non-tumorous tissues (Fig. 1A). Figure 1B shows representative findings of comparative multiplex PCR of the *p14ARF* gene. One HCC (case 1) showed homozygous deletion in exon 1β and exon 2 of the *p14ARF* gene. All DNA samples were also screened with PCR-SSCP for mutation of the *p14ARF* gene, and two HCCs (cases 2 and 3) showed abnormal mobility in exon 2 (data not shown), but none showed abnormal mobility in exon 1β. Sequencing of the two PCR products with abnormal mobility detected a point mutation causing amino-acid substitution (case 2) and one base-pair insertion causing a frameshift mutation (case 3; Fig. 1C).

Relative mRNA level of p14ARF

Target quantities of mRNA were determined from the standard curve and expressed as an n-fold difference relative to the standard sample; that is, HLF cell line. Relative mRNA levels of $p14^{ARF}$ ranged from 0.11 to 5.4. One HCC showed an extremely low level of $p14^{ARF}$ mRNA, although the quantity of GAPDH mRNA was adequate. In this HCC, homozygous deletion in exon 1β and exon 2 of the *p14ARF* gene was observed, suggesting that the deletion resulted in the defect in mRNA expression (case 1; Table 1). On the other hand, two HCCs with mutations of the *p14ARF* gene showed mRNA expression (case 2 and 3; Table 1). In all but three HCCs, the expression of $p14^{ARF}$ mRNA was higher than that in the surrounding non-tumorous tissues ($P < 0.0001$ by the Wilcoxon signed rank test; Fig. 2A). The overexpression of $p14^{ARF}$ appeared to be associated with a poorly differentiated phenotype, although the association was not statistically significant ($P = 0.0990$ by the Mann-Whitney *U*-test; well differentiated vs poorly differentiated; Fig. 2B).

Relationship between alteration of the $p14^{ARF}$ *gene and p53 grade of differentiation of HCCs*

To investigate the relationship between alteration of the *p14ARF* gene and p53, we performed immunohistochemistry of p53 on 44 paraffin-embedded HCC tissues. Of the 44 cases examined, 12 (27.2%) showed p53 overexpression, which was indicative of alteration of p53. Of the three HCCs with alteration of the *p14ARF* gene, 1 also showed p53 alteration (case 1; Table 1). All but 1 of the HCCs 12 with alteration of p53 were moderately or poorly differentiated ($P = 0.0322$ by Fisher's exact test; Table 2). On the other hand, all 3 HCCs with alteration of the *p14ARF* gene were well differentiated ($P = 0.0423$ by Fisher's exact test; Table 2).

Discussion

To investigate the role of disruption of the p53/ARF pathway in HCC formation, we used TaqMan real-time PCR and epigenetic/genetic analyses to examine the *p14ARF* gene, and compared the results with those of immunohistochemistry of p53 in the same HCC samples. Previously, Baek et al.[22] reported that comparative RT-PCR analysis showed no expression of $p14^{ARF}$ in 5 of 20 HCCs. Of these 5, 3 showed homozy-

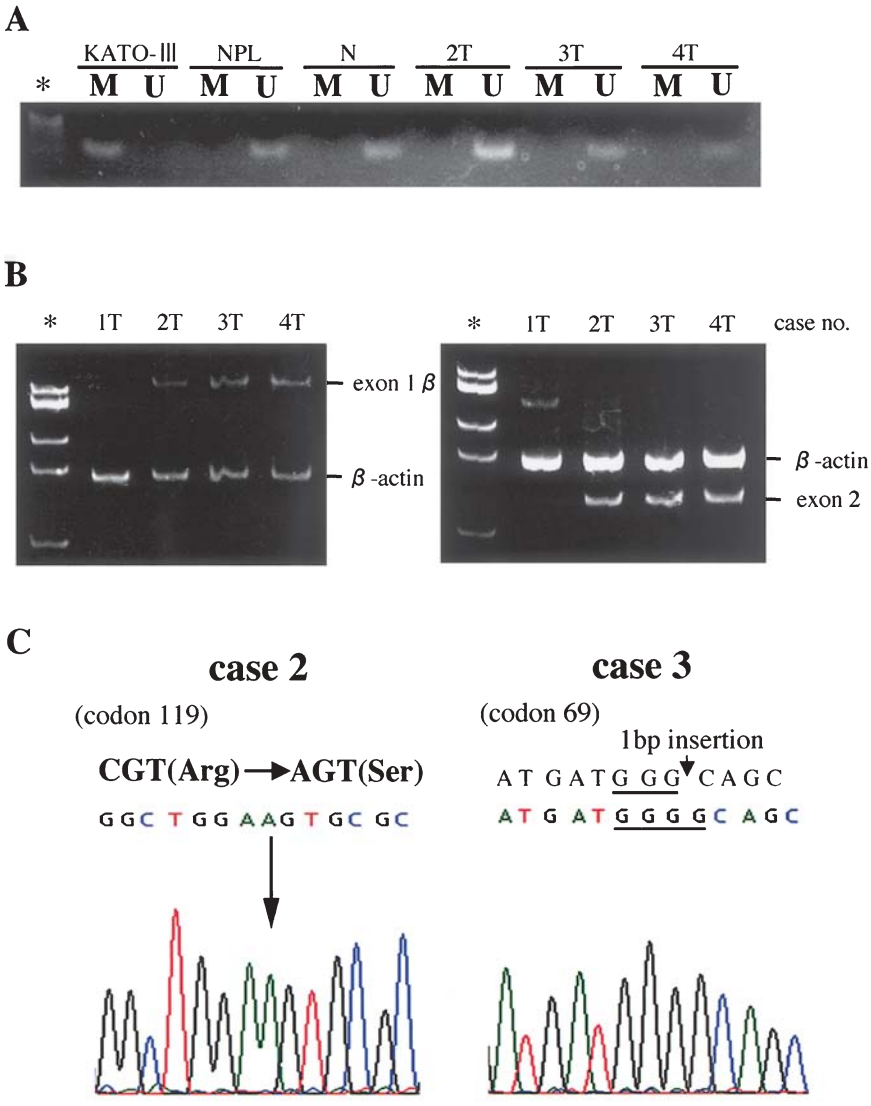

Fig. 1A–C. Examples of analysis of the p14ARF gene in hepatocellular carcinoma (HCC). **A** Representative findings of methylation-specific polymerase chain reaction MS-PCR analysis of the *p14ARF* gene promoter. *Asterisk*, molecular weight marker of ΦX174/*Hae*III digest; *N*, non-tumorous tissue; *T*, HCC tissue; *U* and *M*, PCR products from, respectively, unmethylated and methylated DNA of the *p14ARF* promoter. *KATO-III*, a cell line which contains the methylated *p14ARF* promoter, was used as a positive control for the methylated sequence, and DNA from normal peripheral lymphocytes (*NPL*) was used as a negative control. The tumors of three cases (*2T*, *3T*, *4T*) show PCR products from unmethylated DNA, but no product from methylated DNA, indicating that the *p14ARF* promoter was unmethylated. **B** Representative findings of homozygous deletions in exon 1β and exon 2 of the *p14ARF* gene. The homozygous deletions were identified by comparative multiplex PCR analysis; β-actin was used as an internal control. The tumor in case 1 (*1T*) exhibits homozygous deletion in exon 1β and exon 2 of the *p14ARF* gene. **C** Direct sequencing of two cases with an abnormal mobility shift on PCR-single-strand conformation polymorphism (SSCP) analysis. In the tumor in case 2, a missense mutation was detected in codon 119. In the tumor in case 3, a 1 base-pair insertion resulting in a frameshift mutation was detected in codon 69

gous deletion of the *p14ARF* gene, but the contribution of promoter hypermethylation of the *p14ARF* gene to HCC formation was not examined. Another report showed that no promoter hypermethylation of the *p14ARF* gene was detected in any of 20 HCCs.[23] Peng et al.[24] also analyzed alteration of the *p14ARF* gene, in 40 HCCs, and found that homozygous deletion was the predominant mechanism of p14ARF inactivation. On the other hand, Tannapfel et al.[25] identified that 9% of HCCs showed hypermethylation of the *p14ARF* promoter. Herath et al.[26] reported that 46% of Australian HCCs and 29% of South African HCCs carried hypermethylation of the *p14ARF* promoter. In our series of 44 HCCs, promoter hypermethylation of the *p14ARF* gene was not detected either, suggesting that it is not the major mechanism of p14ARF inactivation for HCC in Japan. The fact that p14ARF expression was absent in only one HCC, with homozygous deletion in exon 1β and exon 2, also supports this idea. The *p14ARF* promoter methylation has been shown to be somewhat complex, and a heterogeneous methylation pattern may result in differences in the frequency of *p14ARF* promoter methylation.[18] In this respect, we might overlook some CpG methylation of the *p14ARF* promoter. However, both the study of Herath et al.[26] and our study applied identical MS-PCR using the same primer sequence, which probed the most frequently methylated site among CpGs examined in a colorectal cell line.[18] In addition, we found that almost all HCCs showed more p14ARF expression than their corresponding non-cancerous tissue. Then, so far as Japanese HCC was concerned, we could conclude that methylation of the P14ARF promoter was not so frequent. According to the report of Herath et al.,[26] risk factors other than hepatitis B or C virus, such as hemo-

Table 1. Clinicopathological findings and alterations of p14ARF and p53 in 44 patients

Clinicopathological parameter				p14ARF			
					TaqMan PCR		
Case no.	Age (years)/Sex	Virus	Differentiation	Genetic/Epigenetic alteration	T	N	p53 IHC
1	68/F	NBNC	W	Homozygous deletion	0.00	0.09	+
2	57/M	C	W	Missense	1.40	0.31	−
3	69/M	C	W	1-bp Insertion	1.30	0.32	−
4	45/M	C	P	−	5.10	0.49	+
5	73/M	C	M	−	2.20	0.27	+
6	63/M	C	M	−	0.90	0.02	+
7	59/M	B	M	−	0.58	0.07	+
8	79/M	C	M	−	0.40	0.01	+
9	41/M	B	P	−	2.90	0.29	+
10	58/M	B and C	M	−	0.76	0.68	+
11	66/M	C	M	−	0.77	0.34	+
12	66/M	C	W	−	3.80	0.13	−
13	65/F	C	M	−	5.40	0.03	−
14	50/M	B	W	−	0.62	0.13	−
15	71/M	C	P	−	2.30	0.21	−
16	67/M	C	M	−	2.60	0.07	−
17	74/M	NBNC	W	−	0.70	0.07	−
18	57/M	C	P	−	0.29	0.05	−
19	65/M	C	W	−	0.83	0.07	−
20	67/M	NBNC	W	−	0.11	0.14	−
21	72/M	C	P	−	0.98	0.02	−
22	60/M	C	M	−	0.14	0.03	−
23	43/M	B	M	−	0.19	0.28	−
24	68/M	C	W	−	0.33	0.17	−
25	50/M	C	P	−	2.00	0.01	−
26	70/M	C	M	−	0.29	0.05	−
27	59/M	C	M	−	0.71	0.37	−
28	68/M	NBNC	M	−	1.70	0.06	−
29	67/M	B and C	M	−	ND	ND	+
30	67/M	NBNC	M	−	ND	ND	+
31	62/M	C	P	−	ND	ND	+
32	63/M	B	M	−	ND	ND	−
33	61/M	C	W	−	ND	ND	−
34	62/M	B	W	−	ND	ND	−
35	63/M	B	W	−	ND	ND	−
36	72/M	C	P	−	ND	ND	−
37	75/M	C	W	−	ND	ND	−
38	66/M	C	P	−	ND	ND	−
39	55/F	NBNC	W	−	ND	ND	−
40	51/M	C	W	−	ND	ND	−
41	65/F	C	M	−	ND	ND	−
42	72/M	NBNC	W	−	ND	ND	−
43	48/M	B	M	−	ND	ND	−
44	68/M	C	M	−	ND	ND	−

T, tumor (HCC) tissue; N, Non-tumorous tissue; B, positive for hepatitis B surface antigen (HBsAg); C, positive for hepatitis C virus antibody (HCVAb); B and C, positive for both HBsAg and HCVAb; NBNC, negative for HBsAg and HCVAb; W, well differentiated; M, moderately differentiated; P, poorly differentiated; IHC, immunohistochemistry; +, positive; −, negative; ND, not done. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control of TaqMan polymerase chain reaction (PCR). Data for p14ARF mRNA expression were normalized to that of GAPDH

chromatosis and aflatoxin, were identified in 24 of 37 (65%) Australian and 21 of 24 (88%) South African HCC cases. On the other hand, 37 of 44 HCC cases (84%) in our study showed hepatitis B or C virus infection. Although we cannot describe a clear reason for the difference in the frequencies of methylation, they might be, in part, accounted for by differences in etiology in the patients.

Among the 44 HCCs we examined, mutation of the *p14ARF* gene was found in 2 HCCs, although *p14ARF*

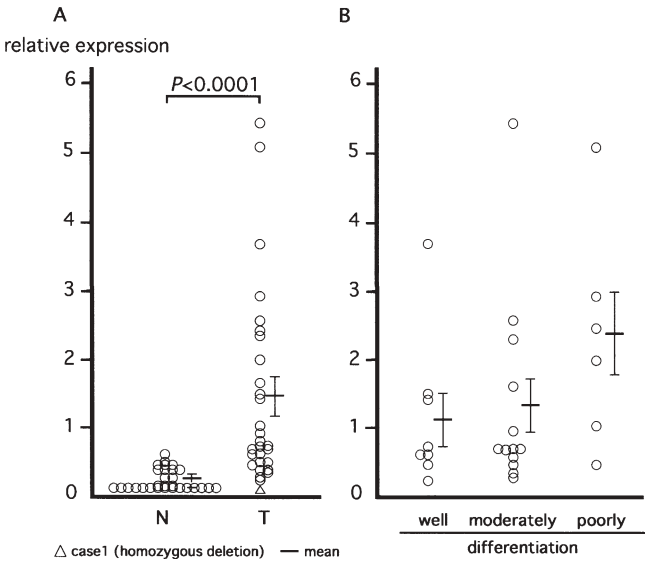

Fig. 2A,B. Relative mRNA levels of p14ARF in HCC. *p14ARF* expression is shown in relation to that of *GAPDH*, and the mean (±SE) for each group is also shown. **A** The mRNA levels of p14ARF in 28 HCCs and corresponding non-tumorous tissues are shown. *N*, non-tumorous tissue; *T*, HCC tissue. In HCC in case 1 (*triangle*), with homozygous deletion of the *p14ARF* gene, no p14ARF mRNA was detected. Expression of p14ARF in HCC was significantly higher than that in the non-tumorous tissue ($P < 0.0001$, Wilcoxon signed rank test) **B** The mRNA levels of p14ARF for three grades of differentiation. The mRNA level in case 1, with homozygous deletion, was excluded. Although the association was not statistically significant, the expression of p14ARF was correlated with poorly differentiated phenotype ($P = 0.0990$ by the Mann-Whitney *U*-test; well-vs poorly differentiated)

Table 2. Alterations of *p14ARF* and p53 and grade of differentiation in HCC

	Grade of differentiation (no. of cases)		
	Well	Moderately or Poorly	Total no.
p14ARF Alteration			
With	3	0	3
Without	13	28	41
Total no.	16	28	
p53 Alteration			
With	1	11	12
Without	15	17	32
Total no.	16	28	

All HCCs with *p14ARF* alteration were well differentiated ($P = 0.0423$ by Fisher's exact test). On the other hand, 11 of the 12 HCCs with p53 alteration were of moderately or poorly differentiated phenotype ($P = 0.0322$, by Fisher's exact test)

mRNA was also detected with TaqMan PCR in these tissues. Recent studies indicate that the C-terminal domain encoded by exon 2 of the *p14ARF* gene, where two mutations were detected in our study, contains an important nucleolar localization signal.[27] Mutations in exon 2 may, therefore, affect the nuclear localization of p14ARF and thereby interfere with p14ARF-regulated Mdm2-dependent stabilization of p53.[28] On the other hand, mutation in exon 1β of the *p14ARF* gene has not been found previously in any HCCs, nor in any other malignant tumor, except for melanoma.[29]

Evidence supporting direct biochemical interactions between p14ARF and p53 has been obtained,[30] leading to the hypothesis that p14ARF inactivation and *p53* mutation in human cancers must be mutually exclusive in the mutational sense, because both genes act on the same pathway. In the present study, the population of HCCs with p14ARF inactivation appears to be too small for an analysis of this relationship. However, all 3 HCCs with alteration of the *p14ARF* gene were well differentiated tumors, whereas only 1 of the 12 HCCs with p53 alteration was revealed to be well differentiated. These results indicated that alteration of the *p14ARF* gene emerges in the early stage of hepatocarcinogenesis compared with p53 alteration, although both molecules are known to play a role in the same p53/ARF pathway.

In HCCs without homozygous deletion of the INK4a/ARF locus, p14ARF overexpression seemed to be associated with a poorly differentiated phenotype. In addition, mRNA levels of p14ARF in most cases were much higher than those of the surrounding non-tumorous tissues. Patients with follicular lymphoma characterized by a high level of p14ARF expression had a significantly shorter overall survival time from the time of diagnosis than other patients.[19] Several mitogenic stimuli, such as E1A, myc, oncogenic ras, and E2F-1 are known to upregulate the *p14ARF* gene, leading to p53 stabilization.[31] Therefore, the high expression of p14ARF mRNA observed in HCCs may reflect oncogenic stimuli and/or inactivation of other tumor suppressor pathways in these tumors, such as that of RB.

In the study presented here, we found that alterations of the *p14ARF* gene were not frequent in HCCs from the Japanese population. However, deletions or mutations of the *p14ARF* gene were detected in some well-differentiated HCCs, suggesting that disruption of the p53/ARF pathway by inactivation of p14ARF may play a role in an earlier stage of HCC than disruption of the pathway by p53 alteration during HCC formation. In addition, overexpression of p14ARF in HCCs tends to be associated with a poorly differentiated phenotype, and may be clinically significant for the prediction of biological behavior. Further study of the relationship between this gene expression and prognosis is now in progress.

Acknowledgments. The authors thank Hidemasa Azechi, M.D., for valuable suggestions regarding MS-PCR and immunohistochemistry. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, and Culture, Japan.

References

1. Serrano M, Hunnon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclinD/CDK4. Nature 1993;366:704–7.
2. Serrano M, Lee HW, Chin L, Cordon-Cardo C, Beach D, DePinho R, et al. Role of the INK4a locus in tumor suppression and cell mortality. Cell 1996;85:27–37.
3. Quella DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encodes two unrelated proteins capable of inducing cell cycle arrest. Cell 1995;83: 933–1000.
4. Cardas C, Hahn SA, Da Costa LT, Redston MS, Schutte M, Seymour AB, et al. Frequent somatic mutations and homozygous deletions of the p16 gene in pancreatic adenocarcinoma. Nature Genetics 1994;8:27–32.
5. Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, et al. Inactivation of the CDKN2/p16/MST1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 1995;55:4525–30.
6. Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, et al. A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994;264:436–40.
7. Kita R, Nishida N, Fukuda Y, Azechi H, Matsuoka Y, Komeda T, et al. Infrequent alterations of the $p16^{INK4a}$ gene in liver cancer. Int J Cancer 1996;67:176–80.
8. Matsuda Y, Ichida T, Matsuzawa J, Sugimura K, Asakura H. $p16^{INK4a}$ is inactivated by extensive CpG methylation in human hepatocellular carcinoma. Gastroenterology 1999;116:394–400.
9. Azechi H, Nishida N, Fukuda Y, Nishimura T, Minata M, Katuma H, et al. Disruption of the p16/cyclin D1/retinoblastoma protein pathway in the majority of human hepatocellular carcinomas. Oncology 2001;60:346–54.
10. Markl ID, Jones PA. Presence and location of TP53 mutation determines pattern of CDKN2A/ARF pathway inactivation in bladder cancer. Cancer Res 1998;58:5348–53.
11. Sanchez-Cepedes M, Reed AI, Buta M, Wu L, Westra WH, Herman JG, et al. Inactivation of the INK4a/ARF locus frequently coexists with TP53 mutations in non-small cell lung cancer. Oncogene 1999;18:5843–9.
12. Iwato M, Tachibana O, Tohma Y, Arakawa Y, Nitta H, Hasegawa M, et al. Alterations of the INK4a/ARF locus in human intracranial germ cell tumors. Cancer Res 2000;60:2113–5.
13. Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB, et al. Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and p53 mutational status. Cancer Res 2000;60:129–33.
14. Iida S, Akiyama Y, Nakajima T, Ichikawa W, Nihei Z, Sugihara K, et al. Alterations and hypermethylation of the $p14^{ARF}$ gene in gastric cancer. Int J Cancer 2000;87:654–8.
15. Xing EP, Nie Y, Song Y, Yang G-Y, Cai YC, Wang L-D, et al. Mechanism of inactivation of $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ genes in human esophageal squamous cell carcinoma. Clin Cancer Res 1999;5:2704–13.
16. Vonlanthen S, Heighway J, Tschan MP, Borner NM, Altermatt HJ, Kappeler A, et al. Expression of $p16^{INK4a}$/p16alpha and $p19^{ARF}$/p16beta is frequently altered in non-small cell lung cancer and correlates with p53 over expression. Oncogene 1998;17:2779–85.
17. Silva J, Dominguez G, Silva JM, Garcia JM, Gallego I, Corbacho C, et al. Analysis of genetic and epigenetic processes that influence $p14^{ARF}$ expression in breast cancer. Oncogene 2001;20: 4586–90.
18. Zheng S, Chen P, McMillan A, Lafuente A, Lafuente MJ, Ballesta A, et al. Correlations of partial and extensive methylation at the p14(ARF) locus with reduced mRNA expression in coloretal cancer cell lines and clinicopathological features in primary tumors. Carcinogenesis 2000;21:2057–64.
19. Taniguchi T, Chikatsu N, Takahashi S, Fujita A, Uchimaru K, Asano S, et al. Expression of $p16^{INK4a}$ and $p14^{ARF}$ in hematological malignancies. Leukemia 1999;13:1760–9.
20. Nishida N, Fukuda Y, Kokuryu H, Toguchida J, Yandell DW, Ikenaga M, et al. Role and mutational heterogeneity of the p53 gene in hepatocellular carcinoma. Cancer Res 1993;53:368–72.
21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor: Cold Spring Harbor Laboratory; 1989. p. 7.6–9.24.
22. Baek MJ, Piao Z, Kim NG, Park C, Shin EC, Park JH, et al. p16 is a major inactivation target in hepatocellular carcinoma. Cancer 2000;89:60–8.
23. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225–9.
24. Peng C-Y, Chen T-C, Hung S-P, Chen M-F, Yeh C-T, Tsai S-L, et al. Genetic alterations of INK4a/ARF locus and p53 in human hepatocellular carcinoma. Anticancer Res 2002;22:1265–72.
25. Tannapfel A, Busse C, Weinans L, Benicke M, Katalinic A, Geissler F, et al. INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas. Oncogene 2001;20:7104–9.
26. Herath NI, Kew MC, Walsh MD, Young J, Powell LW, Leggett BA, et al. Reciprocal relationship between methylation status and loss of heterozygosity at the $p14^{ARF}$ locus in Australian and South African hepatocellular carcinoma. J Gastroenterol Hepatol 2002; 17:301–7.
27. Rizos H, Darmanian AP, Mann GJ, Kefford RF. Two arginine rich domains in the $p14^{ARF}$ tumor suppressor mediate nucleolar localization. Oncogene 2000;19:2978–85.
28. Zhang Y, Xing Y. Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. Mol ell 1999;3:579–91.
29. Rizos H, Puig S, Badenas C, Malvehy J, Darmanian AP, Jimenez L, et al. A melanoma-associated germline mutation in exon 1beta inactivates $p14^{ARF}$. Oncogene 2001;20:5543–7.
30. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression. Cell 1998;92:725–34.
31. Karen H. p53: Death star. Cell 2000;103:691–4.